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Anxiety-like behavior of mice produced by conditional central expression of the HIV-1 regulatory protein, Tat

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Abstract

Rationale—Human immunodeficiency virus (HIV) infection is associated with substantial increases in generalized anxiety. The HIV regulatory protein, transactivator of transcription (Tat), has been implicated in the neuropathogenesis related to HIV-1 infection. However, direct examination of the effect of Tat on behavioral measures of anxiety has not been demonstrated.

Objective—To identify whether expression of the Tat_{1-86} protein exerts dose-dependent and persistent anxiety-like effects in a whole animal model, the GT-tg bigenic mouse.

Methods—GT-tg mice and C57BL/6J controls were administered doxycycline in a dose- (0, 50, 100, or 125 mg/kg, i.p., for 7 days) or duration- (100 mg/kg, i.p., for 0, 1, 3, 5, or 14 days) dependent manner to induce Tat_{1-86} in brain. Mice were assessed for anxiety-like behavior in an open field, social interaction, or marble burying task 0, 7, and/or 14 days later. Central expression of Tat_{1-86} protein was verified with Western blot analyses.

Results—Doxycycline produced no effects on C57BL/6J controls that lacked the Tat_{1-86} transgene. Among GT-tg mice, doxycycline (100 mg/kg for 3, 5, or 7 days) significantly increased anxiety-like behavior in all tasks, commensurate with enhanced Western blot labeling of Tat_{1-86} protein in brain, displaying optimal effects with the 7-day regimen. Greater exposure to doxycycline (either 125 mg/kg for 7 days or 100 mg/kg for 14 days) impaired locomotor behavior; whereas, lower dosing (below 100 mg/kg) produced only transient increases in anxiety-like behavior.

Conclusions—Expression of HIV-1-Tat_{1-86} in GT-tg mouse brain produces exposure-dependent, persistent increases in anxiety-like behavior.

Keywords

GT-tg bigenic; HIV-associated neurocognitive disorder; NeuroAIDS; Open Field; Marble Burying; Transactivator of Transcription; Transcriptional Activator; Strain Differences

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1. Introduction

Among patients infected with human immunodeficiency virus (HIV), combination antiretroviral therapies have greatly reduced the risk of developing acquired immunodeficiency syndrome (Cohen et al. 2011). However, HIV-positive individuals continue to contend with pervasive HIV-associated neuropsychiatric disorders, including deficits in motor coordination, learning and memory, behavioral inhibition, and affective well-being (Bing et al. 2001; Stern et al. 1991; Woods et al. 2009). Of interest, the incidence of generalized anxiety and panic disorder is substantially greater among HIV-afflicted individuals compared to those that are non-afflicted (Atkinson et al. 1988; Bing et al. 2001; Orlando et al. 2002) or patients suffering from mood disorders comorbid with other terminal illnesses, such as cancer (Massie 2004). While psychosocial factors undoubtedly contribute to HIV-related changes in mental health status (Bravo et al. 2010), biological mechanisms may play a role. Supporting this theory, the actions of viral proteins originating from CNS reservoirs (such as macrophages and microglia) are poorly controlled by systemic antiretroviral therapies (Limoges et al. 2000; Masliah et al. 2000; Mollace et al. 2001), and may influence neurological and mood-related status.

One regulatory protein associated with HIV-1 infection that may contribute to HIV-associated mood disorder is the transactivator of transcription (Tat). Tat is a multifunctional, viral regulatory protein that drives HIV transcription (Parada et al. 1999). Tat protein is present in post-mortem brain tissue of HIV-positive individuals with HIV-associated-dementia or – encephalitis (Chang et al. 2011; Hudson et al. 2000; Nath et al. 2000; Wesselingh et al. 1993; Wiley et al. 1996), and brain expression may persist despite highly active antiretroviral therapies (Agbottah et al. 2006). Functional Tat protein is secreted from HIV-1 infected monocytes and glial cells to act at membrane-bound and intracellular targets, promoting cytokine production and infiltration (Nath et al. 1991; Bruce-Keller et al. 2001), oxidative stress (Kruman et al. 1998) and neurotoxicity (Chang et al. 1997; Ensoli et al. 1990, 1993; Rayne et al. 2010). As all of these effects are associated with anxiety (Brocardo et al., 2012; Desrumaux et al. 2005; Hoge et al. 2009), these studies together suggest possible biological mechanisms by which HIV-1 Tat protein may influence neuropsychiatric and affective status. Consistent with this, exogenous intracerebroventricular administration of Tat_{1-86} enhanced central cytokine expression concurrent with an increase in depression-like behavior in BALB/c and C57BL/6J mice (Lawson et al. 2011). However, no investigations to date have examined the effect of Tat protein on anxiety, and an explicit cause and effect relationship has not been established.

In the present investigation, we examined the influence of Tat_{1-86} protein on motor and anxiety-like behavior in a whole-animal model. We and others have previously demonstrated the Tat_{1-86} protein is expressed in a doxycycline-dependent manner, selectively in the brain of GT-tg bigenic mice (Carey et al. 2012; Kim et al. 2003) and another conditional expression mouse model (Fitting et al., 2010). Expression of Tat protein in the GT-tg bigenic mouse model emulates clinical findings of HIV characterized by central macrophage/monocyte infiltration and neuronal cell death (Kim et al. 2003), cognitive impairment (Carey et al. 2012), reductions in limbic gray matter volume (Carey et al. 2013), and increases in psychostimulant reward (Paris et al. 2013). Herein, we verify that Tat_{1-86}

protein expression in the GT-tg brain is consistent with the dose and duration of doxycycline treatment, and extend these findings to investigate changes in Tat protein expression following induction. We hypothesized that expression of HIV-1 Tat_{1-86} protein in the GT-tg mouse brain would increase anxiety-like behavior in a persistent, exposure-dependent manner.

2. Methods

All work was pre-approved by the Institutional Animal Care and Use Committee at Torrey Pines Institute for Molecular Studies, and principles of laboratory animal care were conducted in accordance with U.S. ethical guidelines defined by the National Institutes of Health (NIH Publication No. 85-23) and the "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council, 2003).

2.1. Subjects and materials

2.1.1. Housing—Male GT-tg mice (n = 1,032; originally derived by J. J. He, Indiana University School of Medicine, Indianapolis, IN) were generated in our colony in the Torrey Pines Institute for Molecular Studies vivarium (Port Saint Lucie, FL). GT-tg breeders have been previously back-crossed 7 generations onto the C57BL/6J strain. Male C57BL/6J mice (n = 529) were purchased from The Jackson Laboratory (Bar Harbor, ME) as congenic, negative controls. All mice (approximately 70 days of age) were housed 4-5 / cage and were maintained in a temperature- and humidity-controlled room on a 12:12 h light / dark cycle (lights off at 19:00 h) with *ad libitum* access to food and water. Induction of the neurotoxic Tat_{1-86} transgene was associated with modest attrition rates of < 5 % for all doses/exposures reported in the present experimental series with the exception of the 125 mg/kg/day dose for 7 days regimen (where attrition was ~13%) and the 100 mg/kg/day dose for 14 days regimen (where attrition was ~22%). No doxycycline-related attrition was observed among C57BL/6J control mice.

2.1.2. Chemicals—Doxycycline hyclate (Sigma-Aldrich, St. Louis, MO), was dissolved in sterile 0.9% saline and diluted to concentration (0.1 ml volume administered per 10 g body weight).

2.2. Western blot assays

Full characterization of the dose- and duration-dependent effects of doxycycline treatment on central Tat_{1-86} protein expression in the GT-tg mouse brain with Western blot analysis was previously described (Carey et al. 2012). The effects of dose (25 – 125 mg/kg, i.p.) and duration of doxycycline treatment (1 – 14 days) on Tat protein expression were verified by Western blot analyses in a small number of whole, homogenized brains (n = 6-19/group; see Fig. 1, panels ad) as established previously (Carey et al. 2012). Primary antibodies for β-actin (0.02 μg/ml, Cell Signaling Technologies, Danvers, MA) and Tat protein (1:2000 of the rabbit polyclonal antibody ab43014, lot number 904506, Abcam, Cambridge, MA) were incubated overnight at 4°C with nitrocellulose bound proteins. The present study further examined the persistence of Tat antibody labeling after induction following treatment with saline or an optimal doxycycline dose (100 mg/kg for 7 days), with brain tissue samples

harvested 0, 7 or 14 days after treatment (n = 8-14 observations/group; see Fig. 1, panels e-f).

2.3. Behavioral assays

GT-tg mice were assessed for dose- and duration-dependent effects of central Tat on locomotor and/or anxiety-like behavior in an open field, a social interaction, or a marble burying test during the light phase of the light/dark cycle. Saline-administered (i.e., non-induced) GT-tg mice were used as isogenic, negative controls for experimental groups. C57BL/6J mice, administered saline or a maximal dose of doxycycline, were used as congenic, negative controls (only to rule out non-specific effects of doxycycline on behavior, but not to be directly compared given that some behavioral strain differences between GT-tg and C57BL/6J mice that may have been related to difference in motor behavior have been previously observed; Carey et al., 2012).

2.3.1. Open field test—The open field test assesses anxiety-like behavior and ataxia (Hall and Ballachey 1932). Briefly, mice were placed in the lower left corner of a square Plexiglas box (46 × 46 × 30 cm) and allowed to explore for 10 min. Movement was monitored and digitally encoded by a Noldus (Leesburg, VA) EthovisionPro3 image capture software package. A lesser amount of time spent in the brightly-lit center (~24 cm square) was considered an index of greater anxiety-like behavior. The total distance (cm) traveled was utilized as an index of locomotor behavior.

2.3.2. Social interaction test—The social interaction test utilizes a novel arena and bright lighting conditions to suppress the likelihood of social interaction with a novel conspecific; interaction time is thus used as an index of anxiety-like behavior (File and Hyde 1978). An experimental male mouse was placed in one corner of a square Plexiglass box (46 × 46 × 30 cm) while a weight-matched male GT-tg stimulus mouse was placed in the opposing corner. Mice were allowed to freely explore for 5 min. A lesser amount of time spent engaging in social interaction (sniffing, grooming) was considered indices of greater anxiety-like behavior.

2.3.3. Marble burying test—The marble burying test utilizes spontaneous digging behavior, characteristic of rodents, to assess anxiety-like/compulsive behavior (Broekkamp et al. 1986; Poling et al. 1981). Briefly, mice were individually placed in a standard mouse housing cage (28 × 16 × 13 cm) with 20 marbles (evenly spaced in 5 rows of 4) located on a 5 cm layer of woodchip bedding. Mice remained in the cages with the marbles for a 30-min test, after which the number of marbles that were completely buried were counted. A greater number of buried marbles was considered an index of greater anxiety/compulsive-like behavior. While the compulsive- and anxiety-like components of marble burying have proven difficult to parse (Albelda and Joel 2012), advantages of the task include being largely devoid of test decay effects when administered on more than one occasion (Poling et al. 1981).

2.4. Statistical analyses

β-Actin antibody labeling was measured at the expected weight of 45 kDa, whereas Tat protein labeling was quantified from the band found at 19 kDa as described previously (Carey et al. 2012), with ImageJ 1.62 software (National Institutes of Health). Band values were calculated as percent of control (saline-treated GT-tg mice) sample labeling, and plotted as average values ± SEM across 6-19 experiments. Statistical differences in Western blot labeling were analyzed via one-way ANOVA. Behavioral results with GT-tg bigenic and C57BL/6J control mice were analyzed separately, given that C57BL/6J mice were utilized as negative controls to rule out non-specific effects of doxycycline treatment and were administered only maximal doxycycline dosing. Behavioral measures in within-subjects analyses were assessed via repeated-measures ANOVAs with doxycycline condition as the between-subjects factor, and post-treatment testing day as the within-subjects factor. Between subjects analyses of behavior were assessed via two-way ANOVAs with doxycycline condition and post-treatment testing day as factors. Fisher's PLSD *post-hoc* tests determined group differences following main effects. Interactions were delineated via simple main effects and main effect contrasts with alpha controlled for multiple comparisons. Analyses were considered significant when alpha < 0.05.

3. Results

3.1. Magnitude of Tat_{1-86} expression in GT-tg mouse brain is dependent on dose and duration of doxycycline treatment

Confirming previous demonstrations via Western blot (Carey et al. 2012), doxycycline significantly increased labeling of the suggested observed weight of Tat_{1-86} protein (19 kDa) in the whole brain of GT-tg bigenic mice in a manner dependent on both the dose [$F(3,33) = 4.71$, $p < 0.05$] (Fig. 1a-b) and duration [$F(5,70) = 5.09$, $p < 0.05$] (Fig. 1c-d) of treatment. Either 7 days of 50 ($p = 0.003$) or 125 ($p = 0.004$) mg/kg doxycycline (Fig. 1a-b), or 5 ($p = 0.01$), 7 ($p = 0.0004$), or 14 ($p = 0002$) days of 100 mg/kg doxycycline (Fig. 1c-d), significantly enhanced Tat protein content in the brain compared to 7 or 14 days of saline administration, respectively. Furthermore, Western blots were performed on whole homogenized brains isolated from GT-tg mice pretreated with vehicle (0.9% saline, i.e., uninduced) or doxycycline (100 mg/kg/d, i.p. for 7 d) and harvested 0, 7 or 14 days later to characterize the time-course of Tat_{1-86} protein expression after induction. Immediately following doxycycline treatment, central Tat_{1-86} protein labeling was significantly greater than that observed after 7 days of saline administration [$F(3,44) = 5.99$, $p < 0.05$] (Fig. 1e-f). Harvested 7 days after treatment, Tat_{1-86} protein labeling was reduced from post-treatment day 0 and did not significantly differ from other groups (Fig. 1f, grey bar). Fourteen days after doxycycline treatment, Tat_{1-86} protein labeling had returned to basal concentrations, significantly lower than that observed immediately after treatment ($p < 0.01$; Fig. 1f, dotted bar). Together, these results confirm that Tat protein expression is dependent on the dose and duration of doxycycline exposure, but that the labeling of Tat protein so induced returns to basal levels within 14 days.

3.2. Induction of Tat_{1-86} protein increases anxiety-like behavior

To assess the magnitude of Tat expression on anxiety-like behavior, GT-tg mice were administered saline or a dose of doxycycline (25, 50, or 125 mg/kg) once daily for 7 days, and were tested repeatedly for anxiety-like responding 0, 7, and 14 days later (Fig. 2a).

Among GT-tg mice, the magnitude of Tat expression interacted with repeated testing to produce anxiety-like responding in an open field [$F(6,184) = 2.97$, $p < 0.05$] (Fig. 2b) or a social interaction [$F(6,152) = 2.51$, $p < 0.05$] (Fig. 2c) task. Immediately following doxycycline treatment (post-treatment day 0), Tat expression via the highest dose of doxycycline (125 mg/kg/d, 7 days) significantly reduced the amount of time spent in the brightly-lit center of an open field [$F(3,92) = 5.08$, $p < 0.05$] (Fig, 2b), or interacting with a same-sex conspecific [$F(3,76) = 3.07$, $p < 0.05$] (Fig. 2c), compared to mice treated with a lower doxycycline dose [doxycycline-25 mg/kg: $p_{open\ field} = 0.01$; $p_{social\ interaction} = 0.02$; doxycycline-50 mg/kg (open field only): $p = 0.04$] or uninduced mice administered saline ($p_{open\ field} = 0.0002$; $p_{social\ interaction} = 0.006$). However, responding in both tasks significantly decreased with repeated testing such that overall performance was significantly reduced on post-treatment days 7 ($p_{open\ field} = 0.0003$; $p_{social\ interaction} < 0.0001$) or 14 ($p_{open\ field} < 0.0001$; $p_{social\ interaction} < 0.0001$), compared to initial assessments on post-treatment day 0. The distance GT-tg mice travelled in the open field was also significantly reduced 14 days after Tat induction ($p = 0.0002$; Table 1). As such, group differences were transient in the following weeks of testing with no significant differences observed in the open field task on post-treatment day 7, but all treatment groups accumulated significantly less center-time than saline-administered controls on post-treatment day 14 ($p_{\text{Dox 25mg/kg}} = 0.002$, $p_{\text{Dox 50mg/kg}} = 0.0004$, $p_{\text{Dox 125mg/kg}} = 0.003$; Fig. 2b). Group differences were similarly transient in the social interaction task with doxycycline-administered (50 or 125 mg/kg for 7 days) GT-tg mice spending significantly less time interacting on post-treatment day 7, compared to controls, but no differences were observed by post-treatment day 14 (Fig. 2c).

Given the presence of test decay with repeated testing (day 7 and 14 observation) in the open field or social interaction tasks, a cohort of mice was then assessed for marble-burying behavior, which is reported to be resilient to habituation (Poling et al. 1981). Doxycycline dose-dependently interacted with post-treatment delay in testing which increased marble burying behavior among GT-tg mice [$F(6,184) = 2.53$, $p < 0.05$]. Overall task performance did not decay with repeated testing (Fig. 2d). Induction of Tat protein with the highest doxycycline dose (125 mg/kg/d for 7 days) significantly increased the number of marbles buried on post-treatment day 0 [$F(3,92) = 15.32$, $p < 0.05$; compared to any other group, $p_{\text{saline}} < 0.0001$, $p_{\text{Dox 25mg/kg}} < 0.0001$, $p_{\text{Dox 50mg/kg}} = 0.0002$], post-treatment day 7 [$F(3,92) = 4.21$, $p < 0.05$; compared to any other group, $p_{\text{saline}} = 0.0008$, $p_{\text{Dox 25mg/kg}} = 0.02$, $p_{\text{Dox 50mg/kg}} = 0.03$], and post-treatment day 14 [$F(3,92) = 2.46$, $p < 0.05$; compared to saline, $p = 0.01$, or doxycycline-25 mg/kg, $p = 0.04$) (Fig. 2d). Only initially (post-treatment day 0) did lower doses of doxycycline (50 mg/kg/d) elevate burying behavior above control levels ($p = 0.03$; Fig. 2d).

Despite apparent increases in anxiety-like behavior, the highest doxycycline dose (125 mg/kg for 7 days) reduced responding in marble burying (an anxiety task highly dependent on motor function) and significantly impaired locomotor performance in the open field [$F(3,184) = 3.60$, $p < 0.05$] compared to all other groups ($p_{\text{saline}} = 0.001$, $p_{\text{Dox 25mg/kg}} = 0.02$, $p_{\text{Dox 50mg/kg}} = 0.02$) which otherwise did not differ from each other (Table 1).

3.3. Prolonged exposure to$Tat_{1\text{-}86}$ protein increases anxiety-like behavior

GT-tg mice were administered saline once daily for 14 days or doxycycline (100 mg/kg) once daily for 1, 3, 5, or 14 days, and then were repeatedly tested for anxiety-like responding 0, 7, and 14 days later (Fig. 3a). The duration of Tat induction interacted with repeated testing to promote anxiety-like responding in an open field [$F(8,230) = 2.38$, $p < 0.05$] (Fig. 3b) or a social interaction [$F(8,190) = 3.65$, $p < 0.05$] (Fig. 3c) task. Immediately following induction, Tat protein significantly reduced the amount of time spent in the brightly-lit center of an open field [$F(4,115) = 3.99$, $p < 0.04$ for all durations of induction; Fig. 3b) or in social interaction [$F(4,95) = 5.72$, $p < 0.0003$ for 3, 5 or 14 days of induction; 1 day treatment not significant; Fig. 3c) below control levels. Observed social interaction was lower after 3 days of doxycycline treatment compared to 1 day of treatment ($p = 0.04$), suggesting that brief expression of Tat protein was insufficient to induce acute social anxiety. As before, responding in both tasks significantly decreased with repeated testing, 7 ($p_{\textit{open field}} < 0.0001$; $p_{\textit{social interaction}} = 0.01$) or 14 ($p_{\textit{open field}} < 0.0001$; $p_{\textit{social interaction}} = 0.003$; Table 1) days after the completion of Tat induction, and only transient effects were observed. By post-treatment day 7, only mice with extended, greater exposure to Tat protein (caused by 5 or 14 d doxycycline treatment) interacted less than saline-administered controls (5-day treated mice only: $p = 0.02$) or less than those treated for 3 days ($p_{\textit{Dox 5 days}} = 0.009$; $p_{\textit{Dox 14 days}} = 0.048$; Fig. 3c). Fourteen days after Tat induction, no significant anxiety-like differences remained in either task, and GT-tg mice were travelling significantly shorter distances compared to their earlier performance (post-treatment day 0; $p = 0.03$; Table 1).

To overcome potential test decay attributed to repeated testing (day 7 and 14 observations), a cohort of mice was assessed for marble-burying behavior. The duration of Tat induction produced a main effect to influence marble burying [$F(4,230) = 7.58$, $p < 0.05$], irrespective of post-treatment testing day (Fig. 3d). Induction of Tat protein for 5 days resulted in a significant increase in marble burying compared to saline administration ($p = 0.0005$) or induction for only 1 day ($p = 0.001$), as did a prolonged induction of Tat protein (for 14 days), with significantly more marbles buried than mice treated with saline, or doxycycline for 1 or 3 days ($p \leq 0.01$; Fig. 3d). As such, Tat-induced anxiety-like behavior was greatest following induction of Tat protein for 5 or 14 days. However, the longest induction (14 days) produced significant locomotor deficits [$F(4,230) = 2.66$, $p < 0.05$] compared to mice treated with saline (i.e., uninduced mice; $p = 0.007$), or short durations of doxycycline (1 day, $p = 0.006$; 5 days, $p = 0.02$; Table 1).

Separate cohorts of C57BL/6J control mice (which lack the $Tat_{1\text{-}86}$ transgene) were assessed in the open field, social interaction, or marble burying tasks to rule out non-specific effects of doxycycline treatment. Like GT-tg mice, C57BL/6J mice demonstrated significant test decay when tested repeatedly in the open field [Total Distance: $F(2,92) = 127.26$,

$p_{post\text{-}treatment\ day\ 7\ or\ 14} < 0.0001$; Time in Center: $F(2,92) = 19.67$, $p_{post\text{-}treatment\ day\ 7\ or\ 14} < 0.0001$] or social interaction [$F(2,76) = 39.65$, $p_{post\text{-}treatment\ day\ 7or\ 14} < 0.0001$] tasks, but not the marble burying task (Table 2). However, no significant effects of doxycycline (100 mg/kg for 14 days) were observed in any task, compared to mice administered saline for 14 days (Table 2).

3.4. Anxiety-like effects of Tat_{1-86} endure for at least two weeks after induction

To circumvent the limitations caused by repeated testing, a series of between-subjects experiments were conducted wherein Tat-induced GT-tg mice (administered doxycycline, 100 mg/kg for 7 days) or uninduced GT-tg mice (administered saline daily for 7 days) were assessed only once for anxiety-like behaviors on days 0, 7, or 14 following treatment (Fig. 4a).

Induction of Tat protein significantly reduced the time spent in the center of a brightly-lit open field [$F(1,138) = 20.61$, $p < 0.05$] (Fig. 4b), reduced the amount of time spent socially-interacting with a same-sex conspecific [$F(1,90) = 54.63$, $p < 0.05$] (Fig. 4c), and increased the number of marbles buried in a marble burying test [$F(1,138) = 29.83$, $p < 0.05$] (Fig. 4d) as compared to uninduced GT-tg mice. Effects were not influenced by post-treatment delay in testing, nor were any effects on locomotor behavior observed (Table 1). Notably, there was no difference in performance between C57BL/6J mice treated with doxycycline (100 mg/kg for 7 days) or saline (7 days) on any measure examined (Table 2).

4. Discussion

Induction of Tat_{1-86} protein resulted in significant reductions in the amount of time GT-tg bigenic mice spent in the brightly-lit center of an open field or interacting with a novel mouse, and increased marble burying behavior as compared to saline-administered controls. As both saline and doxycycline treatments produced similar measures of anxiety-like behaviors in C57BL/6J mice lacking the transgene, the present findings support the hypotheses that it was the expression of Tat_{1-86} protein in mouse brain that increased anxiety-like behaviors in an exposure-dependent manner. These data recapitulate clinical observations of elevated anxiety among HIV-afflicted individuals (Atkinson et al. 1988; Bing et al. 2001; Orlando et al. 2002), suggesting the actions of HIV-1 Tat protein may be sufficient to induce affective disorders.

Another aim of this study was to confirm expression the long-term effects of doxycycline administration on central Tat_{1-86} protein expression. Western blot analyses of brains isolated from GT-tg bigenic mice verified doxycycline dose- and duration-dependent increases of Tat_{1-86} protein, consistent with previous findings (Carey et al. 2012). Furthermore, expression of Tat_{1-86} protein was transient, as antibody labeling declined one week after induction and did not differ from control levels two weeks after induction. Despite the known difficulties in conducting protein analyses of in vivo Tat expression, we and others have previously demonstrated similar effects (Carey et al. 2012; Fitting et al. 2010; Kim et al. 2003). Many of the available Tat antibodies generate nonspecific labeling at the predicted weights of Tat protein (14 kDa; Fitting et al. 2010 and Carey et al. 2012, supplemental material), and indeed nonspecific labeling at 14 kDa was again detected herein, even among

control C57BL/6J brain samples that lack Tat protein. The limitations of available detection tools are notable, as Tat protein is typically expressed on arbitrary scales, even in clinical tissues (Wesselingh et al. 1993). However, the 19 kDa band co-varies with expected Tat expression. The discrepancy in molecular weight may be attributed to post-translational modification of Tat and is consistent with other weights so reported (Carey et al. 2012; Fitting et al. 2010; Park et al. 2000). Despite the need for better antibodies to Tat, the expression of this protein in the GT-tg mouse (Kim et al. 2003) is generally consistent with unbound concentrations observed in HIV-positive sera (Westendorp et al. 1995; Xiao et al. 2000). Thus, the present Western blot results confirm expression of the protein, consistent with the progression of affective sequelae observed in the GT-tg model.

There are several potential mechanisms by which Tat_{1-86} may influence neurological and mood-related behavior. Once secreted from CNS reservoirs, Tat protein is known to rapidly act at neuronal membrane-bound targets such as NMDA receptors and calcium channels in cortex, by which it might promote cognitive disruption and excitotoxicity (Haughey et al. 1999; Starling et al. 1999; Wayman et al. 2012). Tat also dysregulates intracellular Ca^{2+} influx, altering mitochondrial function to promote oxidative stress (Mattson et al. 2005; Norman et al. 2008), and induces the up-regulation of pro-inflammatory cytokines and NF-κB-regulated chemokines (Grove et al. 1993; Norman et al. 2008; Yadav et al. 2009). All of these actions have been associated with an increase in anxiety-like behavior (Brocardo et al., 2012; Desrumaux et al. 2005; Hoge et al. 2009). Additionally, recent *in vitro* data also suggest that Tat can acutely impair exocytotic activity of neuroendocrine cells via binding and sequestering intracellular phosphatidylinositol-(4,5)-bisphophate, thus depleting cellular neurotransmission to disrupt endocrine signaling (Tryoen-Tóth et al. 2012) in a manner consistent with anxiogenesis (Gormanns et al. 2011). Regardless, the present findings extend the *in vitro* understanding of Tat-mediated mechanisms of cellular dysfunction to a behavioral phenotype that is potentially clinically significant. Moreover, the multitude of mechanisms suggests a number of possible interventions that might be tested in the GT-tg model for therapeutic potential.

Neurotoxic effects *in vitro* demonstrate the potential for neurodegeneration *in vivo* that could contribute to behavioral aberration. We have previously observed doxycycline-Tat induction for 5 or 7 days, which enhanced anxiety-like behavior in the present paper, to significantly decrease grey matter volume in GT-tg mouse brain, notably in regions critical for affective behavior (including hippocampus and amygdala; Carey et al. 2013). Moreover, we now find that antibody labeling of central Tat_{1-86} protein returns to basal (control) levels within 14 days after doxycycline treatment but anxiety-like behavior persists. While further study is needed, these findings suggest that even acute exposure to Tat protein may result in persistent behavioral deficits beyond the physical presence of the protein which may involve central degenerative process. This suggestion is broadly consistent with emerging data regarding the broader effects of Tat protein on the brain in intact, whole-animal systems. Prolonged transgenic expression of Tat protein in a similar transgenic mouse model that may express fewer copies of Tat, resulted in synaptic remodeling with functional effects for long-term potentiation as well as learning and memory deficits; albeit, substantial neurodegeneration was not observed (Fitting et al. 2013). Together, these data suggest that

acute exposure to Tat may result in remodeling and neuronal degeneration with chronic behavioral consequences.

It is important to note that strain differences in motor behavior were observed in the present study. The founder mice of the GT-tg bigenic line were bred onto a C57BL/6J background (Kim et al. 2003), and back-crossed 7 generations; yet, C57BL/6J mice demonstrated greater locomotor behavior than did GT-tg mice. These findings are consistent with past reports demonstrating C57BL/6J mice to have a greater motor phenotype than other inbred mouse strains (Holmes et al. 2002) including the initial GT-tg founder strain (Kallnik et al. 2007; Schneider et al. 2006). In support, when the total distance travelled was factored in as a covariate in an ANCOVA for center time in the open field, strain differences were no longer significantly detected in the present dataset. Given that motor behavior is largely inter-correlated with anxiety-like performance in the open field and marble burying tasks (Deacon 2009; Gould et al. 2009), these data demonstrate the potential influence of locomotor phenotypes on affective measures and highlight the importance of isogenic (i.e., uninduced) and congenic controls.

The utility of the GT-tg mouse model to titrate Tat protein exposure is evidenced with the increased efficacy of escalating doxycycline treatments on affective behavior. Notably, an upper limit to this flexibility was established with the development of pronounced motor deficits, although this does emulate clinical observations of locomotor deficits in HIV-positive patients (Woods et al. 2009). The magnitude of responses observed among Tat-induced GT-tg mice was commensurate with prior findings utilizing unconditioned anxiogenic stimuli to assess anxiety-like behavior in the light phase of the circadian cycle (Bolivar et al. 2007; Jansen et al. 2010; Rattazzi et al. 2013). Determining the response to conditioned anxiety or unavoidable stimuli would be expected to confirm the present data as well as offer insights into the affective processes that are influenced by Tat_{1-86} protein. Given the multi-regional complexity of anxiety-processing in the brain (see Fanselow and Ponnusamy 2008) and the neurodegenerative involvement of Tat_{1-86} that we have observed in central limbic regions (Carey et al. 2013), tasks such as fear-potentiated startle (Fendt and Fanselow 1999) or reflexive acoustic startle (Ralph and Caine 2005) might differentiate additional deficits in conditioned hippocampus/amygdala-mediated fear and are targets of future investigation.

5. Conclusions

The brain-selective expression of Tat_{1-86} protein in an animal model significantly increased anxiety-like behavior in the open field, social interaction, and marble burying tasks in an exposure-dependent manner, highlighting a potentially important therapeutic target for HIV-positive individuals.

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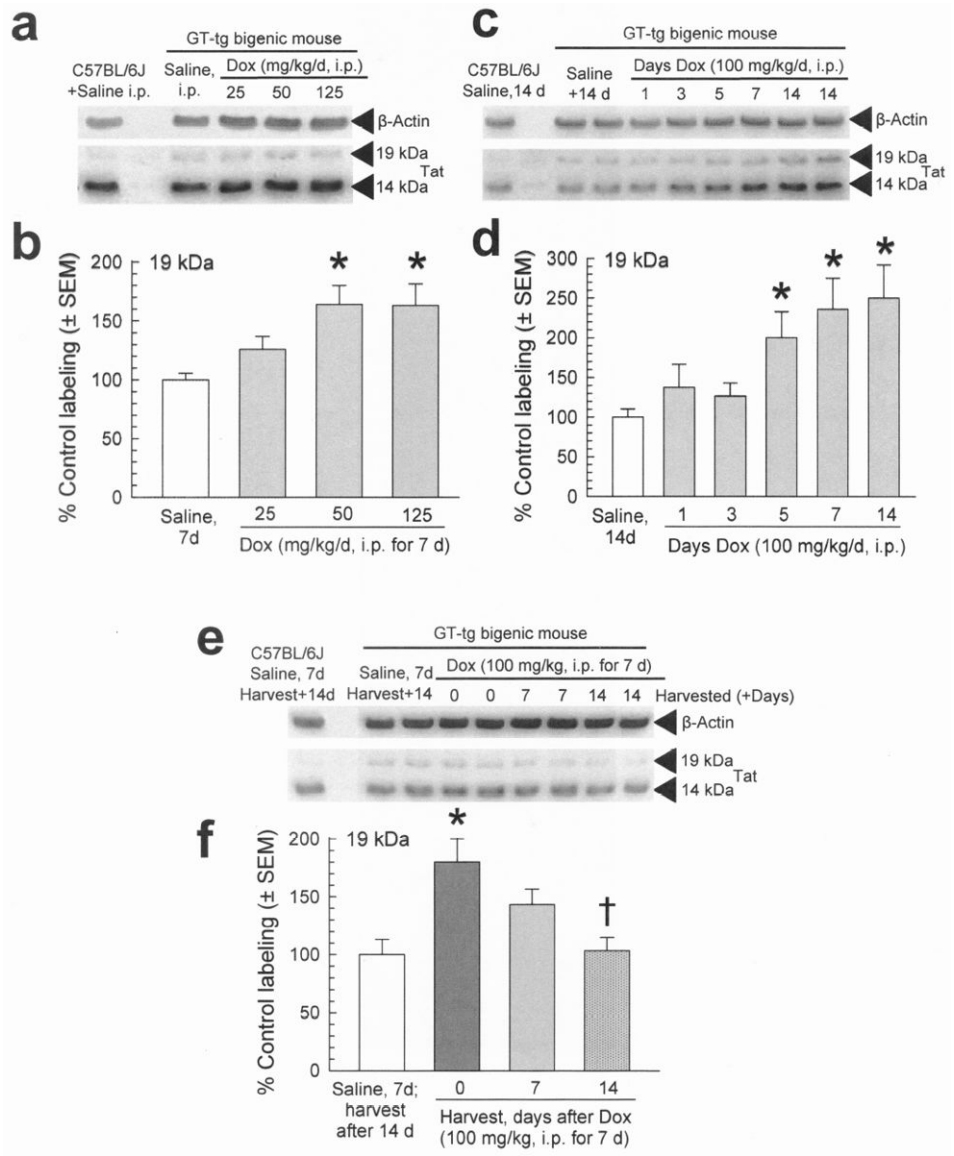

Fig. 1.

Doxycycline-induced Tat_{1-86} protein expression in GT-tg mouse whole-brain. The β-actin antibody labeled a single band (upper panels) corresponding to the weight of the β-actin protein of similar intensity across all samples. By contrast, the Tat antibody labeled a number of proteins non-selectively (lower panels), but only demonstrated a difference in labeling intensity at the 19 kDa band that corresponded to the (a) dose and (c) duration of doxycycline, (e) administered for less than 14 days after treatment. Summary graphs of the quantified whole brain Western blots were plotted as a percent of control (saline-treated GT-tg) labeling. Doxycycline treatment resulted in an increase in Tat-antibody labeling in GT-tg mice dependent on the duration of pretreatment. Labeling intensity differences corresponded to the dose (b) or duration (d) of doxycycline administered, or harvest date after induction (f) at 19 kDa, which has been suggested to be the observed weight of expressed Tat protein

in the GT-tg mouse. Symbols indicate significant difference from saline controls (*) or Dox-treated mice that were assessed immediately (0 days) after treatment end (†), $p < 0.05$.

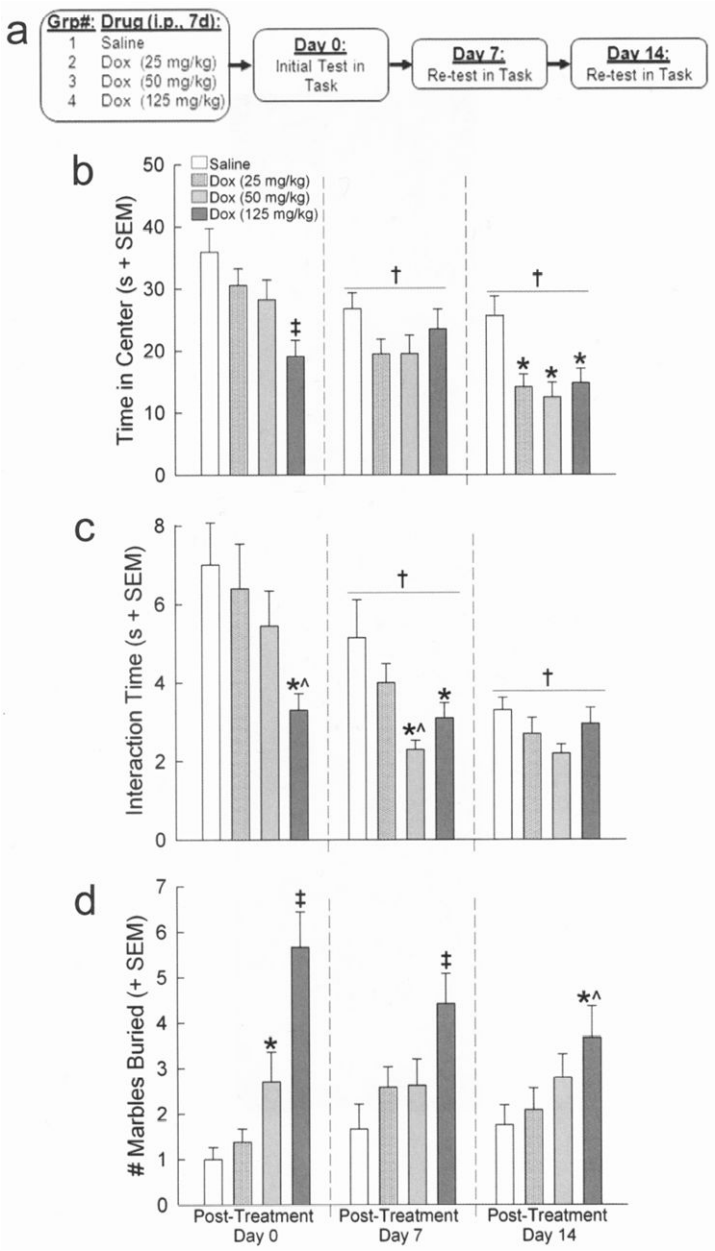

Fig. 2.

(a) GT-tg bigenic mice were administered saline or a dose of doxycycline (Dox) for 7 days. As depicted, mice were repeat-tested 0, 7, and 14 days after treatment and were assessed for anxiety-like response in an (b) open field (n = 24 / grp), (c) social interaction (n = 20 / grp), or (d) marble burying (n = 24 / grp) test at those times. † indicates significant difference from post-treatment day 0, demonstrating habituation due to repeated testing. On respective post-treatment days, symbols indicate significant difference from saline control (*), Dox 25 mg/kg group (ˆ), or all other groups (‡), $p < 0.05$.

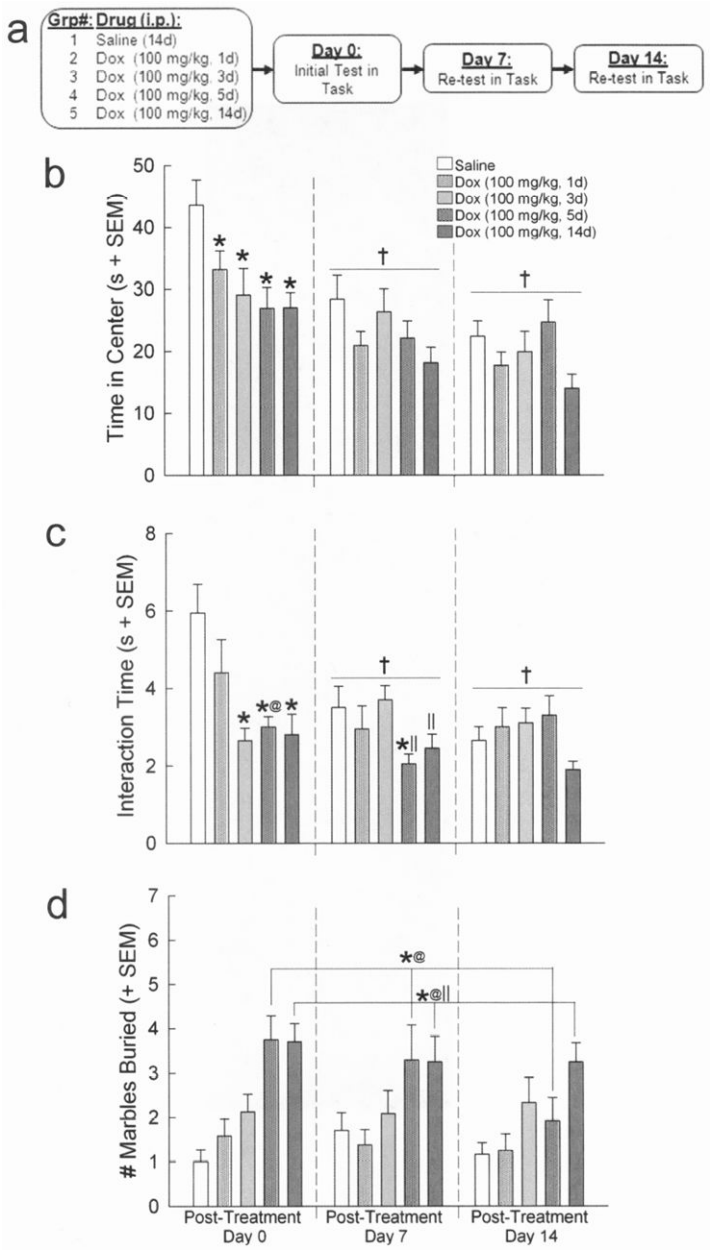

Fig. 3.

(a) GT-tg bigenic mice were administered saline or a given duration of doxycycline (Dox; 100 mg/kg). As depicted, mice were repeat-tested 0, 7, or 14 days after treatment and were assessed for anxiety-like response in an (b) open field (n = 24 / grp), (c) social interaction (n = 20 / grp), or (d) marble burying (n = 24 / grp) test at those times. † indicates significant difference from post-treatment day 0, demonstrating habituation due to repeated testing. On respective post-treatment days, symbols indicate significant difference from saline control (*), Dox 1-day group (@), or Dox 3-day group (‖), $p < 0.05$.

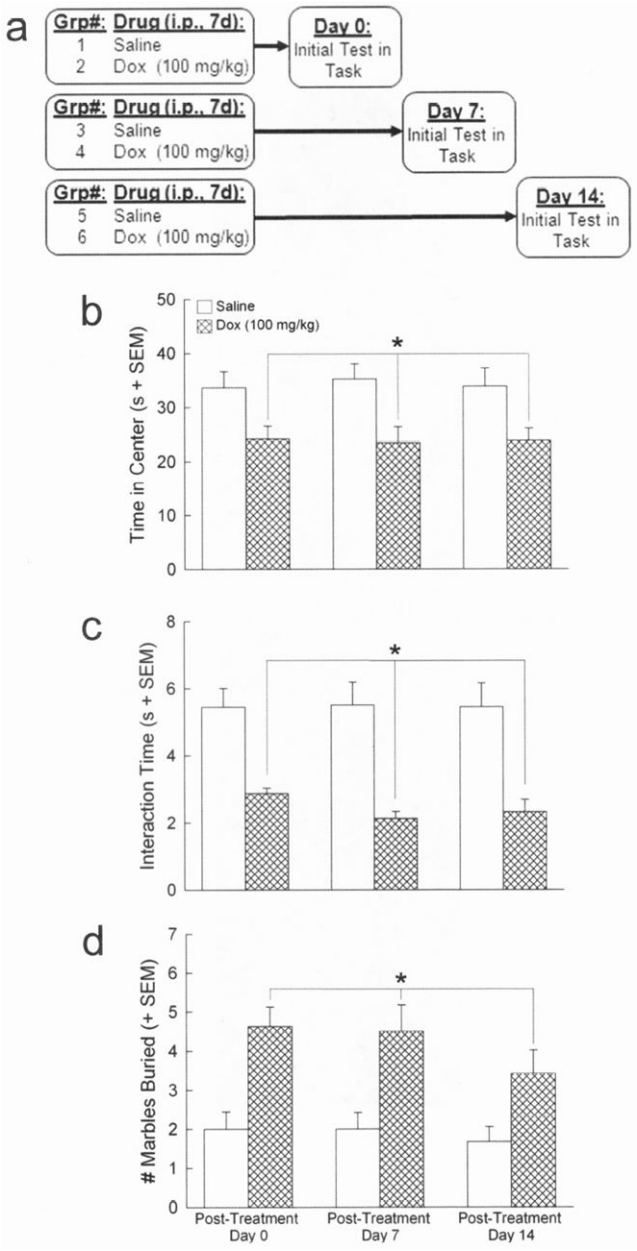

Fig. 4.

(a) To obviate effects of habituation from repeated testing, GT-tg bigenic mice were administered saline or doxycycline (Dox; 100 mg/kg) for 7 days and were tested only once: either 0, 7, or 14 days following treatment. At those times, mice were assessed for anxiety-like response in an (b) open field (n = 24 / grp), (c) social interaction (n = 16 / grp), or (d) marble burying (n = 24 / grp). * indicates significant difference from saline administration, $p < 0.05$.

Table 1

Total distance traveled (m ± SEM) in an open field among GT-tg bigenic mice administered saline or doxycycline (Dox).

	GT-tg Weekly Repeated Measures Locomotion		
	Total Distance (m)		
Treatment Group (n=24/grp)	**Post-Treatment Day 0**	**Post-Treatment Day 7**	**Post-Treatment Day 14**
Saline (0.9 %, 7 d)	46 ± 3	46 ± 4	39 ± 1†
Dox (25 mg/kg, 7 d)	46 ± 2	41 ± 1	36 ± 1†
Dox (50 mg/kg, 7 d)	47 ± 8	44 ± 6	32 ± 2†
Dox (125 mg/kg, 7 d)	34 ± 2‡	37 ± 1‡	31 ± 2‡†
Saline (0.9 %, 14 d)	42 ± 1	41 ± 2	39 ± 2†
Dox (100 mg/kg, 1 d)	42 ± 1	42 ± 2	38 ± 2†
Dox (100 mg/kg, 3 d)	40 ± 3	38 ± 2	37 ± 2†
Dox (100 mg/kg, 5 d)	40 ± 1	40 ± 4	40 ± 2†
Dox (100 mg/kg, 14 d)	35 ± 2*	36 ± 2*	33 ± 2*†
	GT-tg Locomotion 0, 7, or 14 days Post-treatment		
	Total Distance (m)		
Treatment Group (n=24/grp)	Post-Treatment Day 0	Post-Treatment Day 7	Post-Treatment Day 14
Saline (0.9 %, 7 d)	42 ± 1	42 ± 1	39 ± 1
Dox (100 mg/kg, 7 d)	38 ± 2	41 ± 1	41 ± 2

*indicates significant main effect for a motor deficit compared to respective saline-administered group.

‡indicates significant main effect for a motor deficit compared to all other groups.

†indicates significant main effect for reduced motor behavior compared to post-treatment day 0, $p < 0.05$.

Table 2

Anxiety-like response in an open field, social interaction, or marble burying test (mean ± SEM) among C57BL/6J mice treated with saline or doxycycline.

	C57BL/6J Weekly Repeated Measures Anxiety-like Performance					
	Saline (0.9 %, i.p., 14 d)			**Doxycycline (100 mg/kg, i.p., 14 d)**		
Open Field (n=24/grp)	**Post-Treatment Day 0**	**Post-Treatment Day 7**	**Post-Treatment Day 14**	**Post-Treatment Day 0**	**Post-Treatment Day 7**	**Post-Treatment Day 14**
Total Distance (m)	61 ± 3	39 ± 2†	29 ± 1†	58 ± 3	40 ± 3†	29 ± 1†
Time in Center (s)	49 ± 3	38 ± 5†	31 ± 4†	51 ± 5	35 ± 5†	30 ± 3†
Social Interaction (n=20/grp)						
Interaction Time (s)	7 ± 1	5 ± 1†	3 ± < 1†	8 ± 1	4 ± 1†	3 ± < 1†
Marble Burying (n=24/grp)						
# Marbles Buried	4 ± 1	5 ± 1	4 ± 1	4 ± 1	4 ± 1	3 ± 1
	C57BL/6J Anxiety-like Performance 0, 7, or 14 days Post-treatment					
	Saline (0.9 %, i.p., 7 d)			Doxycycline (100 mg/kg, i.p., 7 d)		
Open Field (n=24/grp)	Post-Treatment Day 0	Post-Treatment Day 7	Post-Treatment Day 14	Post-Treatment Day 0	Post-Treatment Day 7	Post-Treatment Day 14
Total Distance (m)	49 ± 2	46 ± 1	44 ± 2	49 ± 2	46 ± 1	47 ± 2
Time in Center (s)	42 ± 3	40 ± 3	37 ± 3	43 ± 4	43 ± 3	37 ± 4
Social Interaction (n=16/grp)						
Interaction Time (s)	6 ± 1	6 ± 1	5 ± 1	6 ± 2	6 ± 1	6 ± 2
Marble Burying (n=24/grp)						
# Marbles Buried	4 ± 1	4 ± 1	4 ± 1	4 ± 1	5 ± 1	4 ± 1

Mice were treated for either 14 days with weekly repeated measures assessments, or were treated for 7 days with assessments only on post-treatment days 0, 7, or 14.

† indicates significant main effect for reduced performance compared to post-treatment day 0, $p < 0.05$.