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# Effects of Chronic Stress on Anxiety-like Behavior and Fear Learning in the TgF344-AD Rat Model of Alzheimer's Disease

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## ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative brain disorder that leads to severe cognitive and functional impairments. Many AD patients also exhibit neuropsychiatric symptoms (NPS) such as anxiety and prior to the clinical diagnosis of dementia. The prodromal manifestation of NPS is highly prevalent among patients with mild cognitive impairment (MCI), and the co-occurrence of preclinical NPS and MCI is associated with an increased risk of developing AD. Prolonged or repeated exposure to stress can result in behavioral disturbances (e.g., anxiety) and accelerated global cognitive decline. Importantly, AD patients exhibit altered stress systems and AD-related neuropathology has been linked to stress in transgenic (Tg+) mice. Collectively, these data suggest that altered stress systems may represent a causal link between NPS and AD. To address this possibility, we examined the effects of chronic

stress in adult male TgF344-AD (Tg+) and wildtype (WT) rats for footshock-induced conditioned fear and anxiety-like behavior in the elevated plus-maze (EPM). Results indicated that Tg+ rats display higher levels of anxiety-like behaviors in the EPM compared to WT controls with no differences in general locomotor activity, but these differences are not exacerbated by chronic stress exposure. Additionally, both stressed and non-stressed Tg+ and WT rats exhibited similar levels of fear-related behaviors with no differences in contextual fear learning. Several methodological issues are discussed that may have prevented our ability to detect a stress effect or differences in fear-related behaviors.

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## Introduction

*Epidemiology*

Alzheimer's disease (AD) is a significant health and societal problem that has no cure or preventative treatments (Sindi et al., 2015). AD is the leading cause of dementia among elderly adults accounting for 60–80% of all dementia cases (2019 Alzheimer's Disease Facts and Figures). Recent reports indicate that AD affects nearly 6 million Americans alone, and nearly 50 million people worldwide (World Alzheimer Report 2019). The societal costs of AD in the U.S. alone are estimated at $290 billion (2019 Alzheimer's Disease Facts and Figures), with a total worldwide cost exceeding 1 trillion dollars (World Alzheimer Report 2019). Tragically, rates of AD are increasing and are expected to reach epidemic proportions by 2050, with prevalence rates reaching 14 million in the U.S. and 150 million worldwide (Alzheimer's Disease Facts and Figures 2019). The resulting global economic burden is estimated to exceed 2 trillion (Alzheimer's Disease Facts and Figures 2019). Thus, there is a critical need for novel treatments which requires a complete understanding of the underlying neurobiological mechanism of AD.

*Clinical Features*

Clinically, AD is characterized by a hallmark gradual and irreversible deterioration in global cognitive function (e.g. language, attention, executive function) and impaired hippocampal-dependent learning and memory (Lazorov and Hollands, 2016). Additionally, AD patients exhibit emotional neuropsychiatric symptoms (NPS) such as anxiety and depression, which often precede the onset of hallmark AD neuropathological features (Geda et al., 2013: Jost and Grossberg, 1996: Lyketsos and Olin, 2002: Tayeb et al., 2014). Due in part to the broad overlap between anxiety and depression, and inconsistencies in self-report measures, there is considerable cross-sectional variance in the incidence of AD patients who have NPS. Indeed, the estimated percentage of AD

patients who exhibit anxiety symptoms ranges between 25% and 75% (Kaiser et al., 2014; Teri et al., 1999; Bergh and Selbæk, 2012), while the estimated percentage of AD patients who exhibit depressive symptoms ranges between 20% and 60% (Muliyala and Varghese, 2010; Tsuno and Homma, 2009; Steffens et al., 2009; Bergh and Selbæk, 2012). Importantly, individuals prone to psychological distress are twice as likely to develop AD (Wilson et al., 2003), while individuals with heightened anxiety have a 48% increased risk of developing AD (Petkus et al., 2016). In addition, NPS are highly prevalent among patients with mild cognitive impairment (MCI), and the co-occurrence of preclinical NPS and MCI increases the risk of developing AD (Gallager et al., 2017; Ismail et al., 2016).

*Genetics*

Typically, AD is diagnosed as either early-onset familial (FAD) or late-onset sporadic. FAD is a rare, autosomal-dominant inherited form of the disorder (~5% of all AD patients) that typically manifests before the age of 65. In contrast, sporadic AD is the predominate, non-heritable form of the disorder (~95% of all AD patients) that usually manifests after the age of 65 (Bird, 1993; Bird, 2008; Ortiz et al., 2015; Bekris et al., 2010). Three genetic mutations have been identified as independent causal factors for developing FAD: amyloid precursor protein (APP), and presenilin 1 (PS1) and presenilin 2 (PS2) genes. Each of these FAD-linked mutations directly alters the amyloidogenic processing of APP to enhance the production, and possibly reduce the clearance, of amyloid-beta (Aβ) peptides, resulting in increased cerebral deposition of "senile" neurotoxic Aβ plaques (O'Brien & Wong, 2011). Additional neuropathological features include formation of intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau proteins (tau-P), glial activation and inflammation, neurodegeneration and cell loss (Newman et al., 2007; Baranello et al., 2015; Zuroff et al., 2017; Nilsson et al., 2013; Sasaguri et al., 2017;

Mawuenyega et al., 2010; Kline, 2012; O'Brien and Wong, 2011; and Wang et al., 2006; Selkoe et al., 2001). There are currently no known genetic causes for most sporadic AD cases.

Although the precise causes of AD are unknown, previous research provides strong evidence for the "amyloid cascade hypothesis". This theory advocates that accumulation of Aβ is the initiating event in AD pathogenesis, and that deposition of cerebral Aβ is both necessary and sufficient for development of AD (for review see Hardy & Selkoe, 2002; Cohen et al., 2013). The strongest support for the "amyloid cascade hypothesis" comes from human genetic evidence, which shows that inheritance of any one or more of the autosomal dominant mutaitons (APP, PS1 or PS2) invariably leads to FAD (Musiek and Holtzman, 2015). Further support stems from human patients with trisomy 21 (i.e. Down's syndrome) harboring triple APP duplications. These patients consequently develop Aβ plaques and exhibit AD-related pathologies (Selkoe and Hardy, 2016). Mutations near the N-terminal of Aβ (Swedish double mutation; APPsw) increase total Aβ production by modulating B-secretase cleavage of APP, while mutations in PS1 and PS2 cause a shift of γ-secretase cleavage, favoring the production of the more AD pathogenic Aβ42 peptide conformation (as opposed to less pathogenic Aβ41; Weggen & Beher, 2012). Initial preclinical animal models of AD were generated based on these FAD-linked mutations.

*Rodent Models*

Transgenic (Tg+) mice have been used extensively in AD research to model the structural and functional abnormalities observed in AD patients. Most Tg+ rodent models of AD overexpress different combinations of FAD-linked mutations (APP or APP/PS1) and exogenous human tau mutations (h-Tau), which alter APP and tau processing respectively, to accelerate certain aspects of AD pathology (Sasaguri et al., 2017). However, as discussed below, none of these Aβ or tau overproducing Tg+ rodent models, or even triple transgenic mice (3xTg-AD) which develop both

Aβ plaques and NFTs, have been able to fully recapitulate in an age-dependent manner the full spectrum of hallmark AD pathological features (Kitazawa et al., 2012).

Overall, APP Tg+ mice (e.g., PDAPP, APP23 and Tg2576 models; Games et. al, 1995; Sturchler-Pierrat et. al, 1997; Hsiao et. al, 1996) and APP/PS1 Tg+ mice (Tg2576 × PS1 and APPswePS1ΔE9; Holcomb et. al, 1998; Jankowsky et. al, 2001) develop age dependent Aβ pathology and cognitive deficits. Specifically, APP and APP/PS1 mice overproduce Aβ resulting in downstream Aβ deposition and cerebral Aβ plaque burden throughout the hippocampus and cortex. They also exhibit impaired hippocampal-dependent behavior, particularly on spatial memory tasks. Additionally, APP/PS1 Tg+ mice exhibit accelerated and more pronounced Aβ plaque pathology compared to only APP mice. However, both APP and APP/PS1 Tg+ mice are limited in their human translational use as they fail to exhibit widespread neurodegeneration (e.g. synaptic connectivity deficits and neuronal loss) and do not develop robust tauopathy including tau-P or NFT formation (Drummond & Wisniewski, 2017). Furthermore, APP/PS1 Tg+ mice expressing five FAD mutations (5xFAD mice) develop Aβ-related pathologies at a very early age (2-3 months), which does not resemble the same pathological time course observed in human AD patients (Oakley et al., 2006; Sasaguri et al., 2017).

Since WT mice do not develop endogenous tau-P or NFTs (Jankowsky & Zheng, 2017), Tau Tg+ mouse models overexpress exogenous human mutant tau (h-Tau) genes to induce tau-related pathology. However, human tau mutations have not been directly implicated as genetic causes of FAD (Bekris et al., 2010; Kitazawa et al., 2012). These Tau Tg+ mice (e.g., P301L, rTg4510 and hTau mice) develop robust tauopathy (e.g. tau-P and NFTs), neurodegeneration, and structural atrophy and functional impairments in AD-relevant brain regions (e.g. hippocampus) (Song et al., 2015; Jankowsky & Zheng, 2017; Gamache et al., 2019). However, Tau Tg+ mice do

not develop robust Aβ pathology unless they additionally co-express mutant APP transgenes as a double (tau/APP) phenotype (Duyckaerts et al., 2008; Yetman et al., 2016; Sasaguri et al., 2017; Jankowsky & Zheng, 2017). These Tau Tg+ models are also limited in their translational use as they require overexpression of non-familial exogenous human tau mutations to produce robust tauopathy, lack endogenous tau-P and NFTs, and lack essential age-dependent Aβ-related pathologies (Roberson et al., 2007; Jankowsky & Zheng, 2017). Lastly, double (tau/PS1) Tg+ mice, which lack APP mutations, do not exhibit either robust Aβ pathology (e.g. cerebral Aβ plaque deposition) or significant neuronal loss (Yetman et al., 2016; Jankowsky & Zheng, 2017).

Recently, a 3xTg-AD mouse model was developed to concurrently express APP and PS1 mutations, as well as exogenous human tau mutations (Oddo et. al, 2003). These 3xTg-AD mice exhibit both age-dependent Aβ and tau-related pathologies, including progressive Aβ plaque deposition, tau-P and NFT formation, and are regarded as the most comprehensive Tg+ mouse model of AD pathology (Sterniczuk et al., 2010a; Sterniczuk et al., 2010b). In addition, the 3xTg-AD mouse model manifests other essential pathological features typically not present in APP/PS1 or h-tau Tg+ models, including neurodegeneration and cognitive impairments. However, 3xTg-AD mice are still limited in their translational capacity to human AD pathology since their observed robust tauopathy and neuronal loss is entirely dependent on the overexpression of exogenous h-tau transgenes which are not genetically linked to human FAD. Even though 3xTg-AD mice show signs of neurodegeneration, the overall levels of synaptic dysfunction and neuronal loss are not comparable to human AD pathology (Drummond and Wisniewski, 2017).

The development of various Aβ and tau Tg+ models have been an invaluable resource in facilitating our understanding of the underlying pathophysiological mechanisms of AD (Epis et al., 2010, Jucker, 2010). However, because these models do not manifest the full spectrum of AD-

related pathophysiology (Casadesus et al., 2010) they may display attenuated behavioral disturbances limiting their ability to effectively model early-stage subtle changes in behavior (Saraceno et al., 2013; Webster at al., 2014; Jankowsky & Zheng 2017). Indeed, previous studies examining anxiety-like behavior in various Tg+ rodent models have reported inconsistent and contradictory findings across different behavioral tests. For example, some studies report decreased anxiety-like behavior in EC-APP (EPM; Harris et al., 2010), Tg2576 (EPM; Gil-Bea et al., 2007, Lalonde et al., 2003, Ognibene et al., 2005), and 5xFAD Tg6799 (EPM; Jawhar et al., 2012) mice, while other studies report increased anxiety-like behavior in APP/hAβ/PS1 (EPM and light-dark test: Guo et al., 2012), Tg2576 (light-dark box test; Dong et al., 2012), PDAPP-J20 (open field test; Beauquis et al., 2014), APPSWE (EPM; Bedrosian et al., 2011), and TgAPP (EPM; Lee et al., 2004) mice. Other studies report no alterations in anxiety-like behavior in TgCRND8 (open field test and EPM; Touma et al., 2004) or Tg2576 mice (EPM; Arendash et al., 2001), or in McGill-R-Thy1-APP rats (EPM; Galeano et al., 2014). These contradictory findings may result from inherent phenotypic differences in behavioral disinhibition (i.e. increased locomotor activity) that appear as reduced anxiety-like behavior (Gil-Bea et al., 2007; Lalonde et al., 2003; Ognibene et al., 2005; Roberson et al., 2007). Additionally, the onset of an anxiogenic phenotype may vary depending on the mouse model and/or stage of amyloid/tau burden. For example, APP/PS1 Tg+ mice develop early behavioral disturbances between 3-6 months of age (Lalonde et al., 2004; Reiserer et al., 2007), while Tg2576 mice manifest behavioral disturbances between 9–11 months of age (Gil-Bea et al., 2007). Further, studies often fail to characterize early anxiogenic-like phenotypes in Tg+ rodents that are examined at the “preamyloid stages” of AD development (Boon et al., 2010, Galeano et al., 2014, Lalonde et al., 2002, Lee et al., 2004).

Collectively, these inconsistent findings highlight limitations of Tg+ mice in accurately modeling preclinical AD behavioral pathology.

*TgF344-AD Rat Model*

In an attempt to overcome some of the limitations associated with Tg+ mice, a rat Tg+ model of AD (TgF344-AD) was recently created that overexpresses the two autosomal dominant mutant human transgenes APPsw and PS1ΔE9. The APPsw and PS1ΔE9 mutations alter the normal processing of endogenous APP to favor the overproduction and increased aggregation of Aβ, which invariably leads to extensive cerebral amyloidosis and subsequent Aβ-driven tau pathology, neuroinflammation, and widespread neuronal loss (Cohen et al., 2013). As a result, TgF344-AD rats are genetically susceptible to both progressive accumulation of Aβ plaques and aggregation of tau-P into NFTs (Cohen et al., 2013; Carmo & Cuello 2013). Importantly, TgF344-AD rats manifest cerebral Aβ plaque deposition in an age-dependent manner that precedes (and possibly initiates) other downstream AD-relevant pathologies. Also, non-pathological levels of (soluble) Aβ and tau-P, as well as increased glial activation, are initially present in male TgF344-AD rats at 6 months of age prior to appreciable extracellular Aβ deposition and cerebral plaque burden (Cohen et al., 2013). By 15-16 months of age, TgF344-AD rats exhibit the full spectrum of hallmark AD pathological features, along with global cognitive disturbances and impairments in hippocampal-dependent learning and memory (Cohen et al., 2013; Do Carmo and Cuello, 2013; Saraceno et al., 2013). Notably, TgF344-AD rats displays early-stage alterations in emotional responses, particularly heightened anxiety-like behavior, prior to appreciable cerebral plaque burden and NFT formation (Pentkowski et al., 2018).

A significant advantage of the TgF344-AD rat model compared to the Aβ and tau-overproducing 3xTg-AD mouse model is that it manifests robust tauopathy without requiring

overexpression of exogenous h-tau transgenes, relying solely on endogenous rat tau protein to facilitate tau-P and NFT pathologies (Cohen et. al, 2013). Moreover, TgF344-AD rats displays progressive learning and memory deficits, as indicated by significantly decreased performance in object recognition at 24 months (Cohen at al., 2013) and spatial memory in the Morris water task at 10-11 months of age (Berkowitz et al., 2018). Importantly, noticeable behavioral (Cohen et al., 2013) and emotional (Pentkowski et al., 2018) disturbances are present in young adult TgF344-AD rats prior to significant cerebral amyloidosis and cognitive impairments, mimicking the progression of NPS commonly observed in preclinical human AD patients (Geda et al., 2013: Jost and Grossberg: 1996, Lyketsos and Olin, 2002: Tayeb et al., 2014). While other Aβ-overproducing Tg+ rodent models also develop similar age-dependent Aβ pathogenesis as the TgF344-AD rat model, they typically fail to entirely recapitulate all the other essential clinical features of human AD pathology, such as NFTs and neuronal loss (Braidy et al., 2012: Do Carmo and Cuello, 2013: Hall and Roberson, 2012: LaFerla and Green, 2012). Indeed, both limited tauopathy (e.g. tau-P & NFTs) and minimal neuronal loss are also observed in APP/PS1 mouse models overexpressing the same (APPsw/PS1ΔE9) transgene mutations as the TgF344-AD rat model (Elder et al., 2010: Hall and Roberson, 2012). In addition, APP/PS1 rat models harbor the same (APPsw/PS1ΔE9) transgene mutations but also utilize a different promoter site to drive transgene expression and fail to exhibit robust tau-related pathologies and neurodegeneration (Do Carmo and Cuello, 2013).

*Stress and AD Studies*

A common physiological risk-factor for developing AD is chronic stress (Carroll et al., 2011). Recently, AD research has focused on the role of the corticotropin-releasing factor (CRF) signaling system in mediating enhanced sensitivity to stress (Campbell et al., 2015: Carroll et al., 2011; Dong et al., 2014; Kang et al., 2007; Rissman et al., 2007; Rissman et al., 2012). CRF is a

41-amino acid neuropeptide that controls the autonomic, behavioral, immune, and endocrine responses to stress (Rissman et al., 2007) via actions at two G protein-coupled receptors, termed $CRF_1$ and $CRF_2$ (Litvin et al., 2007; Spiess et al., 1981; Vale et al., 1981). Chronic stress-induced activation of the hypothalamic pituitary adrenal (HPA) axis leads to an increase in release of CRF and an increase in $CRF_1$ expression (Carroll et al., 2011; Dong et al., 2014; Rissman et al., 2012), resulting in accelerated adrenal glucocorticoid secretion and elevated concentrations of circulating corticosteroid hormone levels (cortisol in humans and corticosterone (CORT) in rodents; Pariante and Lightman, 2008; Kloet et al., 2005;; Herman et al., 2016). HPA axis dysregulation and elevated CRF signaling are associated with impaired CNS functions, including hippocampal-dependent learning and memory, and anxiety-like behavior (Pentkowski et al., 2009; Stephens & Wand 2012; Kasahara et al., 2011; Smith & Vale 2006; Orozco-Cabal et al., 2006). Importantly, increased CRF signaling, HPA axis activity, and increased anxiety-like behavior are reported in Tg+ rodent models of AD overexpressing human mutations in APP or tau processing (Dong et al., 2014: Guo et al., 2012; Touma et al., 2004). These findings suggest that dysregulation of the CRF systems may be one causal mechanism underlying AD-related pathology.

Stress system abnormalities in AD patients have also been linked to impairments in executive function, especially learning and memory deficits (Orozco-Cabal et al., 2006). Both AD and stress-related NPS are characterized by elevated cortisol levels (for review see Justice 2018; Lucassen et al., 2014; Vyas et al., 2016), heightened HPA axis activation (Swaab et al., 2005; Holsboer and Ising, 2008; Pervanidou and Chrousos, 2010), and increased CRF signaling and expression of $CRF_1$ throughout limbic regions (Gold and Chrousos, 2002). In human AD patients, chronic stress induces both structural atrophy and functional dysregulation of critical

brain structures implicated in the HPA axis-mediated neuroendocrine stress response including the amygdala, hippocampus and prefrontal cortex (McEwen, 1999; Kim and Diamond, 2002; Sapolsky, 2002; Bremner, 2006). These neural insults result in reduced hippocampal synaptic plasticity (e.g. decreased LTP) and impaired hippocampal-dependent learning and memory (Landfield et al., 1978; Kerr et al., 1991; Dachir et al., 1993; Bodnoff et al., 1995; Alfarez et al., 2003;). These data suggest that chronic stress may be one of the initial triggers for initiating AD pathogenesis, as the hippocampus is one of the initial brain regions affected in the early stages of AD development (Arnold et al., 1991; Price and Morris, 1999).

Perturbations in endocrine function have also been reported in Tg+ mice. For instance, TgCRND8 (Touma et al., 2004) and Tg2576 (Dong et al., 2008) mice exhibit elevated circulating CORT levels. APP/hAβ/PS1 Tg+ mice, which overexpress two APP mutations with the addition of a PS1 mutation, exhibit both enhanced Aβ deposition and increased CRF levels in crucial, stress-related neural circuits throughout the cortico-limbic system and display an anxiogenic-like phenotype (Guo et al., 2012). Interestingly, cognitive deficits and impaired working memory in the APP/hAβ/PS1 mouse model were not reversed following the partial loss of $CRF_1$ (e.g. $CRF_1$ hemizygosity) but normalized circulating CORT levels, suggesting that elevated $CRF_1$ signaling may account for enhanced anxiety-like behavior at early stages in TgF344-AD rats prior to the onset of Aβ plaque and NFT-related memory and cognitive impairments (Cohen et al., 2013). Importantly, evidence from studies using Tg+ models suggests that heightened stress signaling precedes both Aβ and tau-related AD pathologies (Dong et al., 2008: Guo et al., 2012: Hebda-Bauer et al., 2013: Rothman et al., 2012). Chronic stress-induced elevations in basal CORT levels and increased $CRF_1$ expression lead to enhanced Aβ accumulation and Aβ plaque deposition (Dong et al., 2008; Dong et al., 2012), and increased tau

hyperphosphorylation (Caroll et al., 2011; Campbell et al., 2015). Subsequently, stress-induced neurodegeneration and cognitive impairments involve $CRF_1$-dependent alterations in the pathological production and aggregation of both Aβ (Dong et al., 2004: Dong et al., 2012: Dong et al., 2014: Justice et al., 2015: Kang et al., 2007: Zhang et al., 2016) and tau-P (Carroll et al., 2011: Rissman et al., 2007: Rissman et al., 2012), particularly in the hippocampus and amygdala. Overexpression of CRF mimics the effects of chronic stress and accelerates Aβ deposition, neurodegeneration, and behavioral deficits in Tg2576 mice harboring mutations in APP processing (Dong et al., 2012). Furthermore, in 3xTg-AD mice Aβ deposition was found not only to precede, but also to accelerate, tau-related pathology (Oddo et al., 2003a; Oddo et al., 2003b).

Collectively, these data suggest that the neurobiological mechanisms underlying the anxiogenic-like phenotype in TgF344-AD rats may involve alterations in stress signaling. Specifically, elevated levels of CORT and $CRF_1$ in the hippocampus and amygdala may account for the early heightened anxiety-like behavior observed in TgF344-AD rats during the preclinical stages of AD. Additionally, these results suggest a potential $CRF_1$-dependent mechanism underlying stress-induced AD neuropathogenesis resulting in cognitive and behavioral impairments. These findings add to the growing body of literature which implicate the important role of $CRF_1$ in modulating both early- and late-stage AD pathologies. In the present study, explored the effects of chronic stress on the onset and progression of AD, using the TgF344-AD rat model. We hypothesized that Tg+ rats would exhibit significantly enhanced anxiety-like behavior and conditioned fear compared to WT rats and that chronic stress would further exacerbate anxiety-and fear-related behaviors in both Tg+ and WT rats, with stressed Tg+ rats exhibiting the highest levels.

## Materials & Methods

*Animals*

Subjects were adult male WT (n=37) and Tg+ (n=27) rats from the TgF344-AD rat model of AD (Cohen et al., 2013). TgF344-AD rats were originally generated on a Fischer 344 background by co-injecting rat pronuclei with 2 human genes driven by the mouse prion promoter: "Swedish" mutant human amyloid precursor protein (APPsw) and Δexon 9 mutant human presenilin-1 (PS1DE9). TgF344-AD rats display a progressive increase in the characteristic AD clinical pathology associated with the human condition, including plaques, tangles, and cognitive impairment (Cohen et al.,2013; Tsai et al., 2014), and enhanced anxiety-like behavior prior to significant plaque pathology (Pentkowski et al., 2018). Rats were obtained from the Psychology Department's Animal Resource Facility breeding colony.

Following weaning (~21 days of age), rats were pair-housed with a same-sex littermate maintained under controlled temperature (21-22* Celsius) and illumination (12-hour light/dark cycle, lights off at 09:00 AM) with free access to food and water. Rats were handled 2-3 times per week for 2 minutes beginning 3 months prior to start of behavioral testing. The housing conditions and testing procedures conducted on rats in these experiments were in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and were reviewed and approved by the Institutional Animal Care and Use Committee at the University of New Mexico. All efforts were made to minimize any animal pain and/or suffering.

*Genotyping*

Confirmation of transgene expression for the APP & PS1 mutations was determined by real-time polymerase chain reaction (RT-PCR) amplification of DNA using a specific probe sequence designed to detect the presence (or absence) of the mutated APPsw transgene. The

transgenic mutation assays were outsourced to an automated genotyping services company (Transnetyx, Cordova, TN).

Tissue samples were collected by ear punches (on one or both ears) which were used as a permanent form of study/subject identification and for genotyping. All ear punching was performed on weaned rats (>21 days old) in accordance with standard ear punch/notch identification codes (Harkness & Wagner, 1995) that correspond to specific numbers (within the range of 100-200). Ear punched tissue (1-2 pieces) from each rat was immediately placed into separate wells of a TransnetYX wellplate. Additional tissue (1-2 pieces) was collected from each rat and stored in plastic test tubes at -20 Celsius.

Accurate genotyping was obtained by comparing unidentified/unconfirmed rat tissue samples against a reference tissue sample from a confirmed positive TgF344-AD rat. Following (positive or negative) confirmation of (APPsw) transgene expression, Tg+ and WT rats were randomly assigned to either a control or stress condition; pair-housed rats were assigned to the same stress group and were matched for genotype. Overall, rats were separated into one of four comparison groups based on genotype and stress condition: WT control, WT stress, Tg+ control and Tg+ stress.

*Chronic Restraint Stress*

Previous work on the differential effects of stress across rat species (Rex et al., 1999; Dhabhar et al., 1993; Sternberg 1992) has found that Fischer 344 (F344) inbred rats are hypersensitive to stressful stimuli and exhibit heightened susceptibility to repeated stress compared to other inbred rat strains. Yet a major concern with prolonged exposure to (non-variable) repeated stress is that animals will eventually habituate to the stressful testing environment, resulting in a gradual reduction of the elicited HPA axis stress response. However,

Dhabhar and colleagues (1997) reported that F344 rats display heightened activation of the HPA axis response to acute stress without habituation of the stress response after 10 days of repeated stress. Furthermore, Uchida et al. (2008) reported no significant (or limited) habituation of the stress response in F344 rats following 14 consecutive days of 2-hr restraint stress. Additionally, chronic restraint stress increases anxiety-like behaviors including a decrease in open arm entries and open arm duration in the EPM (Chiba et al., 2012) and fewer entries and less time spent in the light compartment in the LDT (Han et al., 2015). Thus, the TgF344-AD rats generated on a Fischer 344 backbone are an ideal model to examine the effects of chronic restraint stress on the development of AD.

Chronic restraint stress was conducted between 6-7 months of age based on the absence of most AD pathological features at this timepoint, including appreciable cerebral amyloidosis and extensive neuronal loss, and limited cognitive disturbances (Cohen et al., 2013). Rats assigned to the stress condition were exposed to 14 consecutive days of 1-hour restraint stress prior to the start of behavioral testing. For each restraint session, four stress rats (WT and/or Tg+ rats) were transferred to a separate testing room and placed into one of the four individual close-ended transparent plastic cylinder restraint tubes (Harvard Apparatus). Once rats were inside the restraint tube, an adjustable door insert was placed behind the rat's body to prevent rats from escaping during the 1-hour restraint stress period. Restraint stress sessions were conducted in the same testing room each day under white light with a white-noise generator during the entire 1-hour stress period. Rats from both stress conditions were weighed each day to help verify that the restraint stress paradigm was an effective stress-inducing treatment resulting in weight loss. For each testing cohort, on Day 1 the initial/baseline weight measurements were recorded for all rats

prior to the start of the first restraint stress session. For Days 2-14, all animals had their weight measurements recorded immediately after each 1-hour restraint session.

*Behavioral Testing*

Behavioral testing was conducted 24 hours after the final restraint stress session for each rat cohort. Behavioral tests were conducted on three consecutive days between 12:00 and 15.00 hours in the following order: EPM, post footshock (FS) freezing and FS-induced contextual freezing. Animals were tested individually in each of the behavioral tests and all test trials were recorded (Lorex) for subsequent analysis. All non-automated behaviors for each test were scored by a single well-trained observer blind to group assignment using the behavior scoring software SimpleVideoCoder (Barto et al., 2017), while all automated behaviors were analyzed using ANYmaze (Stoelting Co, Wood Dale, IL, USA); rat movements were automatically tracked using the midline as the frame of reference.

The EPM test measured anxiety-like behaviors by assessing exploration and avoidance of the open spaces (EPM). The FS test evaluated post shock and contextual-induced conditioned fear during re-exposure to the threatening test environment by assessing time spent freezing (Blanchard et al., 1969). The EPM test was conducted under red light, whereas FS tests were conducted under white light. A white noise generator was used during all behavioral testing to standardize background noise. Each testing apparatus was thoroughly cleaned between each trial using a 10% ethanol solution.

*Elevated-Plus Maze*

All rats were tested for anxiety-like behavior in the EPM (Handley and Mithani, 1984; Pellow et al., 1985) using previously published protocols (Pentkowski et al., 2009). The EPM apparatus consisted of 4 Plexiglas arms arranged as a plus sign, elevated 75 cm above the floor.

Each arm was 10-cm wide and 50-cm long, and each arm was joined at the center by a 10-cm square platform. The 2 “open” arms contained no walls, whereas the 2 opposite “closed” arms contained 40-cmtall opaque sides. Each individual rat was initially placed in the center arm of the apparatus facing 1 of the 2 closed arms. Tests were 5-min in duration and were conducted under red light. All test trials were recorded from an “overhead view” camera over the center of the EPM apparatus and a “side view” camera facing one of the open arms. Behavioral measures included head dips, head outs, stretch approach, stretch attend and the duration of time spent in the open and closed arms, and total distance travelled.

*Footshock*

The footshock test apparatus (12’’W x 10’’D x 12’’H) was constructed of steel grids with a clear front panel to permit observation and recording. The FS testing paradigm consisted of two phases: A 10-minute FS Acquisition test (Day 1) and a 5-minute FS Re-Test (Day 2). On the FS acquisition day, rats were exposed to the FS chamber for the first time and electric foot shocks were delivered from a (Coulbourn) manual shock device via a steel grid floor. After a 3-min habituation period, three separate foot shocks (1.0 mA, 1 s duration) were delivered at 1-min intervals, followed by a 5-min observation period (post-FS freezing test). No additional shocks were administered during the last 5-min testing period. Behavioral measures (frequency and duration of freezing) were scored only during the last 5 min of the FS acquisition day. Twenty-four hours later subjects were retested for 5 min (FS Re-test day) in the same apparatus without the shock stimulus (context-conditioning test). Behavioral measures were scored throughout the 5-min testing period. Each test session was conducted under white light and the FS testing chamber was cleaned between trails using 10% ethanol solution.

*Behavioral Measures*

Descriptions and scoring criteria of the fear (FS) anxiety-like (EPM) behavioral measures used in this study were previously described (Pentkowski et al., 2009). Briefly, Freezing: complete cessation of movement other than respiration was measured during both FS test trials. Measures during the EPM trials included: Head outs: head movement (up to ears) out from the inherently preferred closed arm without complete entry; Head-dips: extension of the subjects head over the edge of an open arm; Risk assessment composite: combined measure of both *stretch approach*-forward ambulation with flat back and stretched neck, and *stretch attend*-standing on all four paws with flat back and stretched neck orientated toward the threat source; Avoidance**:** proportion of time spent in the open versus closed arms; Number of Open and Closed Arm Entries: measured as any movement from one section of the EPM apparatus to; and Total Distance Traveled: Measured as the total locomotion/movement for the total 5-min testing period.

*Behavioral Scoring & Statistical Analysis*

Each dependent variable was analyzed using separate two-way factorial ANOVAs with genotype (WT, Tg+) and stress (stress, no stress) groups as between subject factors. Significant effects were further analyzed using independent samples t-tests to provide pairwise comparisons. The level of significance for alpha was set at $p < .05$ for all comparisons.

**Results**

*EPM*

Figure 1 presents the effects of genotype and stress on anxiety-like behaviors in the EPM. The ANOVAs failed to detect a genotype by stress group interaction ($F(1,60) = 0.903$, $p = 0.346$) or a main effect of stress ($F(1,60) = 1.101$, $p = 0.298$) for the percent of time spent in the open arms, but there was a main effect for genotype ($F(1,60) = 11.673$, $p = 0.001$). Subsequent

analyses indicated that non-stressed Tg+ rats spent significantly less time in the open arms compared to non-stressed WT rats $t(28) = 2.957$, $p = 0.006$; this same pattern of effects also occurred between stressed Tg+ rats and stressed WT rats but the effect was only marginal $t(32) = 1.825$, $p = 0.077$. The ANOVAs for the number of open arm entries failed to detect a significant interaction ($F(1,60) = 0.243$, $p = 0.624$) or a main effect of stress ($F(1,60) = 0.409$, $p = 0.525$), but the main effect of genotype nearly reached significance ($F(1,60) = 3.043$, $p = 0.086$). Subsequent analyses revealed that Tg+ rats were trending towards significantly fewer open arm entries compared to WT rats $t(62) = 1.761$, $p = 0.083$. However, upon further examination no significant differences in open arm entries occurred between non-stressed Tg+ rats and non-stressed WT rats $t(28) = 1.441$, $p = 0.161$, as well as no significant differences in open arm entries occurred between stressed Tg+ rats and stressed WT rats $t(32) = 0.974$, $p = 0.338$. The ANOVAs for risk assessment failed to detect a significant interaction ($F(1,60) = 0.357$, $p = 0.553$), a main effect of stress ($F(1,60) = 0.424$, $p = 0.517$) or a main effect of genotype ($F(1,60) = 0.388$, $p = 0.536$). The ANOVAs for number of head dips failed to detect a significant interaction ($F(1,60) = 0.086$, $p = 0.771$) or a main effect of stress ($F(1,60) = 1.820$, $p = 0.182$), but there was a main effect of genotype ($F(1,60) = 4.117$, $p = 0.047$). Subsequent analyses indicated that Tg+ rats exhibited fewer head dips compared to WT rats but the effect was only marginal $t(62) = 1.962$, $p = 0.054$. However, upon further examination non-stressed Tg+ rats exhibited marginally significant fewer head dips compared to non-stressed WT rats $t(28) = 1.824$, $p = 0.079$, but no significant differences in the number of head dips occurred between stressed Tg+ rats and stressed WT rats $t(32) = 1.158$, $p = 0.255$. The ANOVAs for total distance travelled failed to detect a significant interaction ($F(1,60) = 0.222$, $p = 0.639$), a main effect of stress ($F(1,60) = 0.059$, $p = 0.809$) or a main effect of genotype ($F(1,60) = 0.177$, $p = 0.676$).

Collectively these results indicate that Tg+ rats display higher levels of anxiety-like behaviors compared to WT controls with no differences in general locomotor activity, and that these differences are not exacerbated by chronic stress exposure.

*Footshock*

Figure 2 displays the effects of genotype and stress during the footshock test trials. The ANOVAs failed to detect a genotype by stress group interaction ($F(1,60) = 0.278$, $p = 0.600$), a main effect of stress ($F(1,60) = 1.562$, $p = 0.216$), or a main effect of genotype ($F(1,60) = 0.079$, $p = 0.780$) during the post footshock test session. Similarly, the ANOVAs for the context conditioning test trial failed to detect a genotype by stress group interaction ($F(1,60) = 0.353$, $p = 0.554$), a main effect of stress ($F(1,60) = 0.201$, $p = 0.655$), or a main effect of genotype ($F(1,60) = 1.502$, $p = 0.225$). These results indicate that both stressed and non-stressed Tg+ and WT rats exhibited similar levels of fear-related behaviors with no differences in contextual fear learning.

*Stress-induced weight loss*

Figure 3 displays the weight loss following 14 consecutive days of restraint stress. The ANOVAs failed to detect a genotype by stress group interaction ($F(1,60) = 2.182$, $p = 0.145$) or a main effect of genotype ($F(1,60) = 0.347$, $p = 0.558$) for total weight loss, but there was a main effect of stress ($F(1,60) = 174.953$, $p < 0.001$). Subsequent analyses revealed that stressed Tg+ rats lost significantly more weight compared to non-stressed Tg+ rats $t(25) = 11.307$, $p < 0.001$. Additionally, stressed WT rats lost significantly more weight compared to non-stressed WT rats $t(35) = 8.304$, $p < 0.001$. The ANOVA for percent total weight loss failed to detect a genotype by stress interaction ($F(1,60) = 1.434$, $p = 0.236$) or a main effect of genotype ($F(1,60) = 0.251$, $p = 0.618$), but there was a main effect of stress ($F(1,60) = 197.223$, $p < 0.001$). Subsequent analyses revealed that percent total weight was significantly decreased in stressed Tg+ rats compared to

non-stressed Tg+ rats $t(25) = 12.644$, $p < 0.001$. Furthermore, percent total weight was significantly decreased in stressed WT rats compared to non-stressed WT rats $t(35) = 8.813$, $p < 0.001$. Collectively, these results indicate that chronic restraint was an effective stressor.

**Discussion**

The present results are consistent with a previous report by that found that TgF344-AD rats exhibit early signs of increased anxiety-like behavior in the EPM at a timepoint where AD-related neuropathology is minimal (Pentkowski et al., 2018; Tournier et al., 2020). Specifically, the non-stressed Tg+ rats in the present study showed increased anxiety-like behavior in the EPM spending significantly less time in the open arms, as well as trends towards fewer head dips and open arm entries compared to non-stressed WT rats (Figure 1). Similarly, stressed Tg+ rats showed a trend toward decreased open-arm time compared to stressed WT rats. Importantly, we observed a null effect on total distance travelled across all groups. This mitigates the possibility that group differences in open arm time between genotypes was due to differences in general locomotor activity. In contrast, stressed Tg+ rats were not significantly different from stressed WT rats on any measures of anxiety-like behavior. Thus, chronic stress exposure did not significantly enhance levels of anxiety for Tg rats. While stress has been linked as a risk factor for AD-related pathology, it appears from the present findings that stress did not further enhance anxiety-like behaviors in these Tg+ rats. Collectively, these data further validate that TgF344-AD rats represent a promising animal model to study AD-related anxiety at the MCI stage of the disease.

In the footshock fear conditioning test, we observed no difference in freezing between Tg+ and WT rats regardless of the stress conditions (Figure 2). These null effects were detected immediately post shock as well as twenty-four hours after the shock trials during the contextual fear conditioning test trial. Therefore, we conclude that elevated levels of "fear" are not present

during the early stages of AD in the TgF344-AD model. These results are consistent with previous reports that found that TgF344-AD rats exhibit normal learning compared to WT rats at early timepoints where cerebral pathology is low (Cohen et al., 2013 and Berkowitz et al., 2018). Importantly, the present results extend the previous reports to include normal contextual fear learning. This observed null effect could be explained by several factors. First, there may be no differences in innate fear (day 1 post shock) or fear learning (day 2). In contrast, there may have been a ceiling effect. Indeed, WT non stressed controls froze nearly 69% during the postshock test and 82% during the conditioned test, greatly minizing our ability to detect an increase in Tg+ rats. Additionally, we failed to find a stress effect among WT rats which was likely also due to a ceiling effect. Future studies examining potential stress effects and/or differences in fear learning in Tg+ models should incorporate a less potent shock regimen or a different stress regimen (e.g., variable stressors, unpredictable stress, etc.).

The present stress effects are partially consistent with prior restraint stress papers. Specifically, as expected, we observed a significant stress effect (Figure 3) of enhaned weight loss in rats assigned to the 14 consecutive days of chronic restraint stress in both Tg+ and WT rats. Interestingly, for most stressed rats we noticed that the greatest amount of weight loss generally occurred between days 7-11. However, anxiety-like behavior was not increased in WT stressed rats compared to WT controls as previously reported (Chiba et al., 2012 & Han et al., 2015). Several reasons may explain the null effects of stress on behavioral outcomes. First, there may have been a ceiling effects as non-stressed WT and non-stressed Tg+ rats spent nearly 75% and 92% of their time avoiding the open arms, respectively, limiting the ability to detect a stress-induced increase in anxiety-like behavior. Additionally, using the same daily stress form for 14 consecutive days may have caused the rats to habituate to this form of stress; however see Uchida

et al., 2008 that found that Fischer 344 rats did not habituate to 14 days of restraint stress. Finally, future studies should stress the rats eariler in life (e.g. 3 or 4 months) to allow time for the stress to interact with genotype and drive subsequent pathology.

**Future directions**

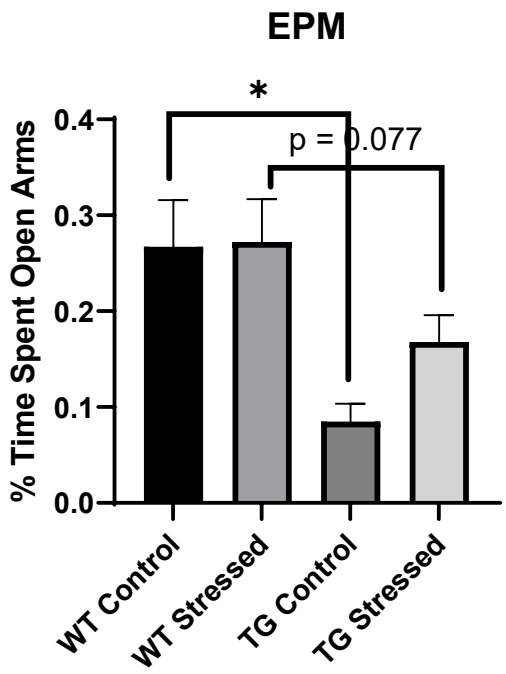
Additional tests of anxiety (e.g. light-dark box test) could be performed following periods of acute and/or long-term stress to further determine if stress is an important risk factor in the onset and progression of AD in TgF344-AD rats. Furthermore, human AD patients also report higher levels of depression and thus depression might be the predominate psychological predisposition (as opposed to anxiety) in determining potential risk for developing the disease. Therefore, future studies should test TgF344-AD rats for elevated levels of depressive-like behavior at early timepoints with and without a history of chronic stress.

**Conclusions**

Overall, we found supporting evidence that genotype does influence anxiety-like behavior in the EPM, with Tg+ rats displaying increased levels of anxiety-like behavior compared to WT rats. However, stress did not have a significant impact on observed anxiety-related behaviors in the EPM or fear-related behaviors during the footshock test trials. While this did not support our initial hypothesis that stress would increase both anxiety and fear-related behaviors, several methodological issues may have prevented our ability to detect a stress effect. The results of this study serve as an important step in understanding early signs (or risk factors) of AD which may help better identify at risk individuals prior to the manifestation of dementia.

**Graphs and Figure Legends**

A

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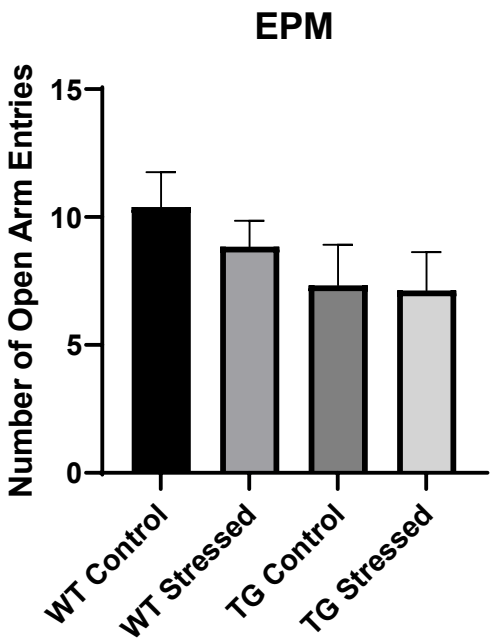


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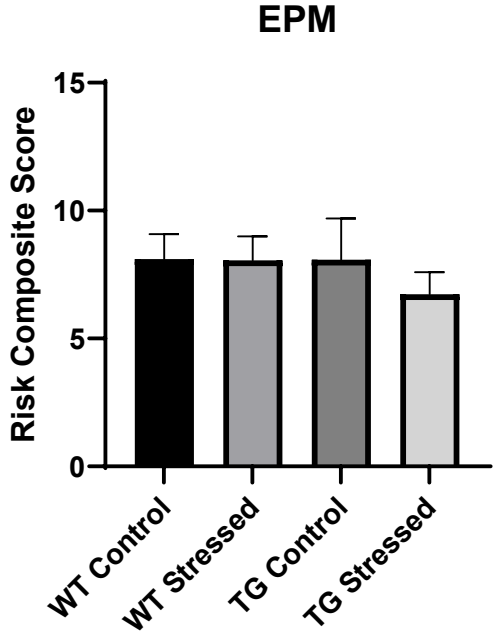


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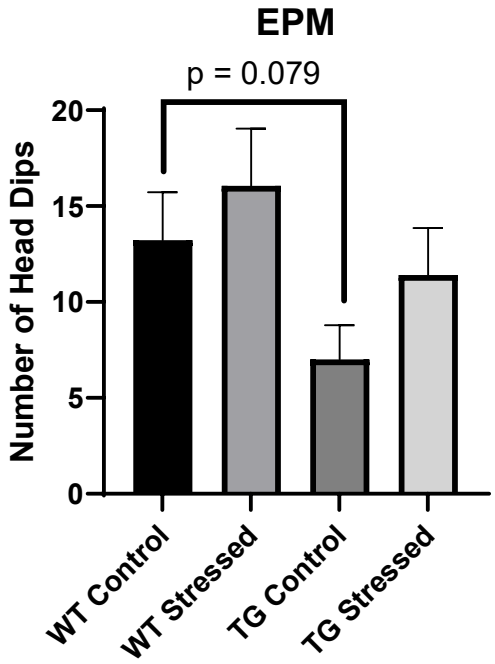

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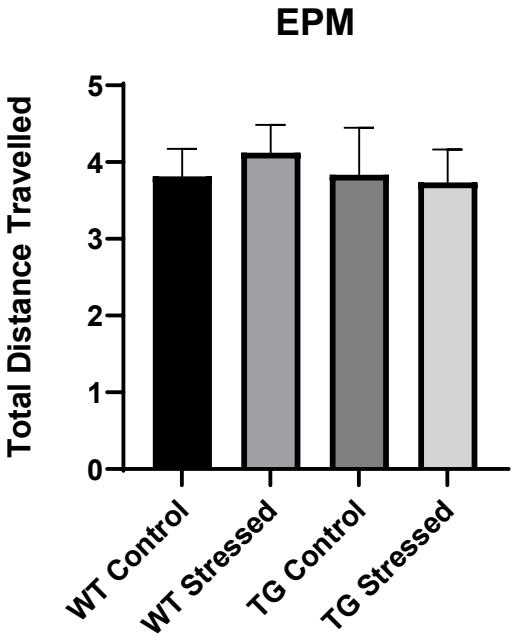


Figure 1. Elevated plus-maze (EPM). Transgenic (Tg+, n=27) and Wildtype (WT; n=37) rats with and without a history of chronic stress were tested for anxiety-related behaviors in the (EPM). Data represent the percent of time in the open arms (a), number of open arm entries (b), risk assessment (c), head dips (d) and total distance travelled (e) during a 5-minute test session (+/- SEM). Asterisk (*) represents a significant difference between genotype ($p<0.05$).

A

B

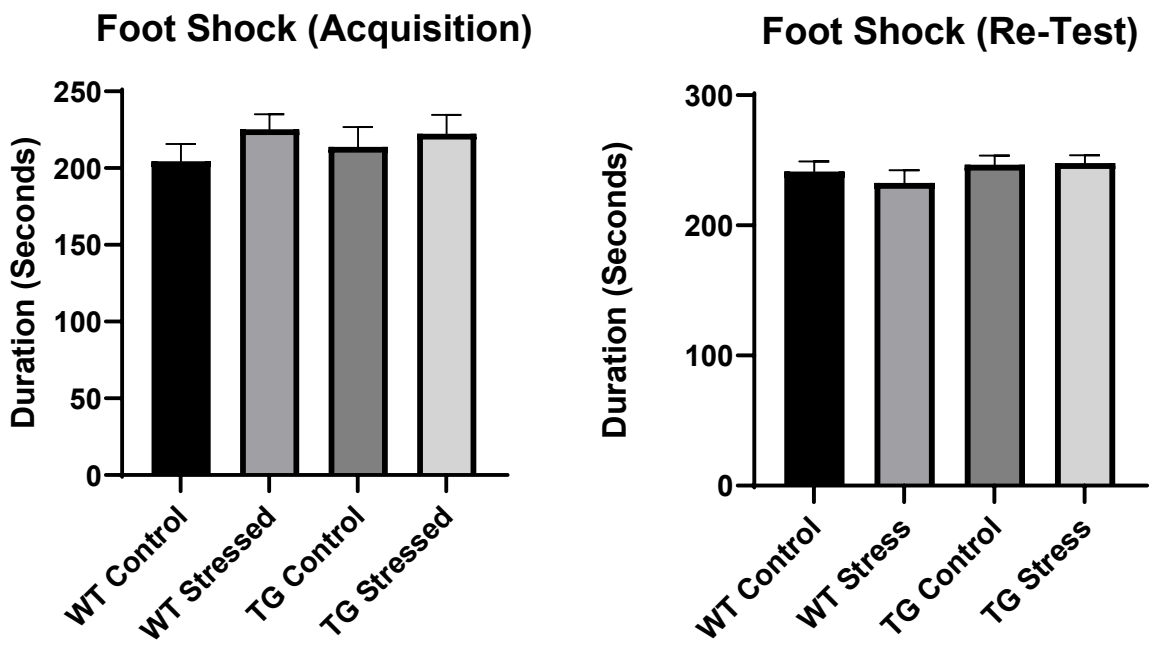


Figure 2. Footshock. Transgenic (Tg+, n=27) and Wildtype (WT; n=37) rats with and without a history of chronic stress were tested fear-related behaviors in the footshock conditioning test. Data represent the duration of freezing immediately following the administration of 3 footshocks (a) or 24 hours following the 3 footshocks during a contextual conditioning test trial (b). Data represent the duration of freezing (+/- SEM) during a 5-minute test session. There were no significant differences between groups ($p > 0.05$ in each case).

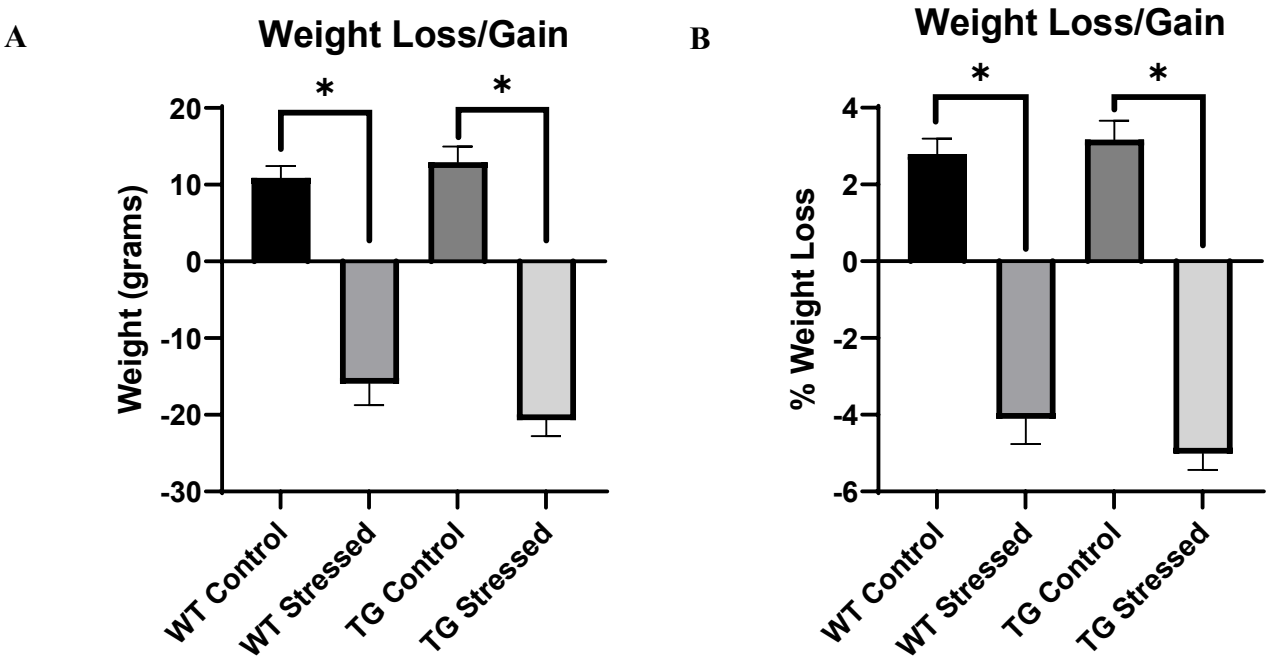


Figure 3. Weight Loss. Transgenic (Tg+, n=27) and Wildtype (WT; n=37) rats with and without a history of chronic stress were tested for total amount and percentage amount of weight loss. Data represent the total amount of weight loss (a) or percentage total weight loss (b). Data represent the amount of weight lost (+/- SEM) after 14 consecutive days of chronic restraint stress. Asterisk (*) represents a significant difference between genotype ($p<0.05$).

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