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Dose-Dependent Neurocognitive Deficits Following Postnatal Day 10 HIV-1 Viral Protein Exposure: Relationship to Hippocampal Anatomy Parameters

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Abstract

Despite the availability of antiretroviral prophylactic treatment, pediatric human immunodeficiency virus type 1 (HIV-1) continues to be a significant risk factor in the post-cART era. The time of infection (i.e., during pregnancy, delivery or breastfeeding) may play a role in the development of neurocognitive deficits in pediatric HIV-1. HIV-1 viral protein exposure on postnatal day (P)1, preceding the postnatal brain growth spurt in rats, had deleterious effects on neurocognitive development and anatomical parameters of the hippocampus (Fitting *et al.,* 2008a; Fitting *et al.,* 2008b). In the present study, rats were stereotaxically injected with HIV-1 viral proteins, including $Tat_{1–86}$ and gp120, on P10 to further examine the role of timing on neurocognitive development and anatomical parameters of the hippocampus (Fitting *et al.,* 2010). The dose-dependent virotoxin effects observed across development following P10 $Tat_{1–86}$ exposure were specific to spatial learning and absent from prepulse inhibition and locomotor activity. A relationship between alterations in spatial learning and/or memory and hippocampal anatomical parameters was noted. Specifically, the estimated number of neurons and astrocytes in the hilus of the dentate gyrus explained 70% of the variance of searching behavior in Morris water maze acquisition training for adolescents and 65% of the variance for adults; a brain-behavior relationship consistent with observations following P1 viral protein exposure. Collectively, late viral protein exposure (P10) results in selective alterations in neurocognitive development without modifying measures of somatic growth, preattentive processing, or locomotor activity, as characterized early viral protein exposure (P1). Thus, timing may be a critical factor in disease progression, with children infected with HIV earlier in life being more vulnerable to CNS disease.

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Keywords

Tat; gp120; Pediatric HIV-1; Spatial Learning; Stereology; Hippocampus

1. INTRODUCTION

Pediatric human immunodeficiency virus type 1 (HIV-1) predominantly results from ``vertical" mother-to-child transmission (MTCT), occurring during active labor and delivery, pregnancy and/or postnatal breastfeeding (Kourtis *et al.,* 2001; AIDSinfo, 2017). Marked decreases in the prevalence of pediatric HIV-1 were observed following the implementation of interventions aimed at preventing MTCT, including treatment with combination antiretroviral therapy (cART; Luzuriaga & Mofenson, 2016). cART is commonly prescribed to HIV-1-infected mothers during pregnancy (Suksomboon *et al.*, 2007; Volmink *et al.*, 2007; Townsend *et al.*, 2008; Briand *et al.*, 2013; Reliquet *et al.*, 2014; Townsend *et al.*, 2014; Wang *et al.,* 2016, Peters *et al.,* 2017) and HIV-1 infected newborns and/or children (Riordan & Bugembe, 2009; Chadwick *et al.*, 2011; van der Plas *et al.*, 2013; Bitnun *et al.*, 2014; Mutwa *et al.*, 2014, Shiau *et al.,* 2017). Nevertheless, despite the availability of antiretroviral prophylactic treatment, vertical MTCT continues to be a significant risk factor in the post-cART era, with approximately 160,000 children being newly infected in 2016, most of whom live in resource-limited settings (UNAIDS, 2017).

Additionally, the advent of cART shifted the global epidemic into a new phase as HIV-1 seropositive children survive into adulthood (Sohn & Hazra, 2013; Crowell *et al.,* 2014; Smith & Wilkins, 2015). By 2020, it is estimated that approximately 1.94 million children will be living with HIV-1 (Penazzato *et al.,* 2014). The chronic nature of pediatric HIV-1 in the post-cART era has enormous implications for children's neurocognition and development (Vijayan *et al.*, 2009; Brady *et al.*, 2010; Paramesparan *et al.*, 2010; Sohn & Hazra, 2013; Crowell *et al.*, 2014). Specifically, high rates of subtle to severe neurocognitive deficits have been reported in HIV-1 infected children on cART that survive to adulthood (Burns *et al.*, 2008; Webb *et al.*, 2009; Paramesparan *et al.*, 2010; Crowell *et al.*, 2014). The causes of these neurocognitive deficits, despite effective cART, are multifactorial and likely include continued viral replication in the central nervous system (CNS), ongoing neuroinflammation, irreversible CNS injury prior to cART initiation, neurotoxic effects of cART, and socioeconomic and psychosocial constraints (Crowell *et al.*, 2014).

Pediatric HIV-1 infection presents a very different clinical picture compared to HIV-1 infection in adulthood (Sohn & Hazra, 2013; Crowell *et al.*, 2014). In the post-cART era, HIV-1 infected children commonly exhibit high rates of chronic neurological impairment, including significant delays in cognitive development, motor skills, and language (Lindsey *et al.,* 2007; Van Rie *et al.,* 2008; Walker *et al.,* 2013). The rates of disease progression within the pediatric HIV-1 population, however, have been shown to dramatically vary and are likely related to differences in viral load, strain of HIV, time of HIV infection, and/or genetic vulnerabilities (Belman, 1997; Rigardetto *et al.*, 1999; Chearskul *et al.*, 2002; Crowell *et al.*, 2014). A relationship between the time of perinatal infection and the course of the disease in infants has also been suggested by other studies (Blanche *et al.*, 1990; Blanche *et al.*, 1994;

Becquet *et al.*, 2012) with a greater sensitivity of the immature brain to the devastating effects of HIV-1 (Meeker *et al.*, 2004; Becquet *et al.*, 2012). To date, however, the relative importance of timing and its effects on neurocognitive development has been relatively understudied in pediatric HIV-1.

In human pediatric HIV/AIDS cases, the time of infection is broadly defined as 'occurring in the period shortly before and after birth', which describes both prenatal (i.e., 20^{th} to 28^{th} week of gestation) and postnatal (i.e., 7 to 28 days after birth) infection (Wiley *et al.,* 1994; Donovan & Palumbo, 2010; CDC, 2014). Technological limitations of HIV-1 testing prevent us from determining either the exact timing or degree of early postnatal transmission of HIV-1. DNA or RNA polymerase chain reactions (PCR), viral culture, and p24 antigen tests, although able to detect the virus itself, are not able to define the route of infection before the age of 2–3 months (Ogundele & Coulter, 2003; CDC, 2014). Due to experimental limitations and ethical constraints in human pediatric AIDS research, an animal model that is (A) translational to the important health issues surrounding pediatric AIDS, and (B) able to determine the timing of MTCT and its effects on the CNS in a precise and controlled manner, is of great clinical relevance in defining the different rates of progression in pediatric HIV/AIDS. In the present study, stereotaxic injections of HIV-1 viral proteins at postnatal day 10 (P10), including the transactivator of transcription ($Tat_{1–86}$) and envelope glycoprotein 120 (gp120), provides an opportunity to elucidate the effect of neurotoxic proteins on neurodevelopment (Carryl *et al.,* 2015).

Neurotoxic viral proteins, including Tat and gp120, are active in the CNS, even when peripheral immune system function is restored under cART, and may be partially responsible for the neuroanatomical alterations commonly observed in HIV-1 seropositive individuals (e.g., synaptodendritic alterations: Ellis *et al.,* 2007; Gelman & Nguyen, 2010; Desplats *et al.,* 2013; neuronal loss: Del Valle *et al.,* 2000; Jones *et al.,* 2000; Nath *et al.,* 2000). Tat, the transactivating protein for retroviral replication (Cann *et al.*, 1985; Li *et al.*, 2010), is produced very early after infection, and is necessary for viral expression, cell-to-cell virus transmission and disease progression (Sodroski *et al.*, 1985; Ensoli *et al.*, 1993; Chang *et al.*, 1997; Karn, 1999; Lin *et al.*, 2003; Ensoli *et al.*, 2006; Richter & Palu, 2006). Gp120, a structural viral gene product, is vital for viral entry into the CNS (Hao & Lyman, 1999) and may cause neurotoxicity by binding to cell surface receptors, ultimately leading to neuronal death (Lipton, 1991; Corasaniti *et al.,* 2001a; Haughey & Mattson, 2002). Preclinical assessments, including both *in vitro* and *in vivo* studies, have extensively examined both Tat and gp120 (Brenneman *et al.*, 1988; Lipton *et al.*, 1995; Nath *et al.*, 1996; Cheng *et al.*, 1998; Aksenov *et al.*, 2001; Corasaniti *et al.*, 2001b; Bruce-Keller *et al.*, 2003; Aksenov *et al.*, 2006; Aksenova *et al.*, 2006; Fitting *et al.*, 2006a; Aksenov *et al.*, 2008; Adams *et al.*, 2010; Zhu *et al.*, 2011; Bertrand *et al.*, 2013; Fitting *et al.*, 2013; Hahn *et al.*, 2013; Bertrand *et al.*, 2014; Bertrand *et al.,* 2015; Marks *et al.,* 2016), providing strong evidence for the role of HIV-1 viral proteins in neurocognitive and neuroanatomical alterations in adulthood.

Although preclinical and clinical studies have characterized HIV-1 associated neurocognitive disorders (HAND) in adults (e.g., review, Woods *et al.,* 2009; Heaton *et al.,* 2010), neurocognitive deficits in HIV-infected children are poorly understood (Crowell *et al.,* 2014). A limited number of preclinical studies have focused on the effects of early exposure

to HIV-1 viral toxic proteins on the development of neurocognitive disorders, independent of the virus itself (Bussiere *et al.*, 1999; Fitting *et al.*, 2008b; Webb *et al.*, 2009; Moran *et al.*, 2014a; Fitting *et al.*, 2015; McLaurin *et al.,* 2017a). Specifically, stereotaxic injections of the viral proteins Tat and/or gp120 on P1 revealed deleterious effects on multiple reflexive, preattentive (e.g., prepulse inhibition (PPI), and neurocognitive assessments (e.g., Morris water maze), as well as alterations in anatomical parameters of the hippocampus (Fitting *et al.,* 2008a; Fitting *et al.,* 2008b; Moran *et al.,* 2014a). A histological dose-response study that injected HIV-1 proteins at P10 indicated that timing may have a differential effect on the anatomical parameters of the hippocampus in adult rats (~ 5 months) compared to the P1 study (Fitting *et al.,* 2008a; Fitting *et al.,* 2010). Stereotaxic injections of HIV-1 viral proteins on P1 in rats were chosen to mimic early transmission (i.e., *in utero*) of the virus, whereas HIV-1 protein delivery on P10 more closely resembles HIV-1 protein entry into the CNS at labor/delivery in humans.

Thus, the present study investigated the effects of perinatal P10 HIV-1 protein neurotoxicity using a series of neurocognitive assessments throughout development and adulthood to address two interrelated aims. First, dose-response functions were used to examine the short and long-term effects of $Tat_{1–86}$ and gp120 on developmental milestones and neurocognition. Measures of somatic growth, including body weight and eye opening, and motor development, including locomotor activity, were used to examine developmental milestones. Neurocognitive assessments included PPI, to examine sensorimotor gating and temporal processing, and the Morris water maze, to examine spatial learning and memory. Assessments were chosen as indexes of neuropsychological assessments in humans that have been shown to be impaired in pediatric HIV-1 infection (Boivin *et al.*, 1995; Newell *et al.*, 2003; Jeremy *et al.*, 2005; Lodha *et al.*, 2005; Martin *et al.*, 2006; Willen, 2006; Lindsey *et al.,* 2007; Baillieu & Potterton, 2008; Van Rie *et al.*, 2009; Webb *et al.*, 2009; Paramesparan *et al.*, 2010; Le Doare *et al.*, 2012; Walker *et al.,* 2013). Second, the relationship between neurocognitive deficits and neuroanatomical alterations (Fitting *et al.,* 2010) was investigated. Thus, the present study provides an opportunity to determine whether the developing CNS is more vulnerable to later HIV-1 viral protein exposure period (i.e., P10) relative to the earlier published findings at P1 (Fitting *et al.,* 2008a; Fitting *et al.,* 2008b; Moran *et al.,* 2014a), aiding in the understanding of the role of timing in chronic neurological impairment in pediatric HIV-1.

2. RESULTS

2.1 Somatic Growth

2.1.1 Body Weight—Figure 1 illustrates the mean (± *S.E.M.*) body weight data across different test days for each treatment group (Tat: A, Gp120: B). Body weight increased significantly with age across all groups. Separate ANOVAs were conducted for each testing set with Tat dose treatment (4 levels) or gp120 dose treatment (4 levels) as a between-subjects factor, and when appropriate, a within-subjects test day factor. For Tat or gp120, ANOVAs demonstrated no treatment effect or treatment by day interaction for any of the test sets. Planned contrast analyses for Tat or gp120 revealed no overall treatment effect, no dose-dependent treatment effect, and hence no threshold effect. Results indicated that the

HIV-1 viral protein doses produced no general growth deficits when injected on P10 consistent with the findings of the previous P1 study (Fitting *et al.*, 2008b).

2.1.2 Eye opening—Figure 2 illustrates the median (± *Interquartile Range*) for eye opening collapsed across P13 – P16. Eye opening began at P13; eyes were fully open for all rats by P16 (maximum rank score was 6 and the minimum rank score was 0). For the Tat treatment, a Kruskal-Wallis test revealed no significant difference among the four dose conditions (Figure 2A). Separate Mann-Whitney U-Tests, for the overall Tat effect and each Tat dose individually compared to the VEH control group revealed no significant difference from the VEH group. Similarly, no significant treatment effects were found across the gp120 dose conditions (Figure 2B). The lack of any P10 HIV-1 protein exposure effect on eye opening contrasts the effect of P1 $Tat_{1–72}$ exposure, but is consistent with the effect of P1 gp120 exposure (Fitting *et al.*, 2008b), suggesting that an early exposure period is more vulnerable to the developing CNS system.

2.2 Sensorimotor Function (PPI of the ASR)

2.2.1 Control Trials (0, 4000 msec Interstimulus Interval (ISI) combined): For preweanlings and adults, one-way ANOVAs on peak ASR amplitude, revealed no significant treatment effect for Tat or gp120 dose treatment. Planned contrast analyses for Tat or gp120 revealed no overall treatment effect, no dose-dependent treatment effect, and no threshold effect, suggesting that the baseline startle response for the HIV-1 viral protein groups were comparable to the VEH condition.

2.2.2 PPI trials (8–120 msec ISI): For preweanlings, at 18 days of age, Figure 3A and 3B illustrate mean peak ASR amplitude (± *S.E.M.*) across ISI (0–4000 msec) for the Tat dose treatment (Figure 3A) and the gp120 dose treatment (Figure 3B).

For the Tat dose treatment groups a 64.4% decrease in response amplitude was found for the PPI trials compared to the ASR control trials, again, indicating effective sensorimotor gating for all four groups. A 4 (Tat) × 4 (ISIs 8, 40, 80, 120 msec) mixed-model ANOVA conducted on peak ASR amplitude demonstrated a significant ISI effect [$F(3, 84) = 20.5$, $p \leq 0.001$] with linear and quadratic components [$F(1, 28) = 15.0$, $p \leq 0.001$ and $F(1, 28) = 44.1$, $p \leq 0.001$, respectively]. No treatment effect or treatment by ISI interaction was noted. Planned contrast analyses revealed no overall treatment effect, no dose-dependent treatment effect, and no threshold effect. For the ISI data, there was no significant shift in the point of maximal peak inhibition for Tat dose treatment compared to the VEH-treated animals; maximal peak inhibition occurred at either the 40 msec or 80 msec ISI.

For the gp120 dose groups, there was a 63.3% decrease in response amplitude for the PPI trials compared to the ASR control trials, demonstrating effective sensorimotor gating for all four groups. A 4 (Gp120) × 4 (ISIs 8, 40, 80, 120 msec) mixed-model ANOVA conducted on peak ASR amplitude demonstrated a significant ISI effect [$F(3, 84) = 23.9$, $p \leq 0.001$] with linear and quadratic components [$F(1, 28) = 17.9$, $p \leq 0.001$ and $F(1, 28) = 48.0$, $p \leq 0.001$, respectively]. No treatment effect or treatment by ISI interaction was noted. Planned contrast analyses revealed no overall treatment effect, no dose-dependent treatment effect, and no threshold effect. For the ISI data, no significant shift in the point of maximal

inhibition occurred for gp120 dose treatment compared to the VEH-treated animals; maximal peak inhibition occurred at either the 40 or 80 msec ISI.

For adults, at 91 days of age, Figure 3 displays (± *S.E.M.*) peak ASR amplitude across ISI (0–4000 msec) for Tat dose treatment (Figure 3C) and gp120 dose treatment (Figure 3D).

For the Tat dose groups, analyses of the data revealed an inhibition of the baseline ASR by 78.9%, again demonstrating effective sensorimotor gating for all four Tat dose groups. A 4 (Tat) × 4 (ISIs 8, 40, 80, 120 msec) mixed-model ANOVA conducted on peak ASR amplitude demonstrated a significant ISI effect [$F(3, 78) = 30.8$, $p \leq 0.001$] with significant linear and quadratic components [$F(1, 26) = 37.9$, $p \leq 0.001$ and $F(1, 26) = 22.5$, $p \leq 0.001$, respectively]. No treatment effect or treatment by ISI interaction was noted. Planned contrast analyses revealed no overall treatment effect, no dose-dependent treatment effect, and no threshold effect. For the ISI data, no significant shift in the point of maximal peak inhibition occurred for Tat dose treatment groups relative to the VEH-treated animals; maximal peak inhibition occurred at either the 80 msec or 120 msec ISI.

For the gp120 dose groups, analyses of the data revealed an inhibition of the baseline ASR by 79.3%, again demonstrating effective sensorimotor gating for all four gp120 dose groups. A 4 (Gp120) × 4 (ISIs 8, 40, 80, 120 msec) mixed-model ANOVA conducted on peak ASR amplitude demonstrated a significant ISI effect [$F(3, 84) = 28.5$, $p \leq 0.001$] with a prominent linear component [$F(1, 28) = 35.6$, $p \leq 0.001$]. No treatment effect or treatment by ISI interaction was noted. Planned contrast analyses revealed no overall treatment effect, no dose-dependent treatment effect, and no threshold effect. For the ISI data, no significant shift of maximal peak inhibition occurred for gp120 dose treatment compared to the VEH-treated animals; maximal peak inhibition occurred at either the 80 or 120 msec ISI.

In sum, as preweanlings and as adults, effective sensorimotor gating was demonstrated by all groups. No significant treatment effect and/or interaction were noted for any of the analyses, indicating that sensorimotor function was not affected by P10 HIV-1 viral protein treatment. The previous P1 study supports the lack of any $Tat_{1–72}$ effect in preweanling rats, but demonstrated significant $Tat_{1–72}$ alterations in the maximum peak inhibition response in adulthood, suggesting long-lasting effects of the HIV-1 protein $Tat_{1–72}$ on the process of sensorimotor gating, when injected at P1 (Fitting *et al.*, 2008b). The findings for sensorimotor function support the conclusion that an early exposure period, specifically to Tat, appears to be more vulnerable to the developing CNS system.

2.3 Locomotor Activity

For weanlings, the mean total activity data (60 min) across P22 – P24 are illustrated in Figure 4A and 4B.

A 4 (Tat) × 3 (Test days) mixed-model ANOVA revealed significant habituation as expressed by a significant effect of test day [$F(2, 56) = 7.3$, $p \leq 0.001$] with a linear component [$F(1, 28) = 10.9$, $p \leq 0.01$] (see Figure 4A). No treatment effect or treatment by day interaction was noted. Planned contrast analyses revealed no overall treatment effect, no dose-dependent treatment effect, and no threshold effect.

A 4 (Gp120) × 3 (Test days) mixed-model ANOVA also revealed significant habituation as expressed by a test day effect [$F(2, 56) = 8.6$, $p \leq 0.001$] with a linear component [$F(1, 28) = 17.8$, $p \leq 0.001$] (Figure 4B). No treatment effect or treatment by day interaction was noted. Planned contrast analyses revealed no overall treatment effect, no dose-dependent treatment effect, and no threshold effect. Thus, neither Tat nor gp120 dose treatment had any significant effect on the activity measure in weanlings.

For adults, mean total activity data (60 min) across P94 – P96 are illustrated in Figure 4C and Figure 4D.

A 4 (Tat) × 3 (Test days) mixed-model ANOVA revealed significant habituation as expressed by a significant effect of test day [$F(2, 52) = 25.3$, $p \leq 0.001$] with a prominent linear component [$F(1, 26) = 35.9$, $p \leq 0.001$] (Figure 4C). No treatment effect or treatment by day interaction was noted. Planned contrast analyses revealed no overall treatment effect, no dose-dependent treatment effect, and no threshold effect.

A 4 (Gp120) × 3 (Test days) mixed-model ANOVA revealed significant habituation as expressed by a significant test day effect [$F(2, 56) = 31.6$, $p \leq 0.001$] with a prominent linear component [$F(1, 28) = 44.7$, $p \leq 0.001$] (Figure 4D). No treatment effect or treatment by day interaction was noted. Planned contrast analyses revealed no overall treatment effect, no dose-dependent treatment effect, and no threshold effect.

Collectively, as for weanlings, neither P10 Tat or gp120 dose treatment had a significant effect on locomotor activity in adulthood, contrary to findings in the P1 study (Fitting *et al.*, 2008b). Although no motoric dysfunction was detected, less habituation was reported for the P1 Tat-treated animals and the P1 gp120-treated animals in adulthood (Fitting *et al.,* 2008b).

2.4 Spatial Learning and Memory

2.4.1 Adolescent Rats (P46 – P51)

2.4.1.1.Acquisition Training: Travel distance during acquisition across the 20 training trials is shown in Figure 5A and 5B. Mixed-model ANOVAs were conducted for each day with either Tat or gp120 dose treatment as the between-subjects factor.

For the Tat dose groups (see Figure 5A), on day 1, a main effect for trial was revealed [$F(7, 182) = 9.2$, $p \leq 0.001$] with a significant linear component [$F(1, 26) = 72.8$, $p \leq 0.001$], indicating that animals performance improved across training trials. Planned contrast analyses on day 1, revealed a linear dose-response treatment effect collapsed across trials [$F(1, 26) = 5.0$, $p \leq 0.05$], with an increase in travel distance by Tat treatment. Planned comparisons indicated a significant deficit in search performance for the high Tat dose group compared to the VEH controls [$F(1, 26) = 4.4$, $p \leq 0.05$]. On days 2 and 3, significant main effects of trial [$F(7, 182) = 9.3$, $p \leq 0.001$ and $F(3, 78) = 14.3$, $p \leq 0.001$, respectively] were characterized by a significant linear component [$F(1, 26) = 63.3$, $p \leq 0.001$ and $F(1, 26) = 34.9$, $p \leq 0.001$, respectively]. No Tat dose treatment effect or treatment by trial interactions were noted on day 2, suggesting that all four groups traveled similar distances to find the target platform location on their second day of acquisition training. As on day 1, planned contrast analyses on day 3 revealed a linear dose-response treatment effect collapsed across

trials [$F(1, 26) = 4.4$, $p \leq 0.05$], with an increase in travel distance by Tat treatment. Planned comparisons failed to detect a threshold effect for either the low or medium Tat dose treatment conditions. As shown in Figure 5A, continued training across days improved performance of all treatment conditions. Neonatal P10 exposure of the viral toxin indicated a dose-response $Tat_{1–86}$-induced deficit in acquisition training in young rats on day 1 and day 3.

For the gp120 dose groups (see Figure 5B), on day 1, a main effect for trial was revealed [$F(7, 196) = 19.0$, $p \leq 0.001$] with a significant linear component [$F(1, 28) = 81.1$, $p \leq 0.001$], indicating that animals performance improved across training trials. No significant treatment effect or treatment by trial interaction was noted, suggesting that all four groups traveled similar distances to find the target platform location on their first day of acquisition. On days 2 and 3, significant main effects of trial [$F(7, 196) = 17.1$, $p \leq 0.001$ and $F(3, 196) = 14.3$, $p \leq 0.001$, respectively] were characterized by a prominent linear component [$F(1, 28) = 120.3$, $p \leq 0.001$ and $F(1, 28) = 31.9$, $p \leq 0.001$, respectively]. No gp120 dose treatment effects or treatment by trial interactions were noted on any of the three training days. Planned contrast analyses on the three training days, revealed no overall treatment effect, no dose-dependent treatment and/or interaction effects, and no threshold effect.

2.4.1.2 Probe Test: Figure 5C and 5D illustrates quadrant preference in the probe test and represents the amount of time the animal searches in each of the four quadrants when no platform was present.

For the Tat dose groups (Figure 5C), a 4 (Tat) × 4 (Quadrant) mixed-model ANOVA conducted on the time spent searching in each of the four quadrants revealed a significant quadrant effect [$F(3, 78) = 41.5$, $p \leq 0.001$]. There was no significant treatment or treatment by quadrant interaction. Planned contrast analyses on each of the four quadrants separately, revealed no overall treatment effect, no dose-dependent treatment effects, and no threshold effect. All four groups spent the greatest time searching in the target quadrant relative to the opposite or the adjacent quadrants [discrimination effect: $F(1, 26) = 72.1$, $p \leq 0.001$ and $F(1, 26) = 85.0$, $p \leq 0.001$, respectively], suggesting they were able to discriminate and remember the prior platform location. A significant effect of evasion was revealed [$F(1, 26) = 5.4$, $p \leq 0.001$], with rats receiving Tat dose treatment spending relatively more time in the opposite quadrant relative to the adjacent quadrants compared to controls. Planned contrast analyses on the discrimination and evasion measure, revealed no overall or dose-dependent treatment effects.

For the gp120 dose groups (see Figure 5D), a 4 (Gp120) × 4 (Quadrant) mixed-model ANOVA conducted on the time spent in each of the four quadrants revealed a significant quadrant effect [$F(3, 84) = 73.4$, $p \leq 0.001$]. No treatment effect or treatment by quadrant interaction was observed. Planned contrast analyses on each of the four quadrants separately revealed no overall treatment effect, no dose-dependent treatment effects, and no threshold effect. All four groups spent the greatest time searching in the target quadrant relative to the opposite or adjacent quadrants [discrimination effect: $F(1, 28) = 127.8$, $p \leq 0.001$ and $F(1, 28) = 112.2$, $p \leq 0.001$, respectively], suggesting they were able to discriminate and remember the prior platform location. In addition, a significant effect of evasion was

revealed [$F(1, 28) = 24.2$, $p \leq 0.001$], with all rats receiving gp120 dose treatment spending less time in the opposite quadrant relative to the adjacent quadrants. Planned contrast analyses on the discrimination and evasion measure, revealed no overall or dose-dependent treatment effects. Thus, all groups avoided the opposite quadrant relative to the adjacent quadrants, suggesting that they had learned where not to go.

In sum, results of the probe test indicated that animals of all treatment groups developed an understanding about where to go and where not to go in the testing environment, suggesting the preservation of spatial learning and memory in the adolescents.

2.4.2 Adult Rats (P104 – P109)

2.4.2.1 Acquisition Training: Travel distance during acquisition training across the 20 training trials is shown in Figure 5E and 5F.

For Tat dose groups (see Figure 5E), mixed-model ANOVAs were conducted on travel distance for each of the three days of training. Day 1 of training revealed a significant main effect of trial [$F(7, 182) = 19.6$, $p \leq 0.001$] with a prominent linear component [$F(1, 26) = 79.9$, $p \leq 0.001$] and a significant treatment effect [$F(3, 26) = 3.4$, $p \leq 0.05$]. Planned contrast analyses revealed no overall treatment effect, but a dose-dependent treatment effect with a cubic component [$F(1, 26) = 6.2$, $p \leq 0.05$]. Planned contrasts revealed significantly longer travel distances for the high dose Tat-treated animals ($M = 666.6$, *S.E.M.* = 108.4) compared to the VEH-treated animals ($M = 462.3$, *S.E.M.* = 60.8) [$F(1, 26) = 4.6$, $p \leq 0.05$]. No significant differences in comparison to the VEH-treated animals were noted for the low dose Tat group ($M = 549.9$, *S.E.M.* = 44.4) or the medium dose Tat group ($M = 368.0$, *S.E.M.* = 41.8). Day 2 of training indicated a significant trial effect [$F(7, 182) = 7.5$, $p \leq 0.001$] with prominent linear and quadratic components [$F(1, 26) = 10.4$, $p \leq 0.01$ and $F(1, 26) = 12.2$, $p \leq 0.01$, respectively]. No treatment effect or treatment by trial interaction was noted, indicating that the Tat-treated groups approximated the performance level of the vehicle control animals on day 2. On day 3, no significant trial effect was noted. Planned contrast analyses for overall treatment revealed a significant difference between the VEH group and the overall Tat dose groups [$F(1, 26) = 5.4$, $p \leq 0.05$]. Further, planned contrast analyses revealed no dose-dependent treatment effect. For the threshold effect, planned contrast analyses indicated that the low Tat group ($M = 250.7$, *S.E.M.* = 45.3) and the high Tat group ($M = 263.3$, *S.E.M.* = 55.2) traveled significantly longer distances compared to the VEH group ($M = 140.4$, *S.E.M.* = 10.4) [$F(1, 26) = 4.5$, $p \leq 0.05$ and F(1, 26) = 5.2, $p \leq 0.05$, respectively]. The medium Tat group ($M = 208.8$, *S.E.M.* = 29.1) was not significantly different from the VEH group.

For the gp120 dose groups (see Figure 5F), mixed-model ANOVAs on travel distance revealed a significant main effect of trial on day 1 [$F(7, 196) = 23.8$, $p \leq 0.001$], with prominent linear and quadratic components [$F(1, 28) = 86.0$, $p \leq 0.001$ and $F(1, 28) = 9.1$, $p \leq 0.01$, respectively]. No treatment effect or treatment by trial interaction was noted. On day 2, no significant effects were noted. Day 3 revealed a significant main effect of trial [$F(3, 84) = 4.4$, $p \leq 0.05$], with a significant linear component [$F(1, 28) = 6.5$, $p \leq 0.05$]. No treatment effect or treatment by trial interaction was noted. Planned contrast analyses on the three training days, revealed no overall treatment and/or interaction effects, no dose-dependent

treatment effects, and no threshold effect. All groups learned the spatial location of the platform, approximating an asymptotic performance level on day 3. No treatment effects or treatment by trial interactions were noted for any of the three training days. As illustrated in Figure 5E and 5F, all groups reached asymptotic performance as adults.

2.4.2.2 Probe Test: Figure 5G and 5H illustrates quadrant preference in the probe test, with the amount of time spent in each of the four quadrants when no platform was present.

For the Tat dose groups (Figure 5G), a 4 (Tat) × 4 (Quadrant) mixed-model ANOVA conducted on the time spent in each of the four quadrants revealed a significant quadrant effect [$F(3, 78) = 141.4$, $p \leq 0.001$]. No treatment effect or treatment by quadrant interaction was observed. Planned contrast analyses on each of the four quadrants separately revealed no overall treatment effect, no dose-dependent treatment effects, and no threshold effect. As illustrated in Figure 5G, all animals were able to discriminate and searched primarily in the target quadrant relative to the opposite or the adjacent quadrants [$F(1, 28) = 230.2$, $p \leq 0.001$ and $F(1, 26) = 216.3$, $p \leq 0.001$, respectively], suggesting that all animals displayed spatial memory for the prior platform location. In addition, a significant effect of evasion was revealed [$F(1, 26) = 12.5$, $p \leq 0.01$], with all groups searching less in the opposite quadrant relative to the adjacent quadrants. No treatment effect or interactions with treatment were noted. Planned contrast analyses on the discrimination and evasion measure, revealed no overall- or dose-dependent treatment effects.

For the gp120 dose groups (Figure 5H), a 4 (Gp120) × 4 (Quadrant) mixed-model ANOVA conducted on the time spent in each of the four quadrants revealed a significant quadrant effect [$F(3, 84) = 128.2$, $p \leq 0.001$]. No treatment effect or treatment by quadrant interaction was observed. Planned contrast analyses on each of the four quadrants separately, revealed no overall treatment effect, no dose-dependent treatment effects, and no threshold effect. As illustrated in Figure 5H, all animals were able to discriminate and searched primarily in the target quadrant relative to the opposite or the adjacent quadrants [$F(1, 28) = 292.2$, $p \leq 0.001$ and $F(1, 28) = 182.6$, $p \leq 0.001$, respectively], suggesting that all gp120 dose groups displayed spatial memory for the prior platform location. In addition, a significant effect of evasion was revealed [$F(1, 28) = 22.7$, $p \leq 0.001$], with all rats searching less in the opposite quadrant relative to the adjacent quadrants. No treatment effect or interaction with treatment was noted. Planned contrast analyses on the discrimination and evasion measure revealed no overall or dose-dependent treatment effects. Thus, all groups avoided the opposite quadrant relative to the adjacent quadrants and remembered where not to go.

In sum, results of the probe test, were consistent with the findings in adolescent animals; all treatment groups developed an understanding about where to go and where not to go in the testing environment, suggesting the preservation of spatial learning and memory in adulthood.

2.5 Relationship Between Behavior and Anatomy

Even though no significant $Tat_{1–86}$ effects were noted for most of the behavioral measures or on estimates of total neuron numbers, correlation analyses were conducted to assess brain-behavior relationships. It was hypothesized that the hippocampus plays an important role in

behavioral measures that correlated with anatomical measures in the previous P1 study, such as locomotor activity in weanlings and spatial memory in adults (Fitting *et al.*, 2008b). Table 1 depicts results of a correlation matrix (Pearson) that was derived for the estimated total number of cells in the five subdivisions of the hippocampus combined (Fitting *et al.,* 2010), expressed as a percentage from the mean of the VEH-treated group, and the behavioral measures in preweanling, adolescent and adult rats. Significant correlations were only noted for estimated number of glia with PPI in adults, and travel distance in acquisition training in adolescent and adult rats (Table 1). The significant correlations were further assessed by separate stepwise multiple regression analyses using percent number of glia as the predictor variable. Approximately 19% – 33% of total variance in the behavioral scores, including prepulse inhibition (Figure 6A), and spatial learning in adolescence (Figure 6B) and adulthood (Figure 6C) was predicted by the anatomical measures (also summarized in Table 2).

Further exploration of the relationships between the five hippocampal subdivisions and PPI during adulthood, the distance traveled in acquisition training in adolescence, and the distance traveled in acquisition training during adulthood was undertaken. Linear multiple regression analyses were conducted to assess how much variance of prepulse inhibition and travel distance was accounted for by the anatomical measures. Starting with the highest parameter models, we then constrained the models by seeking the lowest parameter models that did not cause a significant R^2-change. PPI was examined, despite no significant $Tat_{1–86}$ effects, because of the significant correlation between estimated number of glia and PPI in adults (i.e., 0.573); the most profound correlational relationship observed in the present study. Travel distance was chosen because $Tat_{1–86}$ produced significant dose-response effects on first and last acquisition training day; further, the hippocampus is well known to be involved in spatial learning. Based on the previous P1 study (Fitting *et al.*, 2008b), we used estimates of numbers of neurons and astrocytes in the DGH as predictors for distance traveled during acquisition training (collapsed across the three training days) in adolescents and adults.

For PPI during adulthood, the final model was a 7-parameter model including three intercepts b_0 that varied with condition, and four weights that depended on the Tat dose treatment condition (see Table 3 in the corresponding column). As illustrated in Figure 7A, the model provided a good fit of the peak response amplitude in PPI in adults, accounting for 52% of the variance of the data ($R^2 = 0.517$).

For the distance traveled during adolescence, the final model version was a 6-parameter model including four intercepts b_0 that varied with condition, and two weights that depended on the Tat dose treatment condition (see Table 3 in the corresponding column). As illustrated in Figure 7B, the model provided a good fit of the distance traveled during acquisition training, accounting for 70% variance of the data ($R^2 = 0.695$).

For the distance traveled during adulthood, the final model version was an 8-parameter model including four intercepts b_0 that varied with condition, and four weights that depended on the Tat dose treatment condition (see Table 3 in the corresponding column). As

illustrated in Figure 7C, the model provided a good fit of the distance traveled during acquisition training, accounting for 65% variance of the data (R^2 = 0.651).

3. DISCUSSION

Selective, dose-dependent, neurocognitive deficits were observed following late (i.e., P10) exposure to the HIV-1 viral proteins $Tat_{1–86}$ and gp120, revealing critical information on the role of timing in pediatric HIV-1. Stereotaxic injections of viral proteins into the rat hippocampus at P10 provide a translational preclinical model to examine the effects of neurotoxic proteins on neurocognitive development in children infected during labor or delivery. No neurotoxic effects were observed following exposure to gp120. In sharp contrast, specific, dose-dependent deficits in spatial learning, assessed using the Morris water maze, were observed following exposure to $Tat_{1–86}$. Neurocognitive alterations in spatial learning were primarily (65–70%) explained by an increase in gliosis in the DGH. Compared to the deleterious neurocognitive effects observed following early viral protein exposure (i.e., P1; Fitting *et al.*, 2008b; Moran *et al.,* 2014a), the results of the present study indicated that late viral protein exposure is best described as having task specific effects on neurocognitive development.

The only dose-dependent virotoxin effects observed in young and adult rats following P10 $Tat_{1–86}$ exposure were specific to spatial learning. Specifically, stereotaxic injections of $Tat_{1–86}$ increased travel distance on training days 1 and 3, suggesting a deficit in acquisition, in both adolescent and adult animals. Tat treatment, however, did not alter the animal's spatial discriminability, evidenced by a significant evasion effect (Carman & Mactutus, 2002; Carman *et al.,* 2003). Notably, no dose-response effects were noted for P10 $Tat_{1–86}$ treatment on spatial memory in adolscence or adulthood. Deficits in early training acquisition were also reported in adolescent, but not adult, animals following stereotaxic injections of $Tat_{1–72}$ on P1 (Fitting *et al.,* 2008b). P1 exposure to $Tat_{1–72}$ also altered the distribution of searching behavior in adult animals, suggesting long-lasting deficits in spatial memory (Fitting *et al.,* 2008b); a deficit which was not observed following P10 $Tat_{1–86}$ exposure despite the higher doses. Given the role of the hippocampus in spatial learning and memory (O'Keefe & Nadel, 1978), as well as the effect of HIV-1 Tat on hippocampal function and anatomy (Maragos *et al.*, 2003; Fitting *et al.*, 2006a; Fitting *et al.*, 2008a; Aksenov *et al.*, 2009; Bertrand *et al.*, 2011; Bertrand *et al.*, 2013; Fitting *et al.*, 2013; Hargus & Thayer, 2013; Zucchini *et al.*, 2013; Harricharan *et al.,* 2015; Marks *et al.,* 2016), alterations in spatial learning following Tat exposure are not surprising.

The long-lasting effects of Tat on spatial learning are supported by the long-term dose-response impact of P10 $Tat_{1–86}$ exposure on the anatomical parameters of the hippocampus (Fitting *et al.*, 2008a). $Tat_{1–86}$ treatment increased numbers of astrocytes in the DGH and SUB and numbers of oligodendrocytes in the DGH in a linear dose-dependent manner. No significant Tat dose-response effects were noted on the estimated number of neurons in any of the five hippocampal subfields (Fitting *et al.,* 2010). In sharp contrast, a decrease in neuron numbers in the DGH and the CA2/3 subfield were reported following Tat exposure on P1 (Fitting *et al.,* 2008a). Similarly, a previous study in Tat transgenic mice indicated that Tat exposure later in life caused a variety of different inclusions in astrocytes characteristic

of lysosomes, autophagic vacuoles, and lamellar bodies with minimal dendritic pathology and no evidence of pyramidal neuron death (Fitting *et al.*, 2013). Astrogliosis has been frequently reported in pediatric HIV-1 infection, evidenced by the presence of viral proteins/genes in some astrocytes (Saito *et al.*, 1994; Tornatore *et al.*, 1994), white matter damage (Hoare *et al.*, 2015; Ackermann *et al.,* 2016; Cohen *et al.,* 2016), and/or increases in macrophages and microglia (Vallat *et al.*, 1998). Perhaps most interestingly, regression analyses revealed a significant brain-behavior relationship between virotoxin-induced alterations in spatial learning and/or memory with cell numbers in the DGH; a relationship similar to the one observed following P1 virotoxin exposure (Fitting *et al.,* 2008b). Specifically, the present study demonstrated that the estimated number of neurons and astrocytes in the DGH explained 70% variance of the searching behavior in the MWM acquisition training for adolescents and 65% variance of the searching behavior in the MWM acquisition training for adults. HIV-1 viral protein exposure at P1 indicated that the estimated number of neurons and astrocytes in the DGH explained 81% variance of the distribution in searching behavior in the adult MWM probe test (Fitting *et al.*, 2008b). Thus, the results from both studies suggest that spatial learning and/or memory deficits following HIV-1 viral protein exposure may be due to increased gliosis and/or neuronal loss in the DGH. Previous work has found associations between the DGH and spatial learning and/or memory in multiple disease models, (Zhang *et al.*, 2011; Martinez-Canabal *et al.*, 2013; Cahill *et al.*, 2014; Piazza *et al.*, 2014; Taylor *et al.*, 2015; Zerwas *et al.*, 2015), supporting the findings of the present study.

Dopamine (DA) system dysfunction, observed in both clinical (e.g., Wang *et al.,* 2004; Chang *et al.,* 2008; Kumar *et al.,* 2009; Lee *et al.,* 2014) and preclinical studies (e.g., Webb *et al.,* 2010; Moran *et al.,* 2012; Reid *et al.,* 2016a), may also underlie alterations in spatial learning. Specifically, alterations in the striatal DA system have been reported in pediatric HIV-1 research (Webb *et al.,* 2009; Fitting *et al.,* 2015). Children with pediatric HIV-1 also exhibited improvements in motor function following treatment with levodopa, a DA agonist, suggesting the relevance of the DA system in pediatric HIV-1 (Mintz *et al.,* 1996). The HIV-1 transgenic (Tg) rat, which expresses 7 of the 9 HIV-1 genes (a *gag-pol* deletion renders them noninfectious; Reid *et al.*, 2001) has been used to examine the developmental effects of HIV-1 and its impact on the DA system (Webb *et al.*, 2010; Moran *et al.*, 2012). Findings revealed a significant increase in phosphorylated tyrosine hydroxylase protein expression and a decrease in dopamine transporter (DAT) mRNA, without changes in tyrosine hydroxylase or DAT protein in the HIV-1 transgenic rat midbrain, suggesting selective vulnerability of the DA system in developing brains to HIV-1 infection (Webb *et al.*, 2010). Additionally, HIV-1 Tg rats assessed throughout the lifespan in neurocognitive tasks tapping the DA system, exhibited significant alterations in temporal processing (Moran *et al.,* 2013a; McLaurin *et al.,* 2016; McLaurin *et al.,* 2017a; McLaurin *et al.,* 2017b; McLaurin *et al.,* 2017c; McLaurin *et al.,* 2017d), habituation (Chang & Vigorito, 2006; Moran *et al.,* 2013b; Reid *et al.,* 2016b; McLaurin *et al.,* 2017d), sustained attention (Moran *et al.,* 2014b), working memory (Repunte-Canonigo *et al.,* 2014) and spatial learning (Vigorito *et al.,* 2007; Lashomb *et al.,* 2009).

Results of the present study, following P10 HIV-1 protein delivery, are in sharp contrast to the deficits observed following P1 HIV-1 protein delivery (Fitting *et al.,* 2008a; Fitting *et al.,*

2008b), suggesting the importance of timing in the presence of neurocognitive impairments observed in pediatric HIV-1. Specifically, virotoxin exposure on P10 resulted in dose-dependent-induced deficits for $Tat_{1–86}$ in spatial learning, while HIV-1 protein exposure on P1 resulted in significant alterations in most of the neurobehavioral and neurocognitive assessments (i.e., righting reflex, prepulse inhibition, locomotor activity, spatial memory). A comparative summary of findings in the present P10 study and the previous P1 study is illustrated in Table 4. In preweanling HIV-1 Tg rats, which express viral proteins constitutively throughout development, neurocognitive assessments revealed significant alterations in both prepulse inhibition and locomotor activity (McLaurin *et al.,* 2017a); neurocognitive deficits which were similar to those reported following early viral protein exposure (P1; Fitting *et al.,* 2008b; Moran *et al.,* 2014a). Thus, collectively, these studies provide strong evidence for the effect of timing on the development of neurocognitive impairment, with early viral protein exposure (P1, HIV-1 Tg rat) having a more deleterious effects on the developing CNS than late viral protein exposure (P10).

The rationale for using P1 (Fitting *et al.,* 2008a; Fitting *et al.,* 2008b) and P10 CNS injections as the virotoxin exposure period was based on the human brain developmental perspective, providing an opportunity to extrapolate findings to HIV-1 infected children. The period of rapid brain growth, putatively known as the ‘brain growth spurt’, reflects a period of enhanced vulnerability to nutritional and other growth restriction (Dobbing & Sands, 1979). Timing of the brain growth spurt is different in relation to birth across species and thus, is one of the major factors to be taken into account when any attempt is made to extrapolate results obtained in one species to another (Dobbing & Sands, 1979, see Figure 8). Brain growth velocity curves (Figure 8A) compare the brain growth spurt in humans and rats. Based on the rate of brain development, P10 exposure mimics clinical HIV-1 CNS infection at labor/delivery, as brain growth of P10 rats more closely matches that of human brain growth at birth (i.e., late virotoxin exposure). In sharp contrast, stereotaxic injections at P1, as in Fitting *et al.,* 2008b, mimics clinical HIV-1 CNS infection in pregnancy. Specifically, HIV-1 viral protein exposure at P1 mimics *in utero* infection occurring as the placenta begins to separate from the uterine wall (i.e., early virotoxin exposure).

Further specification of construct validity may be made at the level of hippocampal development (Figure 8B). In both rats and humans the development of the hippocampus, and specifically the development of different regions of the hippocampus, occurs at different time periods in brain maturation (Bayer *et al.*, 1993). Neurons in the subiculum and in the CA1/3 subfields are estimated to be generated from the late 6^{th} week through the 14^{th} week of development in humans, equivalent to embryonic days (E)15 – E20 in rats. In contrast, the granule cells in the dentate gyrus are generated at a time point exceptionally late in development. Approximately 85% of these cells are estimated to be generated from the 12^{th} week up to birth and possibly beyond in humans (in rats: after birth, mainly during the first two postnatal weeks, P1 – P13; Bayer *et al.*, 1993). Thus, from a hippocampal development point of view, stereotaxic injections of HIV-1 viral proteins at P1 interferes with the development of dentate granule cells. In sharp contrast, by P10, most of these cells would already be generated.

Behavioral and anatomical findings provide strong evidence for the role of timing in the development of CNS disease in HIV-1 infected children. Late viral protein exposure (P10), occurring at the peak of brain growth in rats, selectively altered spatial learning and memory in adolescence and adulthood. In sharp contrast, early viral protein exposure (P1), modeled in our previous study (Fitting *et al.,* 2008b), preceded the postnatal increase in the rate of brain growth and had more deleterious effects to the developing CNS. Thus, timing may be a critical factor involved in disease progression, with children infected with HIV earlier in life being more vulnerable to CNS disease.

4. EXPERIMENTAL PROCEDURE

4.1 Animals

Pregnant dams (Sprague-Dawley, Harlan Laboratories Inc., Indianapolis, IN; n=8) were delivered to the animal vivarian prior to embryonic day seven. Dams were individually housed with food (Pro-Lab Rat, Mouse Hamster Chow #3000, NIH diet #31) and water available *ad libitum*. P0 was designated as the day pups were found in the cage. The next day (i.e., P1), litters were culled to 10 offspring with a male: female distribution of 7:3. Seven treatment conditions were used in the experimental design to allow for simultaneous dose-response assessment of both gp120 and Tat. To control for independence of observations, one rat from each litter was represented in each treatment group (i.e., seven male rats per litter). On P21, animals were weaned and separated by sex. On P28, animals were pair- or group- housed with their littermates for the duration of experimentation. Target conditions for the animal vivarium were: 20 ± 2 °C, 50 ± 10 % relative humidity and a 12 h light: 12 h dark cycle with lights on at 0700 h (EST). Guidelines established by the National Institutes of Health (NIH) were used to keep animals in AALAC-accredited facilities. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina, Columbia (Federal Assurance: # A3049–01).

4.2 HIV-1 Proteins

Commercially available recombinant biologically active full-length $Tat_{1–86}$ (Diatheva s.r.l., Italy) was used for the Tat treatment condition. Purified gp120 LAV (T-tropic; Protein Sciences Corp., Meriden, CT) was used for the gp120 treatment condition. Doses of both Tat and gp120 were chosen based on previously established dose-response studies (Tat: Aksenov *et al.,* 2001; gp120: Fitting *et al.,* 2006b; Fitting *et al.,* 2007) and to bracket those used to examine the effect of intrahippocampal injections on P1 (Fitting *et al.*, 2008b). Thus, the design paralleled the previous P1 study and provided the opportunity to capture potential lower or higher toxicity of P10 injections.

4.3 Surgical Techniques and Protein Treatment

Tat, gp120 and vehicle treatments were delivered using standard stereotaxic surgery techniques. Modifications to standard procedures were made for neonates. At P10, pups were individually removed from the dam. Pups were anesthetized using sevofluorane (Abbott Laboratories, Inc.) in 3% oxygen and placed in a stereotaxic mold modified for surgery of neonates (Kopf, Inc). Throughout the duration of the surgery, pups were maintained in an anesthetic state using sevofluorane, which was delivered through a nose

cone. A heating pad was used to maintain body temperature at ~37 °C. The skull was held in place using rubber head bars while bilateral microinjections were made directly into the hippocampus using stereotaxic coordinates for the left and right hippocampus (2.3 mm posterior to the bregma, ±1.0 mm lateral to bregma, −2.5 mm dorsal from dura) and a microsyringe (Hamilton Co., Nevada, USA). Injection coordinates used in the present study were confirmed during pilot work with Nissl-stained sections through the hippocampus. An injection volume of 2.0 μl, a higher volume dose due to solvability issues, was released over two-min after a one-min resting period that allowed the tissue to return to its original conformation. Intrahippocampal bilateral injections were made with one of the seven protein treatments: sterile buffer [vehicle (VEH); 10 mM Tris HCl, 300 nM NaCl, ph = 7.58, sterile)], one of the three gp120 doses: 20 ng (low), 100 ng (medium), 200 ng (high), or one of the three Tat doses: 5 μg (low), 25 μg (medium), 50 μg (high). The injection needle was withdrawn over two-min to prevent reflux. Surgical glue and surgical polypropylene sutures (Ethican, Inc.) were used to close skin piercings in the head. Animals recovered from anesthesia in a chamber on a heating pad (~37 °C) prior to being returned to the dam. Pups were closely monitored for any indications of rejection, which were not observed.

4.4 Experimental Design

Seven male rats per litter ($n = 8$) were randomly selected and assigned to their treatment groups (i.e., VEH; low-, medium-, high gp120; and low-, medium-, high Tat) and received bilateral hippocampal injections on P10. Assessments of somatic growth, including body weight and eye opening, began at P10. Specifically, all rats were weighed at each of the specific test days and eye opening was assessed on P13 – P17. Neurobehavioral assessments began on P18. All rats were tested for (1) sensorimotor gating, indexed by PPI of the acoustic startle response (ASR) prior to weaning (P18) and in adulthood (P91); (2) locomotor activity, an index of motor function, in weanling rats (P22 – P24) and in adulthood (P94 – P96); and (3) spatial learning and memory, indexed by performance in the MWM (P46 – P51 and P104 – P109). Procedures used for neurocognitive testing followed a similar procedure as reported in the previous P1 study (Fitting *et al.*, 2008b), but will be outlined briefly in the following sections.

4.5 Somatic Growth

In the pre-cART era, HIV-1 infected children exhibited slower growth relative to HIV-1 seronegative children; differences which were exacerbated after the second year of life (Newell *et al.*, 2003). However, findings in the post-cART era suggest that HIV-infected children on antiviral prophylaxis treatments displayed a significant improvement in growth parameters (Lodha *et al.*, 2005; Guillen *et al.,* 2007; Parachure *et al.,* 2015). To examine the specific effect of HIV-1 proteins on developmental milestones, somatic growth was assessed by body weight and eye opening.

4.5.1 Body Weight—Pups were weighed on a weekly basis starting on P10, as well as on the days animals were tested.

4.5.2 Eye Opening—A second index of somatic growth, eye opening, was assessed from P13 – P16. The right and left eyes were assessed separately between 0800 – 1000 h (EST)

and coded as ‘eye not open’ (0), ‘slit’ (1), ‘half-open’ (2) and ‘eye open’ (3). Rank scores, with a maximum of 6 and a minimum of 0, were calculated by collapsing across P13--P16 to examine the frequency of eye opening.

4.6 Sensorimotor Function (PPI of the ASR)

PPI of the ASR is a translational experimental technique commonly used to examine preattentive processes, which may underlie deficits in higher-order cognitive functions. Significant alterations in PPI were observed following stereotaxic injections of HIV-1 viral proteins at P1 (Fitting *et al.,* 2008b; Moran *et al.,* 2014a) and in preweanling and adult HIV-1 Tg rats (e.g., Moran *et al.,* 2013a; McLaurin *et al.,* 2017a; McLaurin *et al.,* 2017b; McLaurin *et al.,* 2017c; McLaurin *et al.,* 2017d). PPI also revealed alterations in sensorimotor gating in HIV-1 seropositive adults (Minassian *et al.,* 2013). In the present study, auditory PPI was used to determine whether timing (i.e., P10) and dose of HIV-1 viral proteins altered preattentive processes. All rats were assessed as preweanlings on P18 and as adults on P91.

4.6.1 Apparatus—A 10 cm thick double-walled, 81 × 81 × 116 cm isolation cabinet (external dimensions; Industrial Acoustic Company, Inc., Bronx, NY) enclosed the startle chamber (SR-Lab Startle Reflex System, San Diego Instruments, Inc.). All assessments were conducted individually in the dark. A high-frequency loudspeaker, which was affixed in the chamber 30 cm above the Plexiglas cylinder, delivered all auditory prepulse and startle stimuli. A Plexiglas cylinder (preweanling rats: 3.9 cm internal diameter; adult rats: 8.75 cm internal diameter), resting on a 12.5 × 20 cm Plexiglas stand, was deflected by the animal’s response to the startle stimulus. Deflections were converted into an analog signal by a piezoelectric accelerometer integral to the bottom of the cylinder. Both the startle stimuli (100 dB(A)) and auditory prepulse stimuli (85 dB(A)) were 20 msec in duration. A SR-Lab Startle Calibration System was used to calibrate acoustic stimulus intensities and response sensitivities. A sound level meter (model #2203, Bruël & Kjaer, Norcross, GA) was used to measure and calibrate sound levels with the microphone placed inside the Plexiglas cylinder. The signals were then digitized (12 bit A to D) and saved to a hard disk on a Pentium class computer.

4.6.2 Testing Procedures—Animals were assessed for auditory PPI using a 20-min test session, which began with a five-min acclimation period, including 70 dB(A) background white noise. The test session began with six adaptation trials, which included pulse only ASR-trials. Subsequently, 36 PPI trials, with interstimulus intervals (ISIs) of 0, 8, 40, 80, 120, and 4000 msec were presented according to a Latin-square design. Control trials (i.e., 0 and 4000 msec ISIs) provided the baseline ASR within the PPI test session. The dependent measures analyzed were (1) the baseline ASR indexed by control trials, (2) percent inhibition of the PPI trials (8–120 msec ISIs) relative to the baseline ASR (control trials), and (3) ISI in which the maximal peak response inhibition occurred across the PPI trials (8 – 120 msec ISIs).

4.7 Locomotor Activity

Significant alterations in motor development have been frequently reported both in the pre- (Boivin *et al.*, 1995; Baillieu & Potterton, 2008) and post-cART eras (Foster *et al.,* 2006; Ferguson & Jelsma, 2009; Whitehead *et al.,* 2014). To test whether HIV-1 proteins themselves induce effects on motor activity, locomotor activity was assessed in weanlings and adults for 60-min on three consecutive days, at 21, 22, and 23 days of age and 94, 95 and 96 days of age, respectively.

4.7.1 Apparatus—The square (40 × 40 cm) activity monitors (Hamilton-Kinder Inc., Poway, CA) were converted into round (~ 40 cm diameter) compartments by adding clear perspex inserts. An infrared photocell grid (32 emitter/detector pairs), tuned by the manufacturer to handle the extra Perspex width, detected the animal's free movement. All activity monitors were located in an isolated room. A 60-min test session was conducted between 1500 – 1700 h (EST) under dim light conditions, in the absence of direct overhead lighting (< 10 l×). The dependent variable was the animal's total activity (i.e., the sum of the recorded basic movements and x and y ambulation).

4.8 Spatial Learning and Memory

Despite antiretroviral prophylactic treatment, HIV-1-infected children exhibit high rates of chronic neurological impairment, including neurodevelopmental delays (Franklin *et al.,* 2005; review, Van Rie *et al.,* 2007; Baillieu & Potterton, 2008; Paramesparan *et al.,* 2010). Neurocognitive assessments in HIV-1-infected children have revealed significant deficits in memory, including visuospatial working memory (Boivin *et al.,* 1995; Koekkoek *et al.,* 2008). Previous preclinical assessments, using adult HIV-1 Tg rats, also provide evidence for alterations in spatial learning and memory (Vigorito *et al.,* 2007; Lashomb *et al.,* 2009). Thus, animals were tested in the Morris water maze, to assess spatial learning and memory, as both adolescents and adults over four consecutive days between 49 – 55 days of age and 113 –121 days of age, respectively.

4.8.1 Apparatus—Spatial learning and memory was assessed using the Morris water maze, an aluminum tank measuring 1.8 m in diameter by 0.60 m high. The water depth inside the Morris water maze was 0.40 m. The temperature of the water was maintained at 28 ± 1 °C throughout experimentation. A 10 × 10 cm escape platform was located 2.5 cm below the surface of the water level on a radius of 45 cm in one of the four locations within the north (N), south (S), east (E), or west (W) arbitrarily designated quadrants. Two sets of cues, including four distinct background curtains and 12 objects outside the tank perimeter, surrounded the tank, which was located in a 3 × 3 m room. The four background curtains were: solid navy (north), solid white (south), vertical navy and white stripes (7.6 cm wide; east), and horizontal navy and white stripes (7.6 cm wide; west) Four hanging objects, in a variety of sizes (i.e., from a 20 cm coffee can to a 60 cm mailbox) were suspended from the ceiling. Eight objects, including circles and quadrangles, were placed on the curtains. The starting points used were NW, NE, SE, SW and varied by using two different entry points diagonal to each other in a counterbalanced order, ABBAABBA. Swimming behavior was recorded using a closed circuit video system, which was recessed mounted in the ceiling above the center of the pool.

4.8.2 Procedure—Acquisition training consisted of a total of 20 training trials across 3 days (Day 1: 8 trials; Day 2: 8 Trials; Day 3: 4 Trials). Throughout the duration of training, the escape platform was located in a fixed location for each subject; however, platform location (N,S,E or W) was balanced across subjects. A different platform location was used for training in adolescence and adulthood. The rat was placed into the pool using one of the consecutive entry points, at which point the training trial began. Swimming behavior was recorded for 60 sec, at which point the trial was terminated. If an animal failed to find the platform within the allotted time (i.e., 60 sec), the animal was directed to the escape platform. No animal was removed directly from the pool, only from the platform. Animals were allowed to remain on the platform for 15 sec, at which point they were returned to a holding cage. Due to the high correlation between escape latency and travel distance ($r = 0.96$, $p \leq 0.001$), only travel distance is reported.

Following the last acquisition trial on day 3, a probe test, which involved removal of the escape platform, was conducted. The visual-spatial environment was not altered during the probe test. The probe test was conducted like the acquisition training trials, with swimming behavior recorded for 60 sec prior to terminating the trial. However, a novel (i.e., not used in training) starting position was used. At the termination of the trial, the escape platform was placed in the pool. Animals were allowed to find the escape platform and remain on it for 15 sec.

For the probe test, quadrant preference (i.e., the relative distribution of swimming time in each of the quadrants) provided an index of the animal's spatial discriminability. To analyze quadrant preference, the target and opposite quadrant areas were compared. An evasion measure (Carman & Mactutus, 2002, Carman *et al.,* 2003) provided an additional comparison, by examining the opposite and adjacent quadrant areas. Both quadrant preference and the evasion measure provide an index of spatial discriminability. In the quadrant preference measure, discriminability is evidenced by spending more time in the target quadrant relative to the opposite quadrant (i.e., rats have learned where to go in the pool). In sharp contrast, in the evasion measure, discriminability is evidenced by animals learning where not to go in the pool. Thus, the probe test assesses the rat's more 'implicit' knowledge of the escape platform location.

4.9 Stereology

Stereology methods used to quantify total neuron number and glial estimates in the five hippocampal cellular layers (i.e., granular layer (GL), DGH, cornu ammonis fields (CA)2/3. CA1, and subiculum (SUB)) were reported in Fitting *et al.* (2010). In brief, brain tissue was sectioned into 50 μm thick slices using a microtome cryostat (Microm HM500M, Walldorf, Germany). Cresyl violet (Nissl) staining [1 g cresyl violet acetate (Sigma, St. Lous, MO) in 400 ml distilled water (pH 4.0)] was employed on every 6^{th} section in the series as a method of systematic-random sampling. Neurons were identified morphologically and defined by features including a larger, pale nuclei surrounded by darkly stained cytoplasm containing Nissl bodies. Astrocytes and oligodendrocytes were also identified morphologically in the DGH and SUB.

A Nikon eclipse E800 microscope (Nikon, Melville, NY) equipped with a motorized LEP MAC 5,000 XYZ stage controller (Ludl Electronic Products, NY) and StereoInvestigator software 7.0 (Microbrightfield, Williston, VT) was used for stereology. Unbiased estimates of the total number of neurons and astrocytes were made using the optical fractionator, a stereological technique (West et al., 1991; Gundersen *et al.,* 1999). Cells were counted in a known fraction of the five different hippocampal subregions using a sampling scheme for the optical fractionator. Based on the sample from a known fraction of the entire hippocampus (fractionator sampling), an estimate of the total number of neurons and astrocytes was obtained (West *et al.,* 1991).

4.10 Data Analysis

As appropriate, the data were expressed as mean (± *S.E.M.*) or cumulative frequency (%). Separate analyses were conducted for the dose-response groups of Tat and gp120. The VEH group was used as the common vehicle control group. All statistical analyses were completed using SYSTAT 11.0 (for Windows, SYSTAT Inc.). An alpha level of $p \leq 0.05$ was considered significant for all statistical tests.

Analysis of variance (ANOVA) techniques were used to analyze continuous data (i.e., body weight, locomotor activity, Morris water maze). Either Tat dose treatment (4 levels) or gp120 dose treatment (4 levels) was included as the between-subjects factor. Within-subjects terms (i.e., day, trial) were included in the analyses as appropriate. Orthogonal decompositions were preferentially used to handle potential violations of sphericity (Winer, 1971). Specific planned contrasts were employed to answer three basic questions: *First*, whether a Tat (or gp120) effect exists (overall treatment effect)? In other words, comparison of VEH vs. Tat or gp120 dose groups. *Second,* whether there was a Tat (or gp120) dose-response effect (dose-dependent treatment effect)? In other words, were specific dose-dependent treatment effects for Tat or gp120 revealed by orthogonal component analyses. *Third*, what was the threshold dose for the Tat (or gp120) effect? To answer this question, planned contrasts were performed by comparing VEH-treated animals with each treatment condition of Tat or gp120.

Two non-parametric tests, the Kruskal-Wallis Test and the Mann-Whitney U-Test, were performed on the rank scores of the somatic growth index eye opening for the dose response groups of gp120 treatment or Tat treatment. For categorical data, such as the interstimulus interval (ISI) data in the PPI test, the Fisher's exact test was applied.

To examine the relationship between the behavioral measures and the total number of cells in the five subregions of the rat hippocampus, we employed correlation and multiple regression analyses. The purpose of these analyses was to determine if observed changes in the estimated total number of cells in specific hippocampal areas (Fitting *et al.*, 2010) were significantly predictive of behavioral outcome.

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Highlights

- P10 viral protein exposure was best characterized as having task specific effects
- Spatial learning deficits were observed following P10 HIV-1 viral protein exposure
- DGH neuron and astrocyte number explained 65-70% of the variance in MWM acquisition
- Timing of infection may be a critical factor in progression of pediatric HIV-1

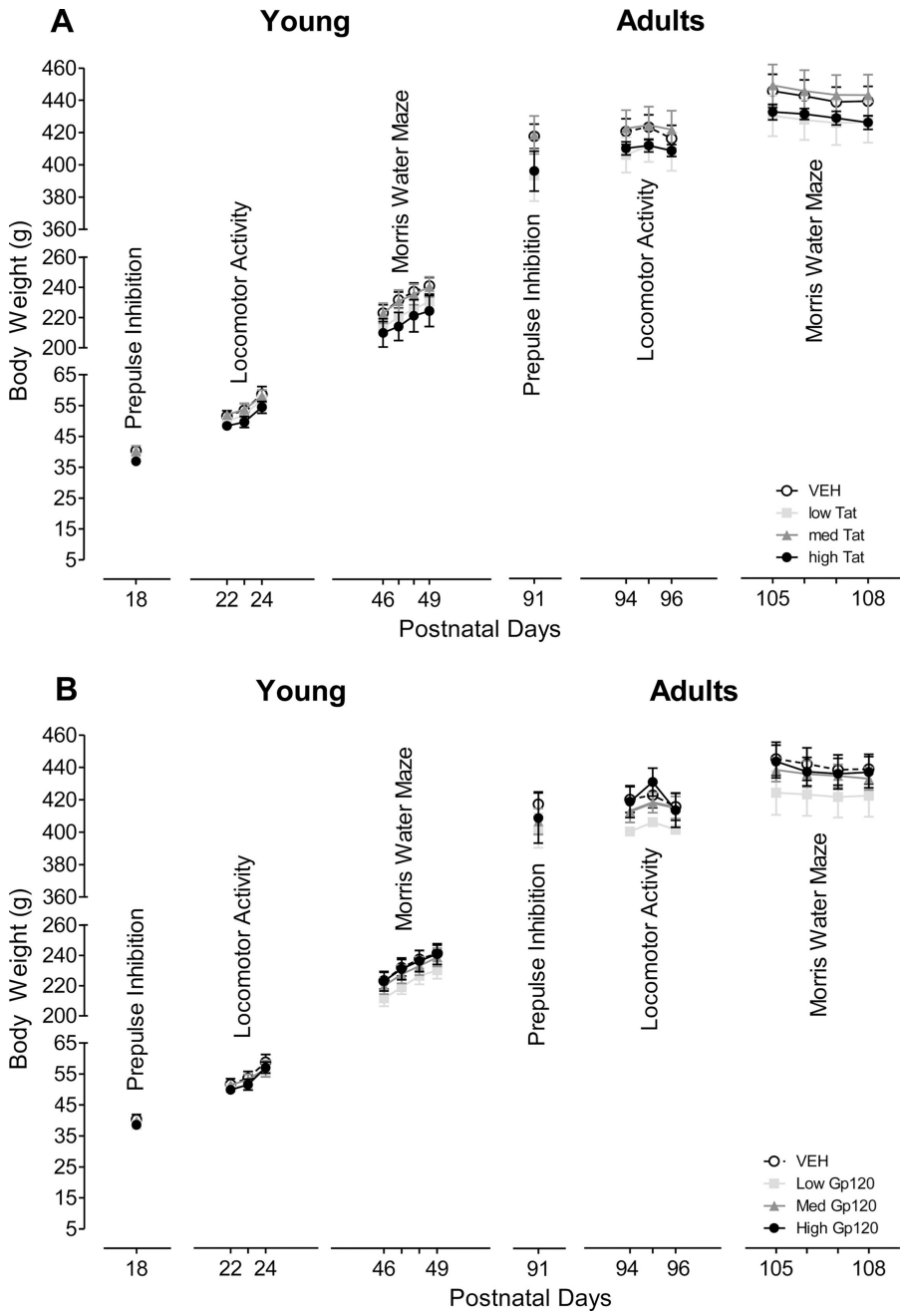

Figure 1.

Mean (± *S.E.M*) body weight across the various test days for HIV-1 proteins (**A**) Tat dose treatment and (**B**) gp120 dose treatment. No Tat or gp120 treatment effects were noted at any test set/day.

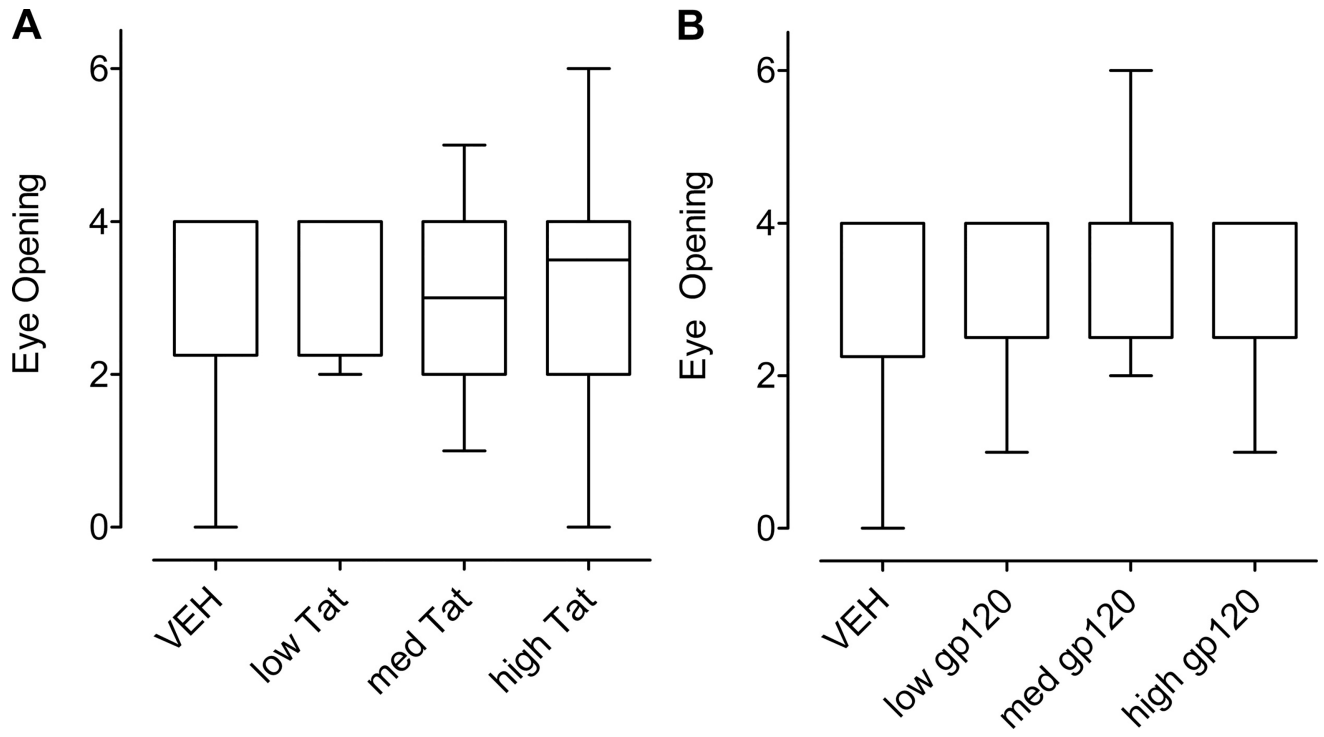

Figure 2.

Median (± *Interquartile Range*) eye opening scores collapsed across P13 – P16 for HIV-1 proteins (**A**) Tat dose treatment and (**B**) gp120dose treatment. No significant treatment effects were noted for either the gp120- or Tat treatments.

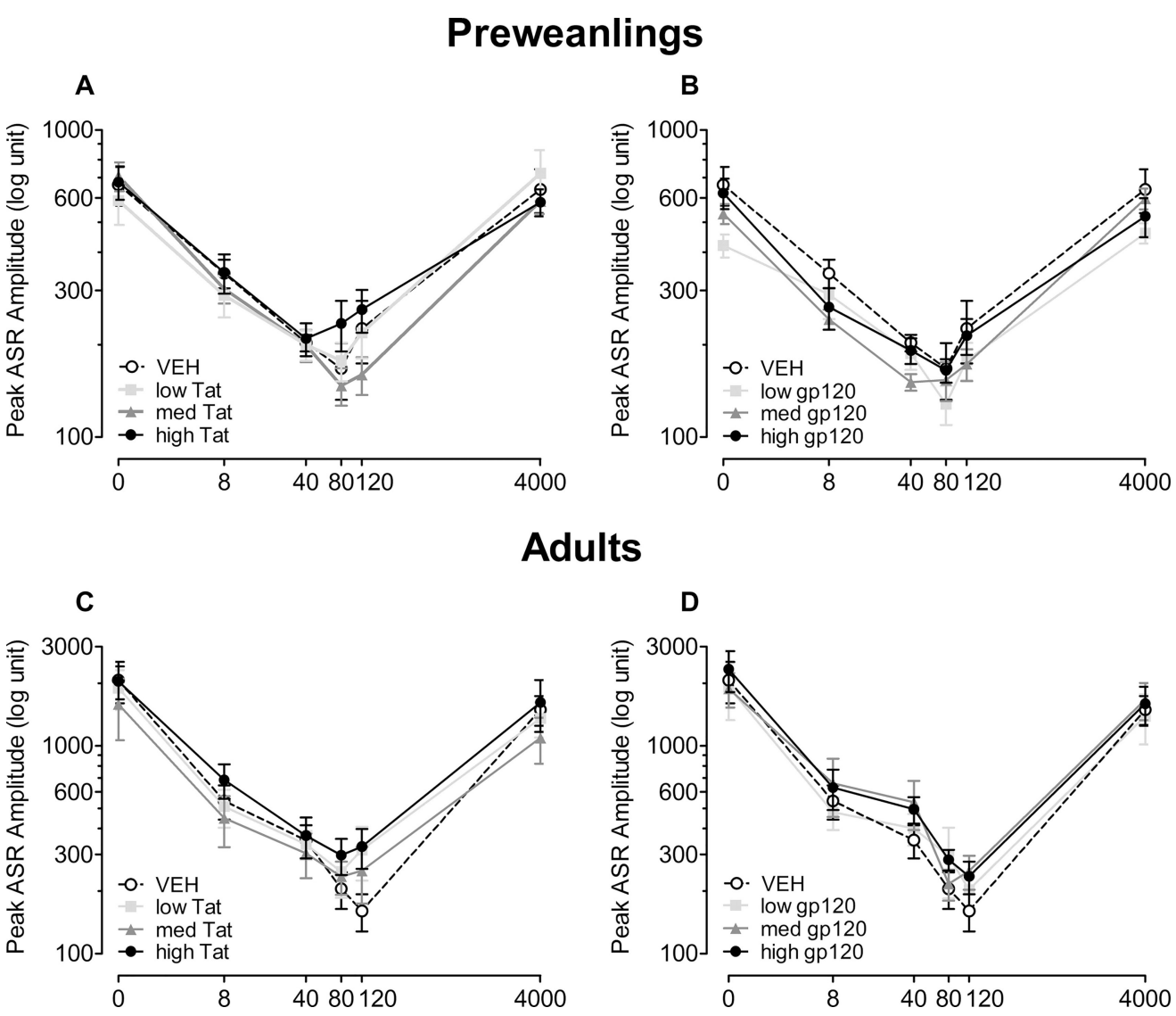

Figure 3.

Peak ASR amplitude data (± *S.E.M.*) across ISI (0–4000 msec) for preweanlings (Tat (**A**) and gp120 (**B**)) and adults (Tat (**C**) and gp120 (**D**)). No significant treatment effect and/or interaction was noted at either age.

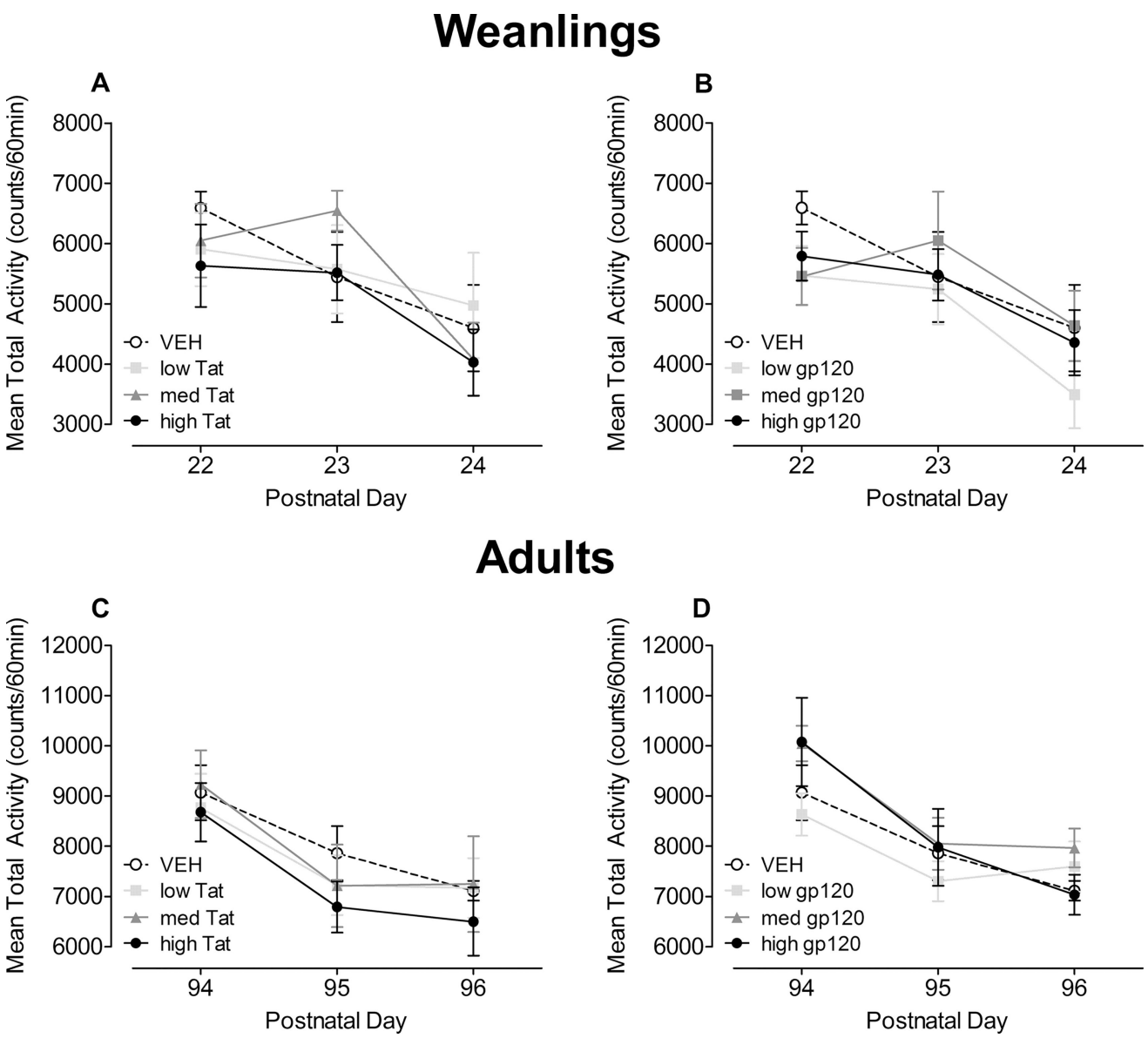

Figure 4.

Mean *(± S.E.M)* total activity across the three test days in weanlings at P22 – P24 (Tat (**A**) and gp120 (**B**)) and adults at P94 – P96 (Tat (**C**) and gp120 (**D**)). For both ages, neither a significant treatment nor treatment by day interaction was noted.

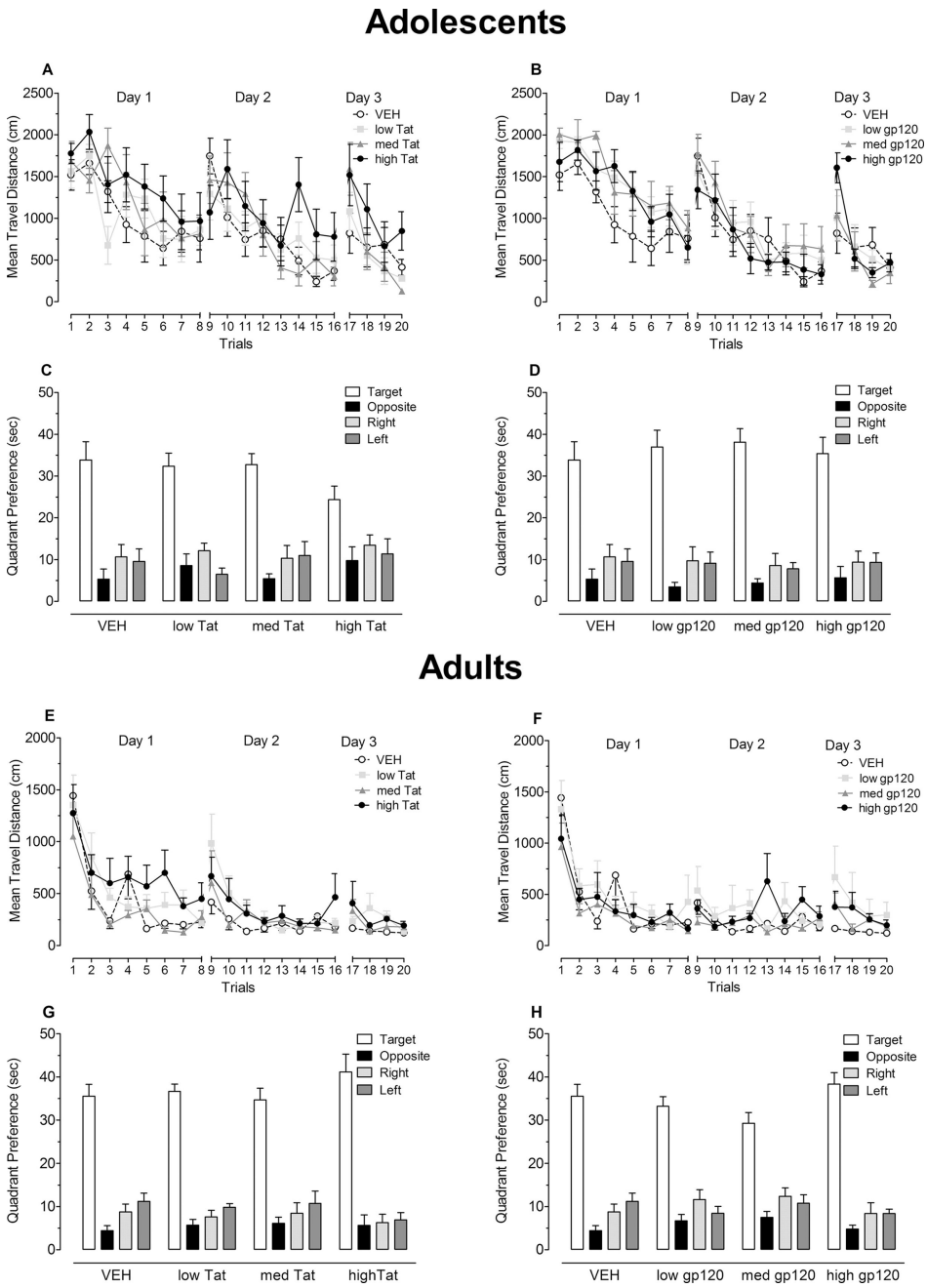

Figure 5.

Acquisition training and probe test assessments for adolescents, at P46 – P51 (Tat(**A, C**) and gp120(**B, D**)), and adults, at P104 – P109 ((Tat (**E, G**) and gp120 (**F, H**)). Mean (± *S.E.M.*) travel distance in acquisition training is illustrated in panels A and E for Tat dose treatment and panels B and F for gp120 dose treatment. Significant treatment effects were noted for Tat dose treatment on days 1 and 3 in both adolescence and adulthood. Mean (± *S.E.M.*) time spent in the four quadrants during the probe test is illustrated in panels C and G for Tat dose treatment and panels D and H for gp120 dose treatment. Despite significant

discrimination and evasion effects, planned contrast analyses revealed no overall or dose-dependent treatment effects for either Tat or GP120 treatment.

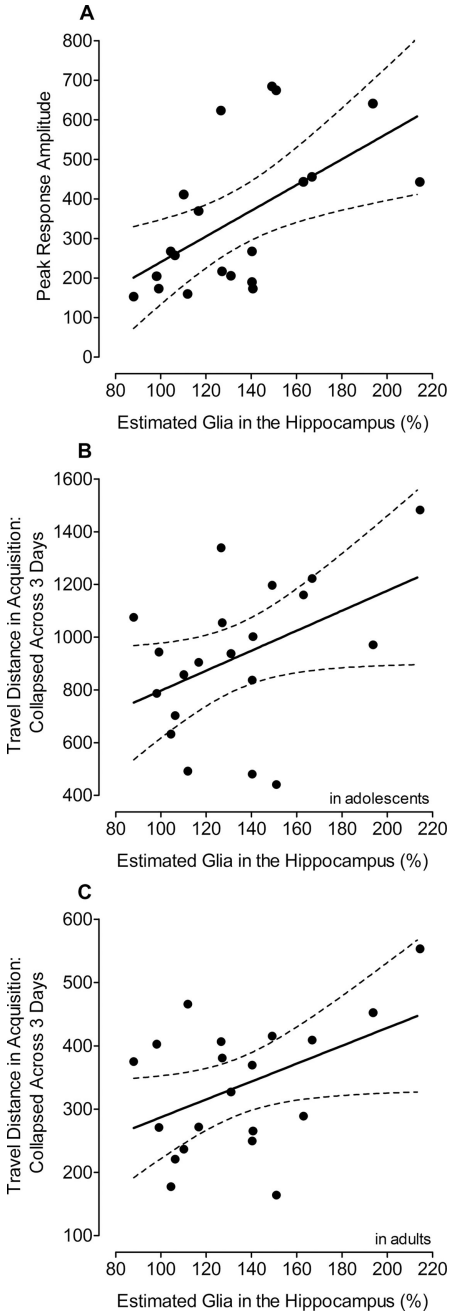

Figure 6.

Simple linear regression models indicating the relationship between, (**A**) peak response amplitude in sensorimotor gating and % glia in the hippocampus, (**B**) distance traveled in acquisition training collapsed across the three training days for adolescents and % glia in the hippocampus, and (**C**) distance traveled in acquisition training collapsed across the three training days for adults and % glia in the hippocampus.

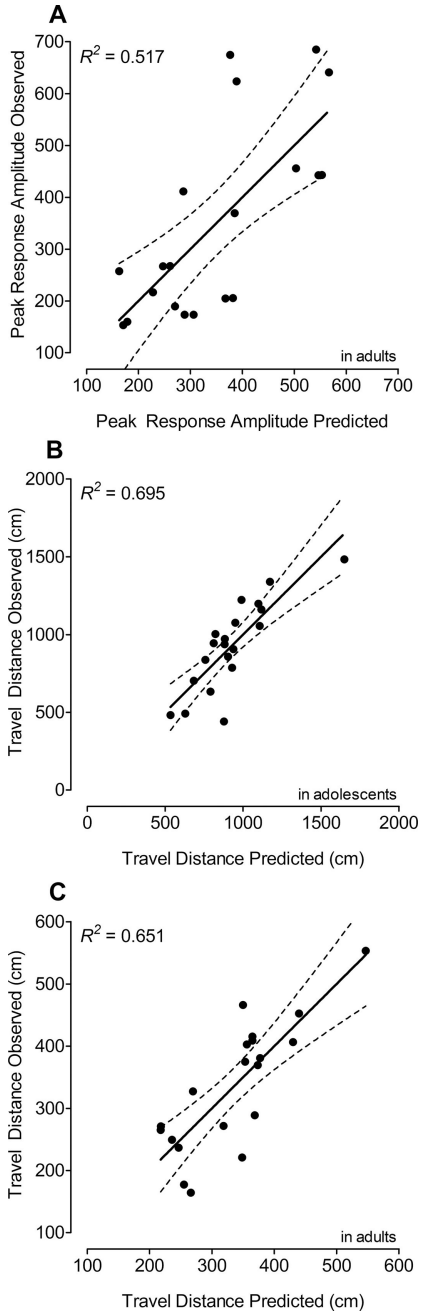

Figure 7.

Actual scores collapsed across the three assessment days illustrated on the y-axis against the predicted scores of travel distance (x-axis). (**A**) Peak response amplitude in sensorimotor gating in adults: Scores were derived from a 7-parameter model explaining 52% of the variance of the data. (**B**) Distance traveled during acquisition training in adolescents: Scores were derived from a 6-parameter model explaining 70% of the variance of the data. (**C**) Distance traveled during acquisition training in adults: Scores were derived from an 8-parameter model explaining 65% of the variance of the data.

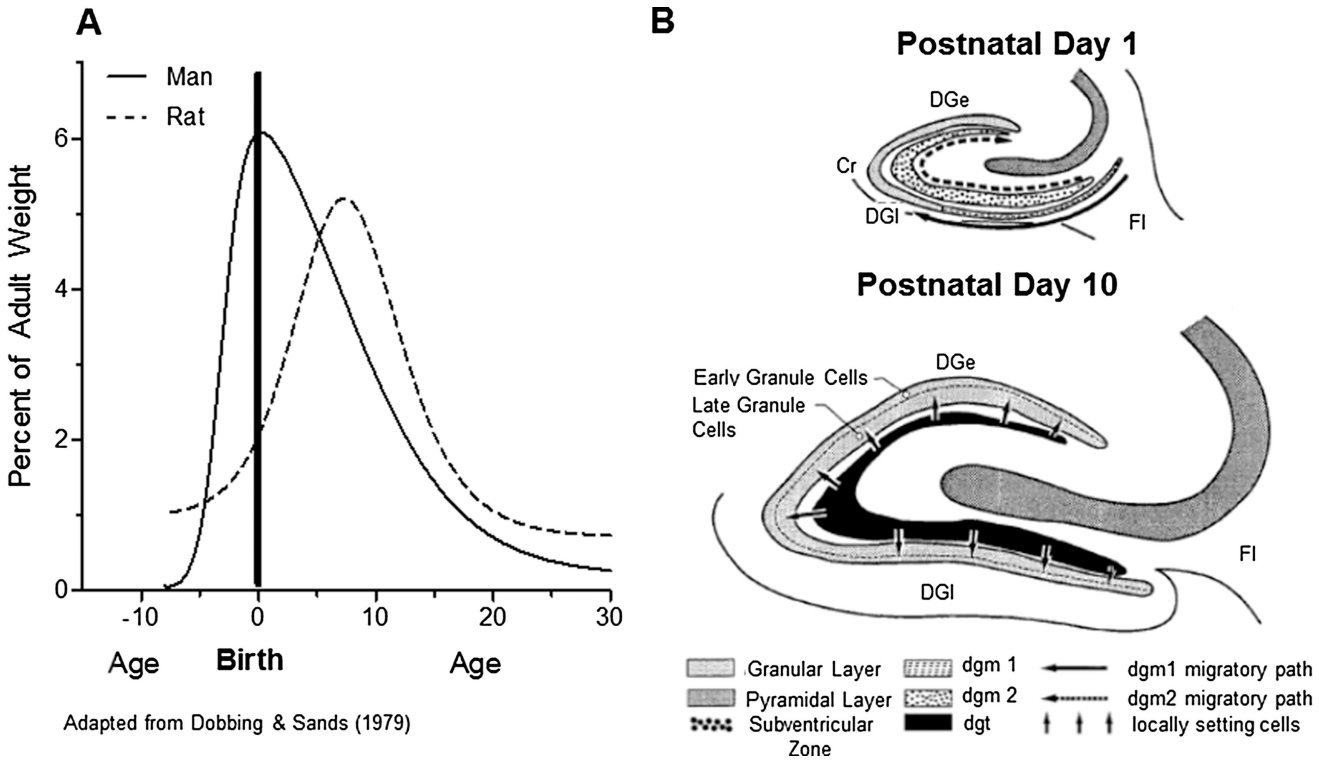

Figure 8.

(**A**) The brain growth spurts of humans and rats expressed as first-order velocity curves of the increase in weight with age. The units of time for humans are in month and for rats in days. Rates are expressed as weight gain as a percentage of adult weight for each unit of time. (**B**) Summary diagram of the morphogenesis of the dentate granular layer.

Table 1

Pearson Intercorrelation Matrix between the Different Behavioral Measures and Percent Cell Number from the Mean of the VEH-treated Animals for the Five Hippocampal Subfields Combined (N = 20).

		Neurons (in %)	**Glia (in %)**
Eye Opening (Ranking) [+]		−0.095	0.015
Prepulse Inhibition (Amplitude)	young	0.333	0.352
	adult	0.066	0.573 **
Locomotor Activity (Counts/60 min)	young	−0.136	−0.184
	adult	−0.213	−0.344
Distance Traveled on Days 1,2, and 3	young	0.014	0.436 *
	adult	0.240	0.445 *
Distribution in searching behavior during the probe test in the MWM ▓ (in sec)	young	0.199	−0.121
	adult	−0.039	0.010

Note: MWM = Morris water maze;

** $p \leq 0.01$,

* $p \leq 0.05$,

+ Spearman Correlation

Table 2

Stepwise Multiple Regression Analyses Predicting Different Behavioral Measures Including Percent Neurons and Percent Glia As the Two Anatomical Predictors.

Behavior	Predictors	Coeff.	*S.E.M.*	t-statistics	Model Fit	R^2
PPI in adults	% glia	0.573	1.096	3.0, $p \leq 0.01$	$F(1, 18) = 8.8$, $p \leq 0.01$	33%
Travel Distance across the 3 days in adolescents	% glia	0.436	1.842	2.1, $p \leq 0.05$	$F(1, 18) = 4.2$, $p \leq 0.05$	19%
Travel Distance across the 3 days in adults	% glia	0.446	0.669	2.1, $p \leq 0.05$	$F(1, 18) = 4.5$, $p \leq 0.05$	20%

Note: MWM = Morris water maze, Coeff. = Coefficient

Table 3

Parameter Values and Fit Indices of the Parameter Models for Prepulse Inhibition and Travel Distance during Acquisition Training (Collapsed Across the Three Training Days) in Adolescence and Adulthood.

	Prepulse Inhibition Adult			**Travel Distance Adolescence Adulthood**					
Condition	b_0	b_{DGHN}	a_{DGHA}	b_0	b_{DGHN}	a_{DGHA}	b_0	b_{DGHN}	a_{DGHA}
VEH	−1698.57	0.027	0.010	3139.4	−0.022	b_{VEH}	2252.6	−0.026	−0.011
Low Dose Tat	−551.95	a_{VEH}	0.005	1444.0	b_{VEH}	$b_{Med\ Tat}$	227.0	0.001	$b_{Low\ Tat}$
Medium Dose Tat	$b_{0\ Low\ Tat}$	$a_{Low\ Tat}$	a_{VEH}	−25.3	0.008	$b_{Med\ Tat}$	−527.5	0.007	$b_{Med\ Tat}$
High Dose Tat	878.46	−0.016	$a_{Low\ Tat}$	190.5	$b_{Med\ Tat}$	$b_{Med\ Tat}$	−24.5	$b_{Med\ Tat}$	$b_{Med\ Tat}$

Note: b_0 = intercept or constant, b_{DGHN} = weight of the total number of neurons in the DGH, b_{DGHA} = weight of the total number of astrocytes in the DGH.

Table 4

Comparative Summary of Findings Following P1 Stereotaxic Injections (Fitting et al., 2008a; Fitting et al., 2008b) and P10 Stereotaxic Injections.

Neuropsychological Deficits	**Neurobehavioral Assessments**	**P1 Results**	**P10 Results**
Somatic Growth Impairments	Body Weight	No	No
	Eye Opening	Yes	No
Early Reflex	Righting Reflex	Yes	-
Developmental Deficits	Negative Geotaxis	Yes	-
Attentional Deficits	PPI in Preweanlings	No	No
	PPI in Adults	$Tat_{1–72}$ Effect	No
Motor Dysfunction	Locomotor Activity in Weanlings	Yes	No
	Locomotor Activity in Adults	Gp120 effect	No
Learning Deficits	MWM in Adolescents	Yes	$Tat_{1–86}$ Dose Effect
	MWM in Adults	No	$Tat_{1–86}$ Dose Effect
Memory Deficits	MWM in Adolescents	No	No
	MWM in Adults	$Tat_{1–72}$ Effect	No

Note: PPI = Prepulse Inhibition, MWM = Morris water maze