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Original Article

TTC9A deficiency induces estradiol-mediated changes in hippocampus and amygdala neuroplasticity-related gene expressions in female mice

Running Title: TTC9A modulates estradiol-mediated neuroplasticity

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Highlights

- Estradiol increases the tail suspension immobility in OVX-*Ttc9a*$^{-/-}$ mice.
- Estradiol inhibits neuroplasticity-related gene expression in OVX-*Ttc9a*$^{-/-}$ mice.
- Anxiety in *Ttc9a*$^{-/-}$ mice is mediated by estradiol and neuroplasticity mechanisms.

ABSTRACT

The involvement of tetratricopeptide repeat domain 9A (TTC9A) deficiency in anxiety-like responses and behavioral despair through estradiol action on the serotonergic system has been reported. Emerging evidence suggests that estradiol is a potent modulator of neuroplasticity. As estradiol and neuroplasticity changes are both implicated in mood regulation and estradiol activity is negatively regulated by TTC9A, we hypothesized that the behavioral changes induced by *Ttc9a*$^{-/-}$ is as well mediated by neuroplasticity-related mechanisms. To understand the effects of TTC9A and estradiol modulation on neuroplasticity functions, we performed a behavioral analysis of tail suspension immobility and neuroplasticity-related gene expression study of brain samples collected in a previous study involving ovariectomized (OVX) *Ttc9a*$^{-/-}$ mice with estradiol or vehicle treatment. We observed that OVX-*Ttc9a*$^{-/-}$ mice had significantly reduced the tail suspension immobility compared to OVX-*Ttc9a*$^{-/-}$ estradiol-treated mice. Interestingly, there was an upregulation in gene expression of tropomyosin receptor kinase B (*Trkb*) in the ventral hippocampus, as well as brain-derived neurotrophic factor (*Bdnf*) and postsynaptic density protein-95 (*Psd-95*) in the amygdala of OVX-*Ttc9a*$^{-/-}$ mice compared to those treated with estradiol. These findings indicate that estradiol plays an inhibitory role in neuroplasticity in *Ttc9a*$^{-/-}$ mice. These observations were not found in the wildtype mice, as the presence of TTC9A suppressed the effects of estradiol. Our data suggest the behavioral alterations in *Ttc9a*$^{-/-}$ mice were mediated by estradiol regulation involving neuroplasticity-related mechanisms in both the hippocampus and amygdala regions.

Keywords: Tetratricopeptide repeat domain 9A (TTC9A), neuroplasticity, estradiol, hippocampus, and amygdala.

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1. INTRODUCTION

Mood disorders are debilitating neuropsychiatric diseases that are predicted to become the second leading cause of disability worldwide by 2020 [1]. Increasing evidence suggests mood disorders are involved in the disruption of neuroplasticity, which is a mechanism of neuronal adaptation, neurogenesis, and synaptogenesis [2]. Studies on brain imaging have revealed both size and functional alterations in the hippocampus, amygdala, and prefrontal cortex of depressed subjects [3-5]. The neuroplasticity hypothesis of mood disorders is further supported by studies that found neurotrophic factors are the potential main targets of antidepressants. In particular, decreased mRNA and protein levels of brain-derived neurotrophic factor (BDNF) were observed in the prefrontal cortex and hippocampus of postmortem brains of depressed subjects [6], whereas increased BDNF levels were found in the dentate gyrus of postmortem brains treated with antidepressants [7]. Furthermore, a single bilateral infusion of BDNF into the hippocampus produced antidepressant-like responses in two rat models of depression, the learned helplessness and forced swim test paradigms, that were comparable in effectiveness with multiple intraperitoneal administration of chemical antidepressants when compared to the saline-infused control [8].

A previous study has demonstrated that anxiety and behavioral despair in female mice are regulated by tetratricopeptide repeat domain 9A (TTC9A) through estradiol modulation on serotonergic action in the dorsal raphe nucleus (DRN) [9]. TTC9A is a protein containing three tetratricopeptide repeat (TPR) domains at the carboxyl terminal [10]. These TPR domains form antiparallel α-helical hairpins that serve as the interface for protein interactions in various biological processes, including cell cycle control, transcription, protein folding, and steroid receptor signaling [11]. Although TTC9A is ubiquitously expressed in human tissues, its expression is highest in the brain [9,12]. As a target gene of both estradiol and progesterone, it is upregulated by progesterone in progesterone receptor-transfected breast cancer MDA-MB-231 cells, whereas it is inhibited in

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breast cancer MCF7 cells and induced in estradiol target tissues by estradiol [12,13]. Recent studies have shown that estradiol activity is negatively regulated by TTC9A, as demonstrated by a more significant estradiol response in *Ttc9a* knockout (*Ttc9a*$^{-/-}$) female mice [14]. Estradiol is also strongly linked to the mood in women during puberty, pregnancy, and menopause, and estradiol dysregulation can lead to an increased risk of developing mood and anxiety disorders [15,16]. More interestingly, emerging evidence suggests that estradiol is a potent modulator of neuroplasticity regardless of sex in multiple brain regions, including the limbic system, a group of closely connected structures involved in mood regulation [17-20]. It highlights a potential link between neuroplasticity and TTC9A through estradiol action in emotional control.

Although we know that *Ttc9a*$^{-/-}$ induces anxiety-like behavior through the effect of estradiol on serotonergic system [9], the possible mechanisms of TTC9A on estradiol and neuroplasticity-related changes remain obscure. In this study, we investigated the effects of TTC9A and estradiol modulation on behavior and neuroplasticity functions by performing a behavioral analysis of tail suspension immobility and a gene expression analysis of tissue samples from various brain regions collected in a previous experimental study involving ovariectomized (OVX) *Ttc9a*$^{-/-}$ mice with estradiol or vehicle treatment [9]. As previous studies demonstrate estradiol plays a role in neuroplasticity changes [17-20] and estradiol functions are negatively regulated by TTC9A through a negative feedback mechanism [14], we hypothesized that the anxiety-like behavior manifested in the *Ttc9a*$^{-/-}$ mice is mediated by estradiol modulation through neuroplasticity-related mechanisms. The interactive roles of estradiol and TTC9A on the expression of neuroplasticity-related genes were examined, with the main focus on synaptogenesis by measuring the synaptic markers synaptophysin (*Syp*; presynaptic) and postsynaptic density-95 (*PSD-95*; postsynaptic). Apart from synaptogenesis, we also investigated the neurogenesis-dependent mechanism by measuring the neurogenesis markers, including nestin (neural stem cell marker), doublecortin (*Dcx*, early postmitotic neuronal marker), and neuronal nuclei (*NeuN*; mature neuronal marker). Since *Bdnf* is a crucial mediator of neuroplasticity that contributes to both synaptogenesis and neurogenesis, its relative expression as well as that of tropomyosin receptor kinase B (*TrkB*; receptor for BDNF) were measured. Throughout the manuscript, synaptogenesis and neuroplasticity are collectively referred to as neuroplasticity for simplicity and consistency.

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2. MATERIALS AND METHODS

2.1. Experimental design

We aimed to investigate the interactive roles of TTC9A and estradiol modulation on behavioral neuroplasticity. The tail suspension immobility data and brain samples from a previous experiment [9] were included in the present study. In brief, *Ttc9a*$^{-/-}$ and wildtype female mice were subjected to ovariectomy (OVX) as reported [9,21]. After a 10-day recovery period, mice were administered a subcutaneous injection of either 10 μg/kg 17-β-estradiol benzoate (EB; Sigma-Aldrich) dissolved in sesame oil with 0.25% benzyl alcohol or sesame oil vehicle with 0.25% benzyl alcohol. The ovariectomized animals were divided into four experimental groups: *Ttc9a*$^{-/-}$ with EB injection (OVX-*Ttc9a*$^{-/-}$ EB; n=7); *Ttc9a*$^{-/-}$ with vehicle treatment (OVX-*Ttc9a*$^{-/-}$ VEH; n=7); wildtype with EB injection (OVX-*Ttc9a*$^{+/+}$ EB; n=7); and wildtype with vehicle treatment (OVX-*Ttc9a*$^{+/+}$ VEH; n=6). All mice underwent the 5-min tail suspension test 52 hours after injection. The selected dosage of EB and the time point of conducting tail suspension test were based on previously established protocols, which were shown to be effective in producing physiological plasma level of EB and EB-induced neuroplasticity changes 48 hours after subcutaneous injection of 10μg/kg EB into OVX rodents [22-24]. The tail suspension test data set from the previous study [9] was reanalyzed by presenting the immobility time at each minute time-point of the testing period. Gene expression study was conducted to examine the neurogenesis and synaptogenesis markers in the hippocampus and amygdala. Since the tail suspension test was the last test performed before sacrificing the animals, this test for behavioral despair would be best associated with the gene expression changes in the animals. The time-line for the experimental design is shown in Fig. 1a.

2.2. Real-time PCR

We investigated the roles of TTC9A and estradiol modulation on neurogenesis and neuroplasticity-related mechanisms in specific brain regions of interest. Brain samples from previous studies [9] harvested immediately after the tail suspension test were subject to quantitative real-time PCR (rt-PCR) analysis. The gene expression levels for mRNA abundance in neurogenesis (Nestin; Doublecortin, *Dcx*; and Neuronal nuclei, *Neun*) and neuroplasticity-related (*Bdnf*; Tropomyosin receptor kinase B, *Trkb*; Synaptophysin, *Syp*; and Postsynaptic density protein-95, *Psd-95*)

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mechanisms were examined in the ventral hippocampus (vHipp), dorsal hippocampus (dHipp), and amygdala. The brains were micro-dissected at 300-400 µm thickness in a cryostat (CM3050; Leica Microsystems) into anatomical regions, according to the Mouse Brain Atlas (Franklin and Paxinos, 2007). The brain samples were homogenized in cold TRIzol (Invitrogen). Total RNA for each sample was extracted using TRIzol reagent with chloroform:isoamyl-ethanol (24:1) and phenol:chloroform:isoamyl-ethanol (50:24:1; Sigma-Aldrich), and then precipitated using isopropanol and washed with 75% ethanol in DEPC-treated water (Sigma-Aldrich), and finally re-suspended in 0.2% DEPC-treated water. About 2 µg of total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed in a KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems) on an ABI Prism 7000 sequence detection system (PE Applied Biosystems). The fold changes for individual sample were calculated from the Ct values. The relative expression levels were determined by normalizing the Ct values against GADPH Ct values as previously described [9]. The expression level for each experimental group was obtained by averaging the measures across samples. The specific primer sequences for the rt-PCR experiments are presented in Table 1.

* Table 1 about here *

2.3. Statistical analysis

The data were analyzed using IBM SPSS Statistics 25. Kolmogorov-Smirnov test was used to examine the normality of the data distribution. Gene expression data were analyzed by two-way ANOVA with Bonferroni post-hoc tests for multiple comparisons. The tail suspension immobility across the 5-min testing period was analyzed by three-way mixed ANOVA followed by Bonferroni corrections for multiple comparisons. All outliers were identified and discarded based on box-plot diagrams. Values above the upper quartile or below the lower quartile by 1.5 times the interquartile range were considered as outliers. The figures represent mean + S.E.M. All p-values < 0.05 were considered significant.

3. RESULTS

3.1. Effects of TTC9A$^{-/-}$ and estradiol *modulation on tail suspension immobility.*

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The tail suspension immobility behavior within each minute of the testing period was compared across groups. Using three-way mixed ANOVA (genotype x treatment x time), there were significant differences for time ($F_{(4, 60)}$ = 30.184, p < 0.001 , partial η^2 = 0.668) but no for genotype ($F_{1, 15)}$ = 0.142, p = 0.721 , partial η^2 = 0.009) and treatment ($F_{(1, 15)}$ = 3.413, p = 0.084, partial η^2 = 0.185. Our analysis revealed a significant main effect for interaction time x treatment ($F_{(4, 60)}$ = 3.672, p = 0.010 , partial η^2 = 0.197); however, we found no statistical significant effects for interaction time x genotype x treatment ($F_{(4, 60)}$ = 1.058, p = n.s., partial η^2 = 0.066), as well as the interaction time x genotype ($F_{(4, 60)}$ = 0.862, p = n.s., partial η^2 = 0.054). Interestingly, we observed a significant main effect for interaction genotype x treatment at the 3rd and 4th min time-points of tail suspension test (3rd: $F_{(1, 19)}$ = 8.067, p = 0.01, partial η^2 = 0.298; 4th: $F_{(1, 22)}$ = 11.551, p = 0.003, partial η^2 =0.344). In both the 3rd and 4th min interval, we found that the mean immobility time was remarkably higher in OVX-*Ttc9a*$^{+/+}$ VEH mice compared to the OVX-*Ttc9a*$^{-/-}$ VEH mice (3rd: p = 0.02; 4th: p = 0.019, Fig. 1b). Additionally, a lower tail suspension immobility was also observed in the *Ttc9a*$^{-/-}$ mice with VEH treatment than those animals that received EB treatment (3rd: p = 0.018; 4th: p = 0.001, Fig 1b). Furthermore, in the 4th min time-point, OVX-*Ttc9a*$^{-/-}$ EB mice had significantly increased the immobility time when compared to the OVX-*Ttc9a*$^{+/+}$ EB mice (p = 0.034, Fig. 1b).

* Figure 1 about here *

3.2. *Effects of TTC9A*$^{-/-}$ *and* estradiol *modulation on mRNA levels of neuroplasticity-related genes.*

As disruption of estradiol and neuroplasticity is implicated in anxiety disorder[2] and TTC9A negatively regulates estradiol activity, we investigated whether *Ttc9a*$^{-/-}$ mice exhibited any changes in neuroplasticity-related genes associated with anxiety behavior. The statistical effects of the two-way ANOVA analysis for genotype (wildtype and *Ttc9a*$^{-/-}$), treatment (vehicle and EB), and their interactions in dHipp, vHipp, and amygdala in the gene expression study are presented in Supplementary Table 1.

In the dHipp, two-way ANOVA revealed a significant difference in genotype for *Dcx, Trkb, Syp* and *Psd-95* ($F_{(1,18- 21)}$ > 7.108, p < 0.014, partial η^2 > 0.253). Although no significant main effect was

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observed for treatment ($F_{(1,18-21)}$ < 1.990, p = n.s., partial η^2 < 0.087), we found a significant interaction effect for genotype x treatment for *Syp* ($F_{(1, 21)}$ = 9.248, p = 0.006, partial η^2 = 0.306) and *Psd-95* ($F_{(1, 19)}$ = 4.552, p = 0.046, partial η^2 = 0.193). Bonferroni post-hoc analysis showed significantly higher levels of mRNA for *Syp* and *Psd-95* (p < 0.006, Fig. 2a) in OVX-*Ttc9a*$^{-/-}$ EB mice as compared to the OVX-*Ttc9a*$^{+/+}$ EB mice. Moreover, in the wildtype animals, EB treatment increased the abundance of mRNA level of *Syp* (p = 0.045, Fig. 2a) compared to the OVX-*Ttc9a* $^{+/+}$ VEH group.

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In the vHipp, statistical analysis revealed a significant effect in the gene expressions of *Neun, Dcx, Bdnf, Trkb* and *Psd-95* ($F_{(1,18-21)}$ > 11.624, p < 0.003, partial η^2 > 0.392); estradiol treatment effect for *Trkb* ($F_{(1, 20)}$ = 4.96, p = 0.038, partial η^2 = 0.199); and interaction difference for *Bdnf, Trkb and Syp* ($F_{(1, 20-21)}$ < 4.770, p < 0.040, partial η^2 > 0.185). In OVX-*Ttc9a*$^{-/-}$ EB animals, Bonferroni post-hoc analysis showed increased mRNA abundances for *Neun Syp, and Psd-95* (p < 0.023; Fig. 2b) as compared to the OVX-*Ttc9a*$^{+/+}$ EB group. When compared to the vehicle treatment, EB administration significantly reduced the expression of *Trkb* and *Syp* in *Ttc9a*$^{-/-}$ and wildtype mice respectively (p < 0.017, Fig, 2b).

In the amygdala, there was a main effect in the genotype for *Bdnf* ($F_{(1, 19)}$ = 10.401, p = 0.004, partial η^2 = 0.354) and *Psd-95* ($F_{(1, 17)}$ = 52.013, p < 0.001, partial η^2 = 0.757), treatment for *Bdnf* ($F_{(1, 19)}$ = 8.264, p = 0.010, partial η^2 = 0.303), and interaction effect for *Psd-95* expression ($F_{(1, 17)}$ = 16.006, p = 0.001, partial η^2 = 0.485). Bonferroni post-hoc tests showed the downregulations of *Bdnf* and *Psd-95* (p < 0.019, Fig. 2c) in OVX-*Ttc9a*$^{-/-}$ EB when compared to OVX-*Ttc9a*$^{-/-}$ VEH.

* Figure 2 about here *

4. DISCUSSION

Although a recent study shows that *Ttc9a*$^{-/-}$ female mice exhibited anxiety-like behavior [9], the neuroplasticity-related mechanisms remain unknown. Given that TTC9A plays an important role in estradiol modulation, *Ttc9a*$^{-/-}$ mice were ovariectomized and further treated with either EB or vehicle

injection. In this study, we validated our previous findings [9], showing a substantial increment in tail suspension immobility in OVX-*Ttc9a*$^{-/-}$ EB treated mice as compared to OVX-*Ttc9a*$^{+/+}$ EB mice. This behavior could be reversed by the removal of EB, as demonstrated by the significantly lowered immobility time in the OVX-*Ttc9a*$^{-/-}$ VEH mice (Fig. 1b). This indicates that TTC9A does indeed have an essential role in estradiol modulation on behavioral despair. Although a higher immobility duration was reported in OVX-*Ttc9a*$^{-/-}$ EB as compared to OVX-*Ttc9a*$^{+/+}$ EB mice [9], it is only observed in the 4th min interval of tail suspension test in our present study, possibly because the knockout effect was diluted as we examined the behavior within each minute time-point rather than a total of 5 minutes duration.

We hypothesized that the anxiety-like responses in TTC9A-deficient mice were mediated by estradiol modulation and neuroplasticity-related mechanisms. Our results revealed that *Ttc9a*$^{-/-}$ led to higher mRNA levels of neuroplasticity-related genes, including *Syp* and *Psd-95* in both the dorsal and ventral hippocampus, and *NeuN* in the ventral hippocampus of the OVX-*Ttc9a*$^{-/-}$ EB mice as comparing to OVX-*Ttc9a*$^{+/+}$ EB mice. Despite the increased gene expressions, OVX-*Ttc9a*$^{-/-}$ EB mice had a longer immobility time when compared with the OVX-*Ttc9a*$^{-/-}$ VEH group, implying that the *Ttc9a*$^{-/-}$ mice experienced more depressive-like behavior when EB was administered. Interestingly, we demonstrated significant decreases in mRNA levels for *Trkb* in the ventral hippocampus, and *Bdnf* and *Psd-95* in the amygdala of mice in OVX-*Ttc9a*$^{-/-}$ EB compared to OVX-*Ttc9a*$^{-/-}$ VEH, which may suggest the regulation of neuroplasticity-related genes in OVX-*Ttc9a*$^{-/-}$ EB was inhibited by estradiol. The inhibitory effect of estradiol on these gene expressions was not observed in the wildtype groups, possibly due to the presence of TTC9A, which has been shown to suppress the effects of estradiol function in female mice [14]. A limitation should be noted in the present study. Instead of normalizing the gene expression data to three housekeeping genes, our data is only normalized against a single gene. It may reduce the robustness of our study. Further study should include multiple housekeeping genes to increase the reliability of data or investigate the expression at both the gene and protein levels to reveal the effect of estradiol.

An overview of TTC9A and estradiol effects on behavioral outcomes and neuroplasticity-related mRNA levels is shown in Fig. 3a. Our results demonstrated that TTC9A-deficient mice treated with

estradiol had reduced expressions of neuroplasticity-related genes in the hippocampus and amygdala regions. The repressed gene expression in OVX-*Ttc9a*$^{-/-}$ EB mice is consistent with a higher tail suspension immobility behavior. Importantly, the negative regulation of estrogen action by TTC9A [14] and the involvement of estrogen in neuroplasticity changes that play an influential role in mood regulation [17-20]. Taken together, our findings suggest that TTC9A modulates the anxiety-related behaviors through mediating estrogen action on neuroplasticity-related gene expression in both the hippocampus and amygdala regions.

The hippocampus and amygdala are two independent and interrelated structures of the medial temporal lobe that have been implicated in the regulation of stress and emotion. In particular, the ventral hippocampus has long been thought to play a preferential role in processing anxiogenic stimuli [25]. Similarly, the amygdala is part of the limbic system that plays an essential role in the emotional control of fear and anxiety disorder [25,26]. A wealth of evidence has suggested that structural abnormalities and alteration in neural activity in the amygdala and hippocampus regions contribute significantly to the development of depression [27-33]. In particular, a smaller hippocampal volume and exaggerated neural activity in the amygdala are closely related to anxiety. Taken together, these findings are consistent with our present study, in which the downregulation of neuroplasticity-related genes is corresponding to the anxiety-like responses in TTC9A-deficient mice.

The underlying mechanism regarding the involvement of TTC9A in neuroplasticity function is not fully understood. TTC9A does not appear to interact directly with ER, but it binds tightly to FK506 Binding Proteins (FKBPs) 38 and 51. FKBPs are co-chaperone proteins that are involved in the assembly and maturation of hormone-receptor complexes [11]. It is possible that TTC9A regulates ER-chaperone complex activity via interaction with FKBPs. Previous studies have established a direct link between estradiol and BDNF transcription [34]. As BDNF has known activity in promoting neuroplasticity and has a direct association with estradiol, we propose that the TTC9A-estradiol complex plays a vital role in regulating neuronal plasticity by controlling BDNF expression. Using our present findings, we proposed hypothetical models illustrating the roles of TTC9A and estradiol modulation in the neuroplasticity mechanisms (Fig. 3b – d). In wildtype animals, the presence of

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TTC9A negatively regulates the suppressive action of estradiol on *Bdnf* transcription by modulating the binding of FKBPs with the ER complex. As a result, expressed BDNF then promotes synaptogenesis and activates pro-survival or other growth genes via BDNF-TrkB signaling, which activates a variety of intracellular signaling pathways, including phospholipase C (PLC), phosphoinositide 3-kinase (PI_3 kinase), and mitogen-activated protein kinase (MAPK) signaling pathways (Fig. 3b) [35]. In OVX-*Ttc9a*$^{-/-}$ EB mice, the absence of TTC9A revealed an inhibitory role of estradiol on *Bdnf* gene expression, which in turn inhibits neuroplasticity resulting in anxiety-like behavior (Fig. 3c). Interestingly, in the absence of both TTC9A and estradiol, we found a significant increase in neuroplasticity-related genes with a reduction in tail suspension immobility, which was possibly due to the abolished repression effects of estradiol on gene expressions (Fig. 3d).

* Figure 3 about here *

Our findings showed that a reduction of *Bdnf* mRNA abundance in the amygdala was accompanied by an increase in tail suspension immobility in OVX-*Ttc9a*$^{-/-}$ mice with EB treatment. This finding is in line with a study that reported a decrease in *Bdnf* gene expression in the amygdala resulted in anxiogenic-like behavior in rodents. Interestingly, their study successfully reversed the anxiety-like behavior after BDNF infusion into the amygdala [36]. However, it should be noted that the upregulation of BDNF in the amygdala is not always shown to be anxiolytic. Overexpression of BDNF in transgenic mice was demonstrated to facilitate symptoms of anxiety, concomitant with dendritic growth in the basolateral amygdala [37]. Spinogenesis in the amygdala was also elicited by both chronic and acute immobilization stress together with increased levels of BDNF and higher anxiety [38-40]. Although such findings were contradictory to our findings that BDNF downregulation in the amygdala was associated with anxiety-like behavior in OVX-*Ttc9a*$^{-/-}$ EB mice, the contrasting effects of BDNF and neuroplasticity in the amygdala suggest an optimal level of neurotrophins is more suitable for mental health.

It has been shown that TrkB is essential in activating intracellular signaling pathways necessary for BDNF-dependent neuroplasticity, including PLC, PI_3k, and MAPK signaling [35]. We found that *Ttc9a*$^{-/-}$ mice without EB treatment exhibited decreased tail suspension immobility as well as an

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upregulation of *Trkb* gene expression in the vHipp. Increased expression of TrkB has been shown to cause anxiolytic effects in mice [41]. Conversely, TrkB-deficient mice demonstrated increased anxiety-like behavior and morphological abnormalities in the basolateral amygdala neurons [42]. Similarly, PSD-95, a key scaffolding protein regulated by BDNF signaling in the postsynaptic density [43], was also shown to be related to anxiety [44]. In line with these studies, our observations of anxiety-like responses induced by *Ttc9a* deletion may be possibly linked to the suppression of BDNF-dependent neuroplasticity, particularly in the amygdala and hippocampus.

5. CONCLUSION

Our study showed that the anxiety-like responses and behavioral despair in TTC9A-deficient mice were mediated by estradiol modulation involving neuroplasticity-related BDNF-TrkB-PSD95 pathway in the amygdala and hippocampus regions. Additional experiments on neuroplasticity-dependent mechanisms of TTC9A are needed to further establish the gene-behavior relationship.

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STATEMENT OF ETHICS

Animal experimentation: All procedures were approved by the Institutional Animal Care and Use Committee of Nanyang Technological University (Ref. no.: ARF-SBS/NIE-A0169 AZ), Singapore.

CONFLICT OF INTEREST

All authors declared no conflict of interest.

AUTHOR CONTRIBUTIONS STATEMENT

L.W.L. & V.C.L.L. conceptualized and designed the experiments. W.S.Y., L.G. & L.W.L. acquired and analyzed the behavioral and gene expression data. L.W.L., V.C.L.L., & Y.S.C. contributed to

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the analysis and interpretation of the overall data. V.C.L.L., S.S., Y.Z.O., & T.L. contributed to setting up the TTC9A transgenic animals. L.W.L., V.C.L.L., & W.S.Y. drafted the manuscript, and all authors reviewed and commented on the article.

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Figure 1. Effects of *Ttc9a*$^{-/-}$ and estradiol modulation on tail suspension immobility.

A schematic diagram represents the time-line of the experiments (a). 52 hours after EB or vehicle injection, all OVX mice were subjected to the tail suspension test. Throughout the 5-min test duration, no significant difference in immobility time was observed between the OVX-*Ttc9a*$^{+/+}$ VEH mice and OVX-*Ttc9a*$^{+/+}$ EB mice (b). With EB treatment, OVX-*Ttc9a*$^{-/-}$ mice had increased immobility in the 4th min interval when compared to the wildtype group. Both OVX-*Ttc9a*$^{+/+}$ VEH mice and OVX-*Ttc9a*$^{-/-}$ EB mice exhibited significantly increased immobility duration within the 3rd and 4^{thmin} time-points compared to OVX-*Ttc9a*$^{-/-}$ VEH mice. The data are presented as mean + S.E.M. **p* < 0.05, significant difference from the wildtype group with the same treatment; #*p* < 0.05, significant difference from the OVX-*Ttc9a*$^{-/-}$ EB treated group.

Figure 2. Effects of *Ttc9a*$^{-/-}$ and estradiol modulation on neuroplasticity-related gene expressions.

Relative expressions of *Dcx*, *Nestin, Neun*, *Bdnf*, *Trkb* , *Syp*, and *Psd-95* in the dorsal hippocampus (a), ventral hippocampus (b) and amygdala (c) of the ovariectomized *Ttc9a*$^{-/-}$ and wildtype mice given either EB or vehicle treatments are shown. Note that when comparing with OVX-*Ttc9a*$^{+/+}$ EB mice, *Ttc9a* knockout with EB treatment led to increased expressions of *Syp* and *Psd-95* in the dorsal and ventral hippocampus, as well as *Neun* in the ventral hippocampus. Within the*Ttc9a*$^{-/-}$ groups, EB treatment reduced the mRNA levels of *Trkb* in the ventral hippocampus, as well as *Bdnf* and *Psd-95* in the amygdala of OVX-*Ttc9a*$^{-/-}$ EB mice. However, the inhibitory effect of EB on these gene expressions was not observed in wildtype groups. The data are presented as mean + S.E.M. Significant difference from the respective vehicle treatment group, **p* < 0.05.

Figure 3. Summary of TTC9A and estradiol effects on behavior and neuroplasticity-related gene expressions.

An overview of the *Ttc9a*$^{-/-}$ effects on behavioral outcome and neuroplasticity-related mRNA levels is presented in (**a**). Hypothesis models regarding the roles of TTC9A and estradiol modulation on

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neuroplasticity mechanisms are illustrated in (**b-d**). We hypothesized that TTC9A in neurons binds to FK506 binding protein 38 or 51 (FKBP38/51), whereas estradiol receptor binds to heat shock protein 90 (Hsp90). FKBP38/51 assembles with Hsp90, leading to the binding between TTC9A and estradiol. TTC9A then negatively regulates the inhibitory action of estradiol on *Bdnf* gene expression. BDNF, in turn, promotes neuronal plasticity via BDNF-TrkB signaling, which activates a variety of intracellular signaling pathways such as phospholipase C (PLC), phosphoinositide 3-kinase (PI_3 kinase), and mitogen-activated protein kinase (MAPK) signaling pathways.

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Figure 1

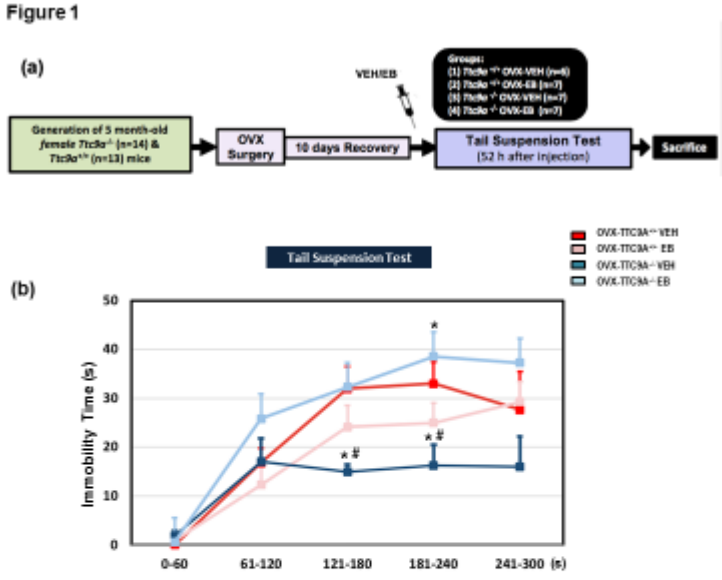

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Figure 2

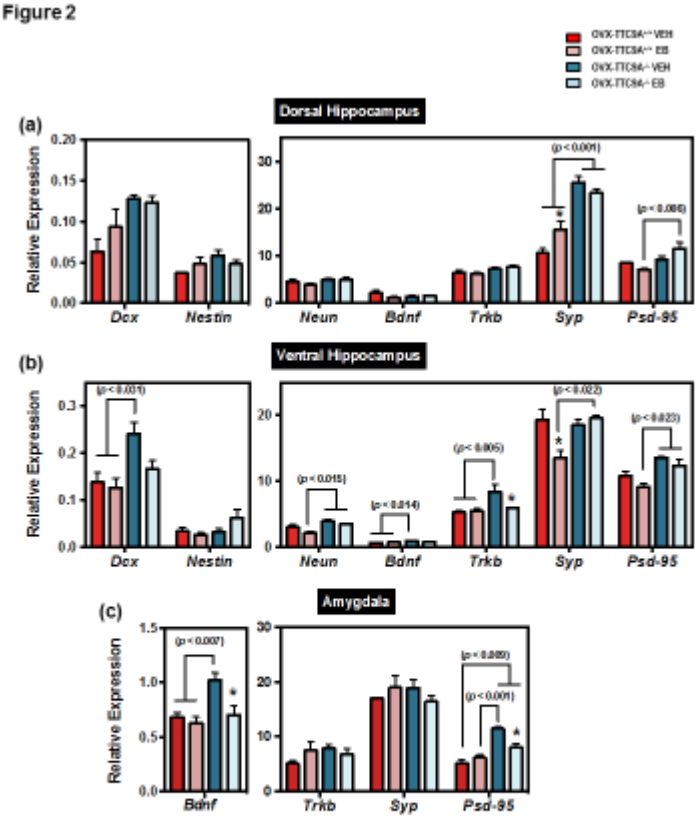

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Figure 3

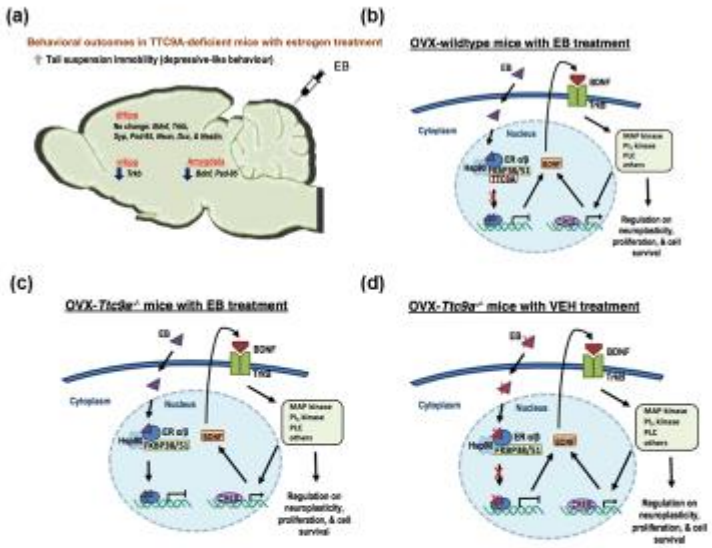

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Table 1. The specific primer sequences of mouse used for real-time PCR studies.

Gene	5′–3′ primer sequence
Nes (1)	Forward: AGGCTGAGAACTCTCGCTTGC Reverse: GGTGCTGGTCCTCTGGTATCC
Dcx (2)	Forward: ACACCCTTGATGGAAAGCAG Reverse: AGGACCACAAGCAATGAACA
Neun (3)	Forward: GAGGAGTGGCCCGTTCTG Reverse: AGGCGGAGGAGGGTACTG
Bdnf (4)	Forward: TGGCTGACACTTTTGAGCAC Reverse: AAGTGTACAAGTCCGCGTCC
Trkb (5)	Forward: CCTCCACGGATGTTGCTGAC Reverse: GCAACATCACCAGCAGGCA
Syp (6)	Forward: TGTGTTTGCCTTCCTCTACTC Reverse: TCAGTGGCCATCTTCACATC
Psd-95 (7)	Forward: GACGCCAGCGACGAAGAG Reverse: CTCGACCCGCCGTTTG
Gapdh (8)	Forward: GTCGGTGTGAACGGATTTG Reverse: AATTTGCCGTGAGTGGAGTC

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