Published in final edited form as:
Prog Neuropsychopharmacol Biol Psychiatry. 2013 June 3; 43: 49–54. doi:10.1016/j.pnpbp.2012.12.018.

Conditional Tat protein expression in the GT-tg bigenic mouse brain induces gray matter density reductions

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Abstract

Tat (Trans-activator of transcription) is implicated in the neuropathogenesis of HIV-1 infection and known to contribute to neuronal damage and learning and memory impairments. However, direct neuroanatomical demonstration of Tat pathobiology is limited. GT-tg bigenic mice with a doxycycline (Dox)-inducible and brain-selective *tat* gene were used to test the hypotheses that conditional induction of Tat activity in brain can induce gray matter density abnormalities. Ultra high spatial resolution *ex vivo* magnetic resonance imaging (MRI) combined with a voxel based morphometry (VBM) analysis revealed gray matter density reductions in the sublenticular extended amygdala, the amygdala, the amygdala-hippocampal area, piriform and peri-/entorhinal cortices, and hypothalamus, in Tat-expressing GT-tg mice compared to Dox-treated C57Bl/6J mice. These neuroanatomical abnormalities are consistent with regions expected to be abnormal based on behavioral deficits exhibited by Tat-expressing mice (Carey et al., 2012). These experiments provide the first neuroimaging evidence that conditional Tat protein expression in the GT-tg bigenic mouse model alters brain structure. The findings warrant future studies to further characterize effects of conditional Tat expression on brain structure. Such studies may improve our understanding of the neurological underpinnings of neuroAIDS and the neurodegeneration associated with HIV-1 infection, potentially leading to new treatments.

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Disclosure/Conflicts of interest

This research is supported by F31 NS064872-01 from the National Institute of Neurological Disorders and Stroke (to ANC), and funds from the State of Florida, Executive Office of the Governor's Office of Tourism, Trade, and Economic Development and R01 MH085607 from the National Institute of Mental Health (to JPM). This project was further sponsored in part by National Institutes of Health grants F32DA026250 (to JWM), S10RR019356 and K02DA017324 (to MJK), by a grant from Varian, Inc., and by gifts from John and Virginia B. Taplin. This project was also sponsored in part by the Counter-Drug Technology Assessment Center, an office within the Office of National Drug Control Policy, via Contract Number DABK39-03-C-0075 awarded by the Army Contracting Agency (to MJK). The content of the information does not necessarily reflect the position or the policy of the Government and no official endorsement should be inferred. The sponsors played no role in study design or in data collection, analysis, or interpretation, or in report preparation or submission. Dr. Kaufman received research support from Varian Inc., Organon/Schering-Plough, NARSAD, the Michael J. Fox Foundation for Parkinson's Research, Air Products and Chemicals, Inc, and from PhotoThera, Inc. The other authors have no conflicts of interest to disclose.

Keywords

HIV-1; neuroAIDS; Tat; magnetic resonance imaging; voxel based morphometry

1.0 Introduction

The activity of accessory proteins produced by HIV-1 viral infection of brain, such as Tat (Trans-activator of transcription), may contribute significantly to the persistence of HIV-related neuropathology and subsequent cognitive and psychomotor slowing (Frankel and Young, 1998; Power et al., 1998; Brack-Werner, 1999; Johnston et al., 2001). It has been suggested that Tat may play a key role in the neurotoxicity and cognitive impairment evident in neuroAIDS (Rappaport et al., 1999; King et al., 2006). Epidemiological reports indicate that clade-specific variation among HIV-1 strains results in differences in the prevalence of HIV-associated dementia (Ranga et al., 2004) and human neuronal toxicity (Mishra et al., 2008; Rao et al., 2008). This variation has been linked to mutations within the neurotoxic epitope of the *tat* gene.

Tat protein is an established inflammatory agent, activating microglia both *in vitro* and *ex vivo* (Bruce-Keller et al., 2001; Bokhari et al., 2009). Tat-induced neuroinflammation in particular brain regions has also been demonstrated *in vivo*, specifically in both the cortex and hippocampus of GT-tg bigenic mice (Zou et al., 2007), the cortex of $CX_3CR_1GFP/+$ mice (Marker et al., 2010) and the striatum of another strain of inducible Tat transgenic mouse (Bruce-Keller et al., 2008). *In vitro* cell culture studies have shown Tat to cause inflammation and excitotoxicity (Aksenova et al., 2005), which have been correlated with an induction of oxidative stress and astrocytosis (Toborek et al., 2003; Zhou et al., 2004). These events are thought to impair astrocyte capacity to support neuronal function (Glass et al., 1993; Zhou et al., 2004), creating a toxic extracellular environment (Aksenov et al., 2001, 2003; Fitting et al., 2010). Tat-induced OS also has been associated with neurotoxicity and loss of brain function, and with pyramidal hippocampal neuronal damage (Aksenova et al., 2005; Askenov et al., 2006). *In vitro* studies demonstrated that exposure to Tat protein induced dysfunction of CA1 hippocampal and entorhinal cells, and the incubation of hippocampal-entorhinal cortex slices or CA1 hippocampal slices with $Tat_{1–86}$ suppressed long-term potentiation (LTP) (Behnisch et al., 2004; Li et al., 2004, respectively). Consistent with the latter finding, intracerebroventricular (i.c.v.) administration of recombinant Tat protein in rats induced the suppression of LTP, the degree of which correlated with increased numbers of eight-arm radial arm maze performance errors (Li et al., 2004). Tat-induced inflammation and neurodegeneration has been further postulated to contribute to HIV-associated dementia (Glass et al., 1993; Sui et al., 2007; Bruce-Keller et al., 2008; Lu et al., 2011).

Although Tat protein has been associated with cell death and degeneration (Kim et al., 2003; Zou et al., 2007; Fitting et al., 2010), no *in vivo* neuroimaging data is available to document regional changes in the intact brain following exposure to Tat protein alone. Accordingly, we conducted initial studies to test the hypotheses that induction of Tat activity can alter brain gray matter densities, as measured with ultra high spatial resolution magnetic resonance imaging (MRI) combined with a voxel based morphometry (VBM) analysis.

This study employed the GT-tg bigenic mouse constructed by He and colleagues (Kim et al., 2003). The GT-tg bigenic mice utilize a “Tetracycline-on” system which becomes transcriptionally active when doxycycline (Dox) is present. As the tetracycline- on system is coupled to the gene that codes for the $Tat_{1–86}$ protein, Tat protein expression is induced by the activation of a *tetracycline-on* promoter site with administration of doxycycline (Kim et

al., 2003; Zou et al., 2007; Carey et al., 2012). Moreover, as the tetracycline-on/$Tat_{1–86}$ system is integrated into the regulator for the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter, the GT-tg mice demonstrate brain specific expression of Tat protein (Kim et al., 2003). Presently, brains from Tat-expressing and control mice were scanned *ex vivo* using ultra-high magnetic field (9.4 Tesla (T)) imaging, to detect gray matter density abnormalities.

2. Methods

2.1 Animals and housing

Subjects were adult male GT-tg bigenic (Kim et al., 2003) and C57Bl/6J wild-type (Jackson Labs) mice, 8–14 weeks of age. GT-tg bigenic mice (a gift of Dr. J.J. He) came from two breeding pairs of bigenic mice homozygous for the Tat allele from a colony established at Northeastern University (NU). Mice were cared for in the NU vivarium in accordance with the 1996 NIH *Guide for the Care and Use of Laboratory Animals* and approved by the NU IACUC. The creation of GT-tg bigenic mice (backcrossed seven generations onto the C57Bl/6J line) and genotype confirmation of inducible, brain-targeted HIV-1 Tat protein were described by Kim et al. (2003). Doxycycline (Sigma-Aldrich) treatment induces expression of the $Tat_{1–86}$ gene via a “tetracycline-on” transactivator located exclusively in GT-tg mouse brain astrocytes. C57Bl/6J mice were used as a comparison group.

2.2 Induction of brain-targeted Tat with Dox treatment

$Tat_{1–86}$ protein was expressed by administering GT-tg bigenic mice Dox via intraperitoneal (i.p.) injection once/day (100 mg/kg in 0.9% saline, 0.3 ml/30 g body weight). The Dox exposure used (100 mg/kg/d, i.p.) for this duration was based on previous findings demonstrating the efficacy of Tat expression (Zou et al., 2007; Carey et al., 2012). Images were acquired and analyzed from Dox-treated C57Bl/6J mice (100 mg/kg/d, 7 days), uninduced GT-tg mice (0.9% saline, 7 days), and Dox-induced GT-tg mice treated (100 mg/kg/day) for 5 or 7 days. Throughout the manuscript, saline-treated GT-tg mice are referred to as “uninduced”.

2.3 Magnetic Resonance Imaging (MRI)

Ex vivo magnetic resonance imaging was conducted on a Varian 9.4 T Direct Drive scanner, using a micro-imaging gradient/shim insert set and a Varian proton (400 MHz) surface coil. Mice (n=8/treatment group) were perfused with 10% buffered formalin and ProHance (Gadoteridol, Bracco Diagnostics, Inc.), in a volume ratio of 20:1 (Cyr et al., 2005). After initial peer review of the manuscript, during which an anonymous reviewer commented on the variability in the 7-day Dox-treated GT-tg mouse group (N=8) and recommended we scan additional mice, we added 4 mice to this group (N=12) and reanalyzed the data. Imaging was performed on extracted fixed whole mouse brains within 72 h of perfusion with the following scan parameters: Repetition Time=3033ms, Echo Train Length=8, matrix size=512×512, Field of View=20×20mm, Echo Time=25.9ms, averages=64, slice number=28, slice thickness=0.5mm, 0mmgap, in-plane resolution=39×39um, scan time=3.33h.

2.4 Statistical Analyses

Voxel based morphometry (VBM)—*Ex vivo* MRI image analyses were performed in FSL v.4.1 (FMRIB Software Library) using the FSL registration and image processing tools. Brains first were extracted from background and then a study-specific template was constructed (Sawiak et al., 2009) iteratively as follows: each of the brain images of uninduced GT-tg and Dox-treated C57Bl/6J mice was normalized (image mean = 1) to

minimize scan-to-scan variance, and co-registered to a selected control brain using a linear approach. These images then were averaged to create an intermediate template. Next, Dox-treated GT-tg mouse brains were co-registered to the intermediate template using a nonlinear approach, and the non-linearly co-registered brain images were in turn averaged to create the final, study-specific template. These images were spatially smoothed using a 0.05 mm Gaussian kernel to reduce voxel-to-voxel fluctuation and to produce more robust cluster-level differences between groups. Then, a non-parametric *t*-test was performed at the image level in FSL using the "randomise" command, which estimates statistical significance based on ranked permutations among groups (corrected for cluster size and for multiple comparisons), resulting in nonparametric maps documenting structural difference distributions. The significance level was set at a threshold of $p \leq 0.05$. The C57Bl/6J mouse brain atlas from the Mouse Brain Library (URL: http://www.mbl.org/atlas/atlas.php) was used to identify regions in which significant gray matter density differences were observed.

An additional post-hoc non-parametric analysis was carried out at the image level. We compared gray matter densities in a composite region of interest (ROI) acquired in four adjacent brain slices anatomically matching those in which bilateral gray matter density differences ($p < 0.05$, corrected) were found in the whole brain analysis that compared 5-day Tat-expressing GT-tg mice versus Dox-treated C57Bl/6J mice (Figure 1, slices 6 –9). Mean gray matter densities were calculated. Significant group differences were determined with the Kruskal-Wallis H-test with post hoc testing (Conover, 1999) using STATA 12 (StataCorp LP, College Station, TX). This ROI analysis revealed a substantial amount of variance within the 7-day Dox-treated GT-tg mouse group, so at the suggestion of one of the manuscript reviewers, we added 4 more mice to this group and reanalyzed the data.

3.0 Results

Significant gray matter density reductions were found in the sublenticular extended amygdala, amygdala, amygdala-hippocampal area, in piriform, peri-/entorhinal cortices, and in hypothalamus, in 5-day Dox-induced GT-tg mice compared to Dox-treated C57Bl/6J mice (N=8/group, whole brain comparison). Areas of difference are shown in Figure 1, corrected $p \leq 0.05$, two-tailed *t*-test). However, only trend group differences in these regions were found when comparing 5-day Dox-induced GT-tg and uninduced GT-tg mice (N's = 8, $p < 0.08$). We also found a trend for a significant gray matter density difference between Dox-treated C57Bl/6J mice and 7-day Dox-induced GT-tg mice ($p < 0.10$). Our failure to detect differences between control groups and the 7-day Dox-treated GT-tg group was an unanticipated finding, given our prior finding of cumulative dose-related Dox effects on learning and memory behavioral tasks (Carey et al., 2012).

To investigate this apparent discrepancy, we conducted a post-hoc non-parametric ROI statistical analysis to compare gray matter densities in 5- and 7-day Dox-induced GT-tg mice. The bilateral ROI we selected was determined from four brain slices exhibiting significant group differences when comparing 5-day Dox-induced GT-tg mice to Dox-treated C57Bl/6J mice (see Figure 1, brain slices 6–9). Inspection of these data after initial analysis revealed substantial variance within the 7-day Dox-induced GT-tg group (N=8), which reduced our ability to detect effects in this group via the whole brain analysis. Following the suggestion of an anonymous reviewer, we induced 4 additional GT-tg mice with Dox for 7 days, perfused and fixed their brains, scanned them *ex vivo* as described above to increase the sample size of this group to 12, and reanalyzed the data. Results from the ROI analysis including the 4 additional brains are shown in Figure 2. We found an overall group difference in gray matter density (Kruskal-Wallis H = 15.505, df = 3, $p = 0.00143$). The 5-day Dox-induced GT-tg mouse brains exhibited a highly significant ROI gray matter density reduction compared to Dox-treated C57Bl/6J controls ($p = 0.000055$,

multiple comparisons-adjusted threshold *p*-value for significance = 0.004167). In addition, 7-day Dox-induced GT-tg mice exhibited reduced ROI gray matter density versus Dox-treated C57Bl/6J controls ($p = 0.003523$). We also detected a trend effect for an ROI difference when comparing 5-day Dox-induced GT-tg mice to uninduced GT-tg mouse controls, although this effect did not survive multiple comparisons correction ($p = 0.0273$, NS).

4.0 Discussion

Using magnetic resonance imaging, we detected significant gray matter density reductions suggestive of neurodegeneration and cell death in the brains of Dox-induced GT-tg bigenic mice. The brain areas we identified as being altered by the presence of Tat include the peri-/entorhinal and piriform cortices, along with amygdala and hippocampus, all of which have been implicated as mediators of learning and memory processes (Murray and Richmond, 2001; Cannistraro and Rauch, 2003; Aggleton et al., 2010; Gavrilovici et al., 2010; Pape and Pare, 2010). Tat protein has been shown previously to be expressed in these brain areas in Dox-treated GT-tg bigenic mice (Carey et al., 2012). The brain areas we found to exhibit Tat-induced abnormalities are thought to subserve the cognitive domains that demonstrate impairment in Dox-induced GT-tg mice (Carey et al., 2012), suggesting that the findings are consistent with behavioral effects of Tat expression. We also found reduced gray matter density in the hypothalamus, a target for HIV (Langford et al., 2011) and Tat protein (Banks et al., 2005; Clark et al., 2005).

Our findings suggest that the GT-tg mouse model may have utility as a limited animal model of the neurobiological consequences of human HIV infection. There are neuroanatomical reasons to believe that this may be the case, as in humans HIV infiltration often is identified in hippocampus and adjacent parts of the entorhinal cortex and temporal cortices (Anthony and Bell, 2008). We found these regions to be affected by expression of Tat protein in this study. In addition, our gray matter density findings parallel those reported in HIV-seropositive humans (Becker et al., 2012). Additional studies of Tat expression in the GT-tg mouse are warranted, to more fully characterize the effects of Tat protein in the intact brain and organism. Future studies utilizing repeated-measures *in vivo* imaging and behavioral methods could focus on progression of Tat-induced brain structural and behavioral changes, as progression of HIV-associated dementia correlates with progression of distinct neuropathological changes, such as decreased synaptic density and increased astrocytosis, neuronal loss and encephalitis (Everall et al., 1993).

Our findings of reduced gray matter density in peri-/entorhinal cortices may be related to the novel object recognition task deficits we observed in Dox-expressing GT-tg mice (Carey et al., 2012), as these brain areas have been linked to visual recognition memory (Murray and Richmond, 2001; Aggleton et al., 2010). Moreover, the amygdala-hippocampal gray matter density abnormalities we detected in Dox-induced GT-tg mice are consistent with the location of lesions known to produce deficits in spatial learning and memory performance (Schwarcz and Witter, 2002; Nadel and Hardt, 2011). The widespread structural deficits we observed may likewise contribute to the impaired spatial ability reported in other animal models of HIV-infection (Sei et al., 1992; Zink et al., 2002; Griffin et al., 2004; Li et al., 2004; Vigorito et al., 2007), including Tat-expressing GT-tg bigenic mice tested in the Barnes maze (Carey et al., 2012). Our finding of reduced hippocampal gray matter is also consistent with findings from *in vitro* Tat exposure studies, which report the suppression of hippocampal CA1 (and entorhinal) LTP (Behnisch et al., 2004; Li et al., 2004) and increased cell death (Aksenova et al., 2005; Askenov et al., 2006). As hippocampal LTP is the neuronal basis for hippocampal-dependent learning (see Teyler, 1987–1988; Sarvey et al.,

1989 for reviews), the loss of cells is a likely contributor to deficits in spatial learning and memory attributed to Tat protein.

Given the greater incidence of mood disorder among HIV-afflicted individuals (Bing et al., 2001), our finding of a hypothalamic gray matter density abnormality could be relevant to previous observations that hypothalamic dysfunction in HIV infection may promote the prevalence of mood disorders (Langford et al., 2011). Tat-induced disruptions of hypothalamic activity were shown to disrupt circadian signaling in mice (Clark et al., 2005), an event associated with depressive symptomology in people and rodents (Lall et al., 2012). Indeed, even a single intracerebroventricular administration of Tat protein has been shown to induce depressive-like behavior in rodents (Lawson et al., 2011). Overall, the present data suggests structural correlates by which Tat-protein may contribute to HIV-mood-related pathology.

There are a number of limitations to this study that merit discussion. First, while our whole brain MRI analysis findings did not detect gray matter density differences when comparing 5-day Dox-induced GT-tg mice to uninduced GT-tg mice, or when comparing 7-day Dox-induced GT-tg mice to either control group, we believe these negative findings are attributable to the relatively small sample sizes we used in this initial study. In fact, when we increased our sample size in the 7-day Dox-treated GT-tg mouse group, we obtained a trend effect ($p < 0.10$) for whole brain analysis significance when compared to Dox-treated C57Bl/6J controls. Further, we found in our post-hoc ROI analysis that increasing the sample size of our 7-day Dox-induced GT-tg mouse group resulted in a statistically significant effect (Figure 2). Thus, the relatively small sample sizes we used (compared to N's of 42 to 47 in an *ex vivo* mouse imaging study by Sawiak and colleagues (2009)) likely were somewhat underpowered to detect all effects. This suggests that the sensitivity of future MRI studies could be increased by including more subjects per group.

Secondly, although no significant differences were detected between uninduced GT-tg bigenic mice and Dox-treated C57Bl/6J mice, we did observe a trend toward reduced ROI density between these groups. Despite backcrossing the GT-tg mice 7 generations onto the C57Bl/6 line, it remains possible that a phenotypic divergence in the ancestry of the mice generated subtle differences in brain structure seen here. While this trend highlights the importance of making direct comparisons between the uninduced GT-tg mouse and the parent strain of mice in future, it does not negate our whole brain analysis finding of Tat-induced structural differences.

Alternatively, "leaky expression" of trace amounts of Tat mRNA transcripts has been reported in the absence of Dox in the GT-tg bigenic mouse model (Kim et al., 2003). Thus, it is conceivable that such "promoter leak" could expose GT-tg mice to trace levels of Tat protein over time, potentially accounting for the observed differences between Dox-treated C57Bl/6J mice and uninduced GT-tg mice. However, the great majority of brain Tat expression in GT-tg bigenic mice is known to be induced and regulated by Dox, as demonstrated by both Northern blot (Kim et al., 2003) and Western blot analyses (Carey et al., 2012), consistent with other uses of this model (Kistner et al.,1996; Chen et al., 1998; Mansuy et al., 1998). Further, in our previous experiments (Carey et al., 2012), we did not find learning or memory impairments in uninduced GT-tg mice exhibiting minimal Tat expression, suggesting minimal brain effects of any promoter leak. Although phenotypic divergence and promoter leak cannot be fully discounted, the present data suggest that the structural deficits we found are related to Dox-induced Tat protein expression.

Although the prefrontal cortex is known to mediate cognitive processes such as learning and memory (Kesner and Churchwell, 2011), no significant gray matter density differences were

detected in the areas comprising the prefrontal cortex. As noted above, this could be a result of small effect sizes that were not revealed with the current sample size. Alternatively, Tat protein is a known neuroinflammatory agent *in vivo.* Exposure to Tat protein increases markers of neuroinflammation in the cortex of GT-tg bigenic mice (Zou et al., 2007), activates astrocytes and microglia in the striatum in the presence of morphine (Bruce-Keller et al., 2008) and microgliosis in the cortex of CX_3CR_1 GFP/+ mice (Marker et al., 2010), and induces microgliosis lasting up to 28 days following a single exogenous administration into the hippocampus (Lu et al., 2011). As neuroinflammation may promote subsequent neurodegeneration over time (Kraft and Harry, 2011) and has been associated with deficits in learning and memory performance (Yirmiya and Goshen, 2011), it is possible that Tat-induced neuroinflammation may exert a significant early impact on brain microstructure segueing into measurable grey matter deficits and gross anatomical changes over time. Further studies comparing the time course of Tat-induced neuroinflammation in the prefrontal cortex with additional longitudinal imaging studies would be of potential interest. Moreover, as the present MRI findings support the idea that multiple brain regions are affected by conditional Tat expression, these data suggest the benefits of future evaluation of additional brain regions.

5.0 Conclusions

In summary, induction of brain-specific Tat expression in the GT-tg bigenic mouse reduced gray matter densities in brain regions subserving learning, memory, and neuroendocrine function. Our findings add to a growing body of evidence supporting the concept that Tat protein expression is sufficient to induce brain structural changes. While further neuroanatomical, behavioral and imaging studies are necessary to more fully characterize Tat effects, the present data support the concept that Tat protein associated with HIV infection may mediate some of the neuroanatomical and behavioral abnormalities found in HIV. If this concept is validated, then therapeutics that target brain Tat protein may be useful for treating HIV and co-morbid mood disorders identified in neuroAIDS patients (Kopnisky et al., 2007).

Acknowledgments

We thank Johnny He for the gift of the GT-tg transgenic breeder mice. We also thank Tim Gillis and Bonnie Adams for their assistance with the MRI scans.

Abbreviations used

AIDS	Acquired Immune Deficiency Syndrome
ANOVA	analysis of variance Dox doxycycline
FSL	FMRIB Software Library
GFAP	Glial Fibrillary Acid Protein
HIV	human immunodeficiency virus
IACUC	Institutional Animal Care and Use Committee
i.c.v	intracerebroventricular
i.p	intraperitoneal
LTP	Long term potentiation
MHz	Megahertz

MRI	magnetic resonance imaging
NU	Northeastern University
PLSD	protected least significant difference
ROI	region of interest
T	Tesla
Tat	Trans-activator of transcription
VBM	voxel based morphometry

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Highlights

- Tat protein is overexpressed in people with HIV
- We used MRI to study effects of conditional Tat protein expression on mouse brain
- We scanned fixed brains at 9.4 Tesla to measure Tat effects on gray matter density
- ; Affected areas are involved in learning and memory, which are abnormal in HIV
- Thus, Tat-induced brain changes could induce cognitive deficits in those with HIV

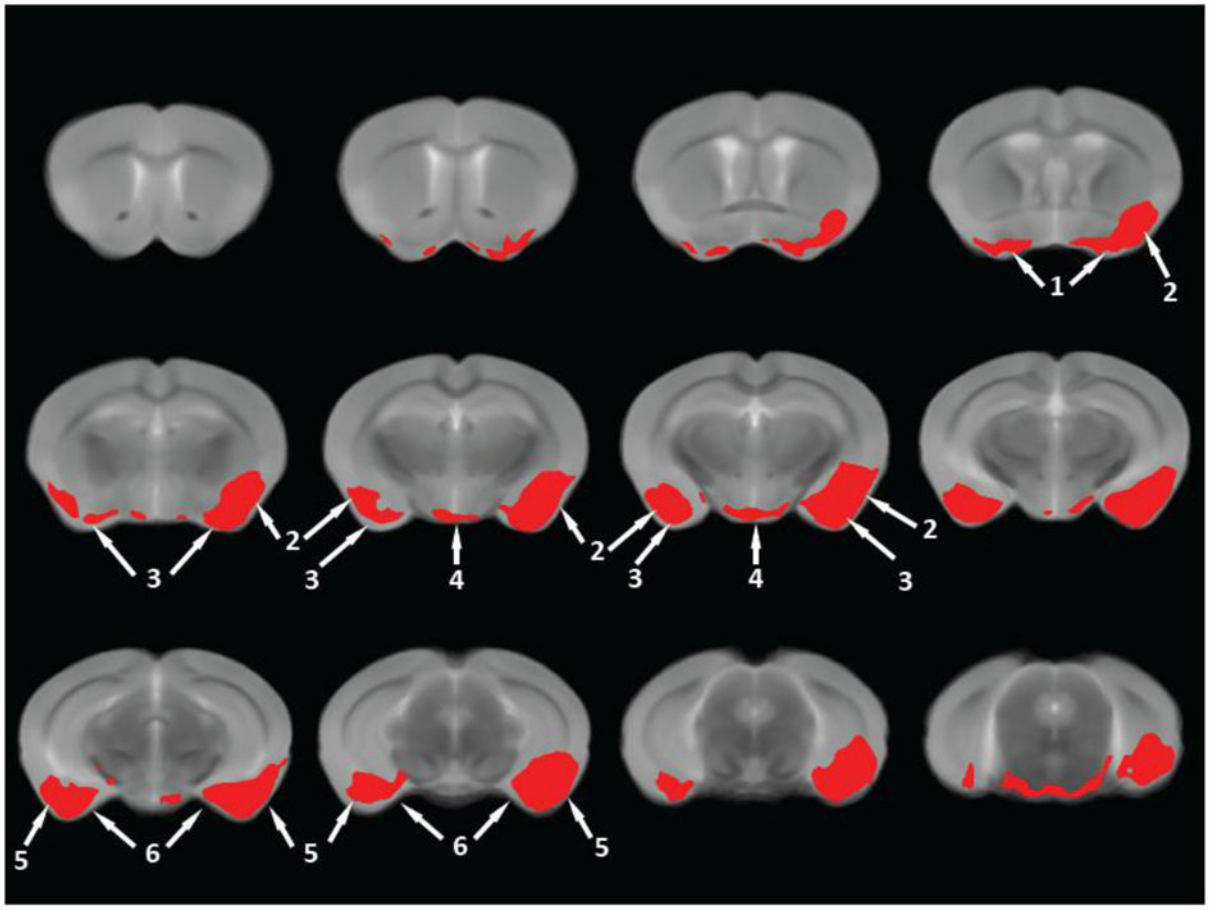

Figure 1. GT-tg mice treated with Dox (5 days) exhibited gray matter density reductions in several brain areas

The brains of the 5-day Dox-induced GT-tg mice exhibited gray matter density reductions versus Dox-treated C57Bl/6J mice. Gray matter abnormalities (red voxels overlaid on composite brain) were identified in the sublenticular extended amygdala (1), piriform cortex (2), amygdala (3), hypothalamus (4) peri-/entorhinal cortex (5), and amygdala-hippocampal area (6) (whole brain $p \leq 0.05$, multiple comparisons and cluster-corrected *t*-tests). Coronal brain sections are shown from roughly +0.74 mm to −4.20 mm Bregma (n = 8/group).

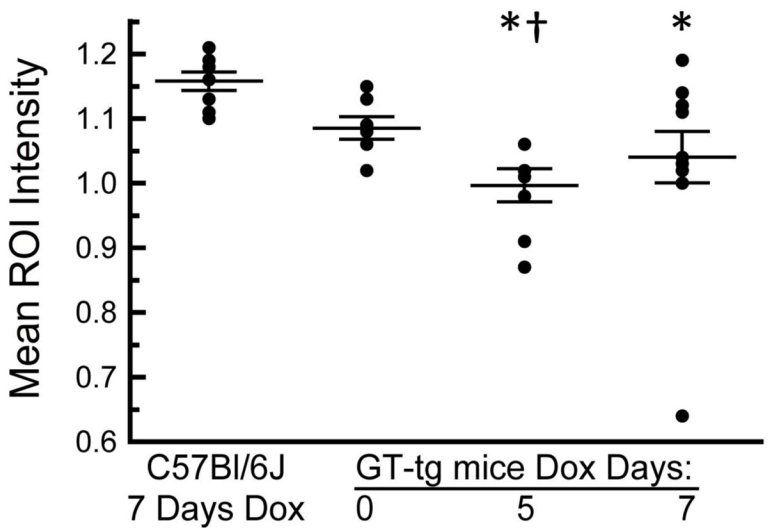

Figure 2. Post-hoc region of interest (ROI) analysis

A composite ROI was identified using the volumes identified in the VBM whole brain analysis in 4 adjacent coronal slices (Figure 1, slices 6–9) as differing between 5-day Dox-induced GT-tg mice and 7-day Dox-treated C57Bl/6J mice. Mean gray matter densities in this composite ROI were calculated for each of the 4 experimental groups. There was a significant gray matter density reduction in the composite ROI of 5-day ($p = 0.000055$) and 7-day ($p = 0.003523$) Dox-induced groups compared to 7-day Dox-treated C57Bl/6J mice (controls). Gray matter densities in control groups (7-day Dox-treated C57Bl/6J and uninduced GT-tg mice) did not differ in the composite ROI. (Legend: * = different from 7-day Dox-treated C57Bl/6J mice at $p < 0.004167$ (corrected); † = different from uninduced GT-tg mice at $p = 0.0273$ (uncorrected); Kruskal-Wallis H-test with post hoc testing).