HHS Public Access

Author manuscript

Gene. Author manuscript; available in PMC 2020 May 15.

Published in final edited form as:

Gene. 2019 May 15; 696: 176–185. doi:10.1016/j.gene.2019.02.013.

Exploring the involvement of *Tac2* in the mouse hippocampal stress response through gene networking

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Abstract

Tac2 is expressed in a number of areas throughout the brain, including the hippocampus. However, knowledge about its function has been only well explored in the hypothalamus in the context of reproductive health. In this study, we identified and validated increased hippocampal *Tac2* mRNA expression in response to chronic mild stress in mice. Expression quantitative trait locus (eQTL) analysis showed *Tac2* is cis-regulated in the hippocampus. Using a systems genetics approach, we constructed a *Tac2* co-expression network to better understand the relationship between *Tac2* and the hippocampal stress response. Our network identified 69 total genes associated with *Tac2*, several of which encode major neuropeptides involved in hippocampal stress signaling as well as critical genes for producing neural plasticity, indicating that *Tac2* is involved in these processes. Pathway analysis for the member of *Tac2* gene network revealed a strong connection between *Tac2* and neuroactive ligand-receptor interaction, calcium signaling pathway, as well as cardiac muscle contraction. In addition, we also identified 46 stress-related phenotypes, specifically fear conditioning response, that were significantly correlated with *Tac2* expression. Our results provide evidence for *Tac2* as a strong candidate gene who likely plays a role in hippocampal stress processing and neural plasticity.

Keywords

Genetics; Genomics; Chronic stress; Hippocampus; QTL analysis; Gene co-expression network; Fear learning; Synaptic plasticity

Disclosure Statement

The authors declare no conflicts of interest.

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1. Introduction

The stress response is the culmination of complex coordination by the brain to deal with stress through body-wide effectors such as adrenal corticosteroids (McEwen et al., 2002). In turn, corticosteroids can feed back to the brain and influence long-term effects through gene transcription by binding intracellular glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) (de Kloet et al., 2005; Heegde et al., 2015). The hippocampus has been of special focus in this model because of its key role in central stress processing (McEwen, 2007; Groeneweg et al., 2011), abundant co-localized expression of both GRs and MRs (Ruel et al., 1985; Ahima et al., 1991; Ahima and Harlan, 1990; Van Steesel et al., 1997; Han et al., 2004), and demonstrated plasticity in response to stress (McEwen et al., 1999; McEwen and Gianaros, 2011; McEwen et al., 2016). While corticosteroids are key components, there are many other mediators involved in fine-tuning of the stress response (Joels and Baram 2009). In addition, many stress-related peptides and hormones are known to influence hippocampal gene expression (Kurumaji, 2011; Maras and Baram, 2012; Gray et al., 2014) and structural remodeling (McEwen et al., 2016)

The duration and context of a stressor also plays a role in shaping stress's effect on the hippocampus (Joels and Baram, 2009; Radly et al., 2011; Karatsoreos and McEwen, 2013, Hunter et al., 2014). While acute stress brings physiologic adaptation, chronic exposure can cause allosteric overload triggering pathologic maladaptation (McEwen et al., 2002; McEwen and Gianaros, 2011). For example, mice exposed to acute restraint stress show induction of genes related to neurogenesis and neuroprotection in the hippocampus (Sannino et al., 2016). In contrast, rat and mouse models have shown that chronic stress induces genes linked with fear learning (Carhuatana et al., 2014), depression (Liu et al., 2010; Andrus et al., 2012), Alzheimer's Disease (Santha et al., 2012), and worsened recovery from novel stressors with increased anxiety behaviors (Gray et al., 2014). This divergent gene expression can be as specific as neuronal sub-populations in the hippocampus, as demonstrated recently by Gray and colleagues (2016) in mouse CA3 pyramidal neurons.

It is well accepted that genes do not act in isolation, but work through influential networks. Previous work has identified a number of hippocampal stress-reactive genes, but interactions between them are less well characterized. Building genetic networks can model these interactions (van der Sijde et al., 2014; Feltus et al., 2014; Schugart and Williams, 2017, Ch. 10), giving deeper insights into mechanisms of stress-induced hippocampal plasticity and how it contributes to stress adaptation and psychiatric disease. In the current study, we explored the stress related gene network in the hippocampus through systems genetics analysis. Recombinant inbred (RI) mice are a powerful tool for systems genetics analysis. The largest panel of these strains—the BXD family—consists of more than 100 strains derived from a cross of the C57BL/6J (B6) and DBA/2J (D2) inbred strains with variable expression of fear and anxiety-like traits (Brigman et al., 2009) stemming from differences in the parent strains' response to stress (Giardino et al., 1997; Gioia et al., 2016). BXD mice have identified a number quantitative trait loci (QTLs) related to fear, anxiety and the stress response (Radcliffe et al., 2000; Sokoloff et al., 2011; Carhuatana et al., 2014; Baker et al., 2017) making them strong models for this study.

Tac2 encodes Neurokinin B (NkB), a member of the tachykinin family of neuropeptides (Beaujouan et al., 2004). NkB has primarily been investigated for its role in central control of puberty onset, menstrual cycling, and reproductive health and pathology (Glidewell-Kenny et al., 2013; Steinhoff et al., 2014; Angell and Steiner, 2015; Navarro et al., 2015; Fergani and Navarro 2017). NkB signaling has also previously been shown to be stress-sensitive, being key player in reproductive suppression during acute systemic stress in female rats (Grachev et al., 2014). Interestingly, NkB has been shown to be expressed in a wide range of brain regions, including the olfactory bulb, cerebral cortex, amygdala, hippocampus, habenula, hypothalamus, and cerebellum, but largely uninvestigated in these regions (Bonner et al., 1987; Mar et al., 2013) Recently, NkB was implicated in amygdala-based fear-learning and post-traumatic stress disorder in chronically stressed mice (Andero et al., 2014; Andero et al., 2016). In this study we examine the genetic underpinnings of *Tac2* expression in hippocampus, and analyze gene pathways and network through in which *Tac2* plays potential roles in hippocampal stress responses.

2. Materials and methods

2.1 Animals

Eighteen female mice from BXD parental stains, C57BL/6J (B6) and DBA/2J (D2), were assigned to two groups with normal housing (NH) (8 mice total and 4 mice per strain) and chronic mild stress (CMS) (10 mice total and 5 mice per strain). All mice were housed as one mouse/cage, and maintained on a 12 h reversed Light:Dark schedule (lights on at 23.00 and out at 11.00) and allowed *ad libitum* access to water and normal laboratory chow diet until sacrificed for tissue harvest, except for the stress treatment experiments described below. All procedures were approved by the University of Tennessee Health Science Center Institutional Animal Care and Use Committee (protocol 14-131).

2.2 Chronic mild stress protocol

For our CMS protocol, we applied a weekly schedule of stressors (**see** Table 1), with one light phase stressor at 9:00AM CST and one dark phase stressor 1:00PM CST. This schedule was repeated for 7 weeks. Stressors consisted of four broad categories: light cycle changes, physical stressors, social stressors, and predatory stressors. Mice were returned to normal housing conditions for few days directly following the CMS protocol, and then were sacrificed to harvest hippocampi for RNA extraction.

2.3 RNA extraction and microarray data sets

Total RNA was extracted from the B6 and D2 hippocampi obtained through the CMS protocol experiment described above using the AllPrep DNA/RNA Mini Kit (Qiagen). Extracted RNA was reverse transcribed into cDNA, then was subsequently hybridized using the Affymetrix GeneChip Mouse Transcriptome Array 1.0 (Thermo Fisher Scientific) according to the manufacturers protocol. The resulting CEL files had outliers identified and removed, and were normalized using the robust multi-array average (RMA) method through Affymetrix Expression Console Software. Finally, the modified Z-scores method (Chesler et al 2005) was used to generate gene level log2 transformed expression profiles. This data set

was used to identify differentially expressed genes in the hippocampus between the NH and CMS treatment groups.

The second hippocampal expression data set that was used for systems genetics analysis in this study was previously generated from B6, D2, B6D2F1, D2B6F1, and 67 BXD mouse strains by our lab. This expression data set was obtained using the Affymetrix Mouse Genome M430 2.0 array, and can be publicly accessed in our GeneNetwork website (www.genenetwork.org) through the following identifier: GN110 (Hippocampus Consortium M430v2 (Jun06) RMA; Overall et al., 2009). Detailed information for this data set, including strain, age, sex, experimental protocol, data quality control, etc. can be found in the "info" pages on the GeneNetwork website.

2.4 eQTL mapping and variant identification of Tac2

We performed simple interval and composite interval eQTL mapping of *Tac2* using the WebQTL module on GeneNetwork. Both analyses yielded a likelihood ratio statistic (LRS) score, indicating linkage strength between *Tac2* gene expression levels and genetic markers. Significance levels were estimated with 2000 permutations tests and loci were considered statistically significant if their genome-wide p-value was < 0.05.

Genetic variation within the *Tac2* gene body and its surrounding 2 kb up-stream or down-stream region was independently assessed using three databases: Mouse Genome Informatics (http://www.informatics.jax.org), the Mouse Genome Project (http://www.sanger.ac.uk/science/data/mouse-genomes-project), and the SNP Variant Browser link on GeneNetwork.

2.5 Gene list filtering and co-expression network construction

To construct the *Tac2* gene co-expression network, the list of genes from the M430 2.0 array data set was sequentially filtered in the following five steps:

1. *Unbiased co-expression*: Genes potentially correlated with *Tac2* were identified in an unbiased fashion through calculating their Pearson correlation coefficient using GeneNetwork. Any genes with p-value < 0.05 and mean expression level > 7.0 were selected for the further analysis.
2. *Literature correlation*: We used the literature correlation tool on GeneNetwork to identify published biological connections between *Tac2* and rest of the genes. This tool searches previously published literature for the queried genes, assigning r values to genes that are mentioned together in the literature. While this filter cannot discover new associations, it does result in very robust associations being highlighted. Genes with and r-value > 0.2 were selected for further analysis.
3. *Identification of protein coding genes*: We uploaded the gene list to the MGI Batch query analysis tool (http://www.informatics.jax.org/batch). Any genes defined as protein coding genes were selected for further analysis.
4. *Weighted gene co-expression network analysis (WGCNA) analysis*: The gene list was then uploaded to R statistical software's WGCNA R package (version 1.61)

for unbiased co-expression clustering. Soft thresholding power β = 6 was chosen based on a scale-free topology, then used to calculate co-expression similarity and adjacency. The adjacency was further transformed into Topological Overlap Matrix (TOM) and corresponding dissimilarity was calculated. Genes were aggregated into modules by hierarchical clustering based on TOM and further refined using the dynamic tree cut algorithm. The gene module that contained *Tac2* was identified (black module, BM), and genes contained within this module were selected for further analysis.

5. *GeneMANIA analysis*: The BM gene list was uploaded to GeneMANIA (http://genemania.org/, an online database that contains co-expression, co-localization, and physical interaction data. The GeneMANIA gene list (GM) resulting from this analysis was used to construct the gene network displayed in Fig. 5.

2.6 Phenotype correlation Analysis

To identify phenotypes that highly correlated with the variation of Tac2 gene expression, we queried the BXD published phenotypes database in GeneNetwork for all behavioral traits, and focused our analysis on stress related traits that were significantly correlated with Tac2 expression in the hippocampus ($p < 0.05$) calculated by Pearson correlation coefficient.

2.7 Gene pathway enrichment analysis

The gene lists of the *Tac2* gene module (black) identified with WGCNA and the *Tac2* co-expression network obtained from GeneMANIA (https://genemania.org/) were annotated using the hypergeometric test tool on WebGestalt (http://www.webgestalt.org/) utilizing the KEGG pathway database and mouse genome reference gene set as a background. Raw p-values were adjusted through the Benjamini-Hochberg procedure (BH). Pathways reaching an adjusted p-value < 0.05 and shared gene numbers > 2 were deemed significant.

2.8 Quantitative RT-PCR

Fourteen female B6 and D2 mice are used for quantitative RT-PCR experiment. Three B6 and four D2 mice from NH and CMS group respectively. Total RNA from hippocampi was extracted using Direct-zol™ RNA Miniprep Plus (Zymo Research, Irvine, CA). RNA was treated with DNase and purified according to the kit instruction. Purification, concentration and integrity of the RNA were examined with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE), and Agilent Bioanalyzer (Agilent Technologies, Foster City, CA), respectively. cDNA was prepared from total RNA using a Superscript™ IV VILO™ Master Mix (Invitrogen, Carlsbad, CA). The gene-specific probe and primer sets for *Tac2* (upstream 5'-agggagggaggctcagtaag-3', downstream 5'-ggcggctgtcgtagagtc-3') were deduced using Universal Probe Library Assay Design software (https://www.roche-applied-science.com). *Tac2* mRNA levels were detected and analyzed on a LightCycler 480 System (Roche, Indianapolis, IN) under the following cycling conditions: 1 cycle at 95°C for 5 min and then 40 cycles at 95°C for 10 sec, 60°C for 30 sec, and 72°C for 10 sec. The PCR mix contained 0.2 μl of 10 μM primers, 0.1 μl of 10 μM Universal library probe, 5 μl of LC 480 master mix (2X), 2 μl of template cDNA, and RNase-free water to 10 μl. TATA box-binding protein (TBP) was selected as the endogenous quantity control. The relative gene expression

of *Tac2* was analyzed with the ΔΔCT method with *TBP* used as the reference gene for normalization. Briefly, the threshold cycle (CT) values for *Tac2* was determined by automated threshold analysis using the LightCycler 480 System and normalized to the CT value of the reference gene *TBP* to obtain ΔCT (*Tac2*) = CT(*Tac2*) – CT(TBP). Then *Tac2* expression (fold change) in each CMS treated B6 mouse relative to average of the corresponding NH B6 mice was calculated as: Fold Change = 2^-[ΔCT(*Tric2* in each B6 CMS treated mouse) – (Mean of ΔCT of NH B6 mice)]. The fold change for D2 CMS treated mice was calculated accordingly. Expression differences between CMS and normal control were evaluated using a two-variable (strain, treatment) Analysis of Variance (ANOVA).

3. Results

3.1 Expression differences of Tac2 between NH and CMS groups

We used the MTA 1.0 array data set to analyze the effect of stress on the *Tac2* expression in the hippocampus. Results showed significant increases ($F_{(1, 17)} = 8.6434$, $p = 0.01$) in *Tac2* expression in the CMS group according to linear regression analysis (Fig. 1). There is also a significant effect of strain on the *Tac2* expression under stress treatment ($F_{(1, 17)} = 8.574$, $p = 0.01$). Average *Tac2* expression for B6 and D2 combined was 7.13 and 7.52, for B6 mice only was 7.19 and 7.82, and for D2 mice only was 7.08 and 7.22 for the NH group and CMS group, respectively.

3.2 Tac2 expression levels in hippocampus of BXD mice

Hippocampal gene expression levels of 67 BXD mouse strains and their corresponding parental and F1 strains were examined using Affymetrix Mouse Genome 430 2.0 Array. The probe set 1419411_at in this array is the only probe set that represents *Tac2* gene and targets last 5 exons and 3' UTR of *Tac2* gene. Its expression shows broad variability across BXD strains with a fold change of 2.25 (Fig. 2). The average expression of *Tac2* is 7.74±0.03 (log2 scale, mean ± SEM). BXD75 shows the lowest expression level of *Tac2* (7.23 ±0.20), while BXD87 the highest level (8.44±0.98).

3.3 eQTL mapping and sequence variants of Tac2

To identify regulatory loci and variants, we treated *Tac2* expression level as a quantitative trait and performed the interval quantitative trait locus (QTL) mapping using 7586 effective SNPs across the mouse genome. With 2000 permutations, one significant expression QTL (eQTL) was identified on chromosome 10 with a likelihood ratio statistics (LRS) of 38.5, directly corresponding with the location of *Tac2* (Fig. 3). Further composite interval mapping controlling for peak SNPs demonstrated that no other significant loci were present, meaning *Tac2* is likely cis-regulated. We then used three databases (MGI, MGP, GeneNetwork) to identify any candidate sequence variants affecting the *Tac2* expression finding 14 SNPs and one 1 basepair deletion (Table 2). Among those, rs29322066 located in exon 3 is a synonymous mutation, rs36312330 is a 3’ UTR variant, and rs229201473 is defined as upstream gene variant. The rest are located in introns. At least one of these SNPs is responsible for Tac2 expression differences in BXD mice.

3.4 Gene co-expression network construction

Expression levels of 5335 probe sets correlate significantly with *Tac2* ($p < 0.05$). After filtering for mean expression level, literature correlation, and protein coding genes from MGI (See Materials and Methods), 2555 probe sets containing 2125 protein coding genes were uploaded into WGCNA for analysis. By performing sample cluster using hclust, two samples (BXD45 and BXD55) were detected as outliers were removed (Fig. 4A). Soft thresholding power ($\beta = 6$) was chosen in this analysis, the lowest power that can approximate a scale-free network topology (Fig. 4B). The initial dynamic tree constructed with WGCNA may have some very similar co-expressed modules. We used module eigengenes and clustered them with their correlation to quantify the co-expression similarity among these modules using a height cut of 0.25 to merge (correlation of 0.75) (Fig. 4C). A total of 10 modules were identified. *Tac2* was present in the black model (BM), which contained 569 transcripts (537 genes) (Fig. 4D). The BM gene list was uploaded to GeneMANIA (http://genemania.org/) to generate the list of 69 genes that are highly co-expressed in the network, noting 48 genes are directly connected with *Tac2* (Fig. 5).

3.5 Phenotype correlation

We correlated *Tac2* expression profiles with all behavioral phenotypes recorded in GeneNetwork, resulting in 104 significant associations ($p < 0.05$), 46 of which are stress related phenotypes, including fear conditioning response, anxiety assay, acoustic startle response, pain response, and locomotor activity. All of 46 stress-related phenotypes are listed in Supplement Table 1.

3.6 KEGG pathway enrichment

The entire BM gene list was submitted into WebGestalt to identify significant involvement in defined metabolic and signaling pathways. KEGG enrichment analysis resulted in 71 significant pathways (adjp< 0.05), the top 20 of which are displayed in Table 3. We then submitted only the GM gene list for KEGG analysis revealing ‘neuroactive ligand-receptor interaction’ (9 genes, adjP=4.69e-10), ‘calcium signaling pathway’ (6 genes, adjP=4.42e-07), and ‘cardiac muscle contraction’ (4 genes, adjp = 9.96e-06) as the top three enriched pathways.

3.7 qRT-PCR validation

The RT-PCR results showed significantly increased expression of *Tac2* ($F = 9.4284$, $P = 0.0134$), with an approximate 50% increase after CMS treatment compared with the control group (Fig. 6). This was consistent with the results from microarray analysis. In addition, we also found significant strain differences between B6 and D2 ($F = 18.804$, $P = 0.0019$). Intrastrain comparisons of *Tac2* expression level showed it to be more inducible in B6 (t-test $p = 0.0091$) than in D2 (t-test $p = 0.0702$).

4. Discussion

In this study, we aimed to elucidate the relationship between stress treatment and the expression of *Tac2* in the hippocampus as well as to identify interacting genes and pathways through which *Tac2* regulates stress responses. We have found *Tac2* expression significantly

increased in the hippocampus after exposure to chronic mild stress. Genetic mapping indicated that *Tac2* is a cis-regulated gene, making it an excellent candidate for study as a modifier of gene expression (Ciaobanu et al., 2010, van Dam et al., 2017) and co-expression network construction showed which genes may be closely interlinked to *Tac2* expression.

Phenotype correlation showed that hippocampal *Tac2* expression and stress response behaviors are strongly linked, specifically in fear conditioning and related activity. While this study is the first to our knowledge to cast *Tac2* for this role in the hippocampus, compelling evidence already exists for *Tac2* in the amygdala working in the same capacity. Andero and colleagues recently concluded that signaling in the central amygdala (CeM) by *Tac2* and its receptor, the Nk3 receptor (Nk3R), were necessary and sufficient for modulating fear memories (Andero et al., 2014; Andero et al., 2016). They demonstrated increased CeM *Tac2* mRNA expression in wild-type B6 male mice following fear conditioning, which utilizes various stressors (methods described in Andero et al., 2011; Andero et al., 2013), mirroring our results in the hippocampus (see Fig. 1). These changes in the CeM were accompanied by enhanced emotional-learning and fear memory consolidation, which were conversely impaired when this pathway was pharmacologically interrupted. Notably, the studies by Andero and colleagues were in male mice only. Given the prominent role of *Tac2*/NkB in central control of the HPA-axis, it is reasonable to investigate potential sexual dimorphism of this system. Our lab has previously documented a modest expression difference in hypothalamic *Tac2* expression, with increased expression under some measures in female mice (Mozhui et al., 2012). This difference is why we chose gender-restrict our subjects. While this makes physiologic sense for the hypothalamus, given its key role in the HPA-axis, this sexual dimorphic effect may be less prominent in other areas of the brain, notably in the hippocampus (Reinius et al., 2010). Additionally, expression of sexually dimorphic transcripts varies less than 10% between the sexes on average (Yang et al., 2006; van Nas et al., 2009; Reinius et al., 2010). This suggests to us that there are still significant effects of stress on *Tac2* expression, even in males (in-which *Tac2* expression may be lower overall). Likewise, pharmacologic activation of the Nk3R in the hippocampus of Wistar rats has shown to enhance learning and facilitate episodic-like memory (de Souza Silva et al., 2013; Chao et al., 2014). Taken together, this suggests that *Tac2* may function similarly to its emerging role in the CeM.

It has been suggested that the *Tac2*-Nk3R pathway does not cause changes directly, but exerts its effects through modifying the signaling of other neuropeptides in an autocrine or paracrine fashion (Navarro 2013; Andero et al., 2016). Because of this, there has been interest in identifying neuropeptides co-localized with *Tac2* to better understand the mechanisms underlying its effects. Indeed, the central amygdala is ripe with an abundance of various co-localized neuropeptides with *Tac2*, as seen through immunohistochemical mapping (Kim et al., 2017; McCullough et al., 2018). However, this sort of mapping has not been done in the hippocampus. In reviewing our network, we identified four genes encoding neuropeptides co-expressed with *Tac2*: *Npy* (neuropeptide Y), *Sst* (somatostatin), *Avp* (arginine vasopressin), and *Hcrt* (hypocretin/orexin). These neuroactive ligands are either expressed in or projected to neurons of the rodent hippocampus (Allen et al., 1983; Gray and Morley, 1986; Wahlestedt et al., 1989; Finley et al., 1981; Johansson et al., 1984; Viollet et al., 2008; Hawthorn et al., 1980; Tiberiis et al., 1983; Peyron et al., 1998), with each of their

corresponding GPCRs expressed as well (Martel et al., 1986; Dumont et al., 1998; Kask et al., 2002; Bruno et al., 1993; Alesco-Lautier & Soumireu-Mourat, 1998; Marcus et al., 2001) indicating both presence and ability to signal. They have also been shown to be stress reactive (Reichmann et al., 2015; Sweerts et al., 2001; Hassan et al., 2014; Conrad and McEwen, 2000; Arancibia et al., 2001; Czeh et al. 2015;Lin and Sibille, 2015; Ma et al., 1997a; Ma et al., 1997b; Ma et al., 1997c; Aubry et al., 1999; Stricker-Krongrad and Beck, 2002; España et al., 2003) and play prominent roles in hippocampal stress processing and stress-related phenotypes (Reichmann & Holzer, 2016; Tasan & Sperk, 2016; Schmeltzer et al., 2016; Stengel & Tache, 2017; Prevot et al., 2017; Beurel and Nemeroff, 2014; Herman & Tasker 2016; Caldwell et al., 2017; Berridge et al., 2010; Johnson et al., 2012; James, Campbell, and Dayas, 2017). While this does not replace traditional methods of expression mapping, co-localized expression of *Tac2* and other neuropeptides suggests roles in related processes.

To broadly identify potential biological processes of our network, we underwent KEGG pathway enrichment which revealed that a number of our genes were involved in pathways related to neuroactive ligand-receptor interactions and the underlying processes of neural plasticity, namely long-term potentiation (LTP), long-term depression (LTD), axon guidance, and regulation of the actin cytoskeleton. In line with these findings, the four co-expressed neuropeptides we identified play major roles in shaping hippocampal neural networks (Decressac and Barker, 2012; Li et al., 2017; Stefanelli et al., 2016; Leguz-Leczenar et al., 2016; Alesco-Lautier et al., 2000; Pagnani et al., 2015; Yang et al., 2013) allowing for adaptation, and sometimes maladaptation, to stressful conditions (McEwen et al., 2012; Leuner and Shors, 2013). Signals resulting from ligand-receptor interactions, like those described above, can trigger intracellular signaling that ultimately modulates gene expression, so called “activity dependent plasticity”, thought to be the underlying basis of memory and learning (Carulli et al., 2011). This and other effects that plastically alter neural networks cause reassignment of existing neural connections, new axonal growth, and alter dynamics of synapse efficacy in response to sensory input and experience, processes reflected in our network’s identified phenotypes (Table 3). One of the families of genes critical in achieving activity dependent plasticity is the neural cell adhesion immunoglobulin superfamily (Fields and Kouichi, 1998; Dityatev et al., 2008). We identified four members of this superfamily in our network: *Ncaml* (Neural Cell Adhesion Molecule 1), *L1cam* (L1 Cell Adhesion Molecule), *Cadm1* (Cell Adhesion Molecule 1) and *Cadm3* (Cell Adhesion Molecule 3).

Ncam1 and *L1cam* are intimately involved in regulating synapse formation as scaffolding to stabilize neuron-neuron connections and as anchors to intracellular machinery for proper synapse maturation and function (Sytnyk et al., 2002; Sytnyk et al., 2006). Both help stabilize of changes results from LTP and NCAM has specifically been shown to accumulate in new neurons and those undergoing LTP to serve this purpose (Luthl et al., 1994; Fux et al., 2003). In the hippocampus, both have been shown to regulate synaptic plasticity, learning, memory, and stress responses during learning (recently reviewed by Sytnyk, Leshchyns’ka, and Schachner, 2017). A post-translationally modified version of NCAM, polysialylated NCAM (PSA-NCAM), has proven just as pivotal in LTP, LTD, spatial learning, and contextual fear learning in the hippocampus (recently reviewed by Varbanov

and Dityatev, 2017). We also identified *Gap-43* (Growth Associated Protein 43), which produces a critical protein that complexes with NCAM to induce neurite outgrowth for synaptogenesis through actin polymerization, altering the neuron cytoskeleton so it can grow toward a new connection (Korshunova et al., 2007; Korshunova et al., 2009). Similar to *Ncam1* and *L1cam*, the CADM family (also known as the SynCAM or Nectin-like family) plays a prominent role in axon guidance, synapse formation, and plasticity (Frei and Stoeckli, 2017). CADM1 (also known as SyNcam1 or Necl2) hippocampal overexpression transgenic mice promotes formation of excitatory synapses, restricts long-term depression (Robbins et al., 2010). CADM3 (also known as SynCAM3 or Necl1) has been less well characterized, but is thought promote myelination through associations between axons and glial cells (Maurel et al., 2007; Spiegel et al., 2007; Park et al., 2008).

Ultimately, our goal is to better understand *Tac2*, its related genes, and their role in producing effects of the stress response to gain insight into stress related disorders. To this end, non-synonymous SNPs are an important source of genetic variation that can underlie phenotypic variation likely contributing to an individual's risk for developing stress-related disease. We identified seven genes (*Arrb1, Ndn, Dom3z, Rangap1, Spock1, Magel2, Mylk2*) with non-synonymous SNPs in our network. One gene of interest is *Arrb1* (Arrestin Beta 1), whose protein product has been previously identified as a mediator of DNA damage various brain structures in response to acute stress (Sood et al., 2014), chronic stress (Hara et al., 2011; Hara et al., 2012) and stress-related catecholamines (Jia et al., 2014). Theoretical effects of *Arrb1* expression have been proposed to play a role in both general development of neuropsychiatric conditions and plastic changes in the hippocampal ventral CA1 region relating to its connectivity to the amygdala and hypothalamus (so called 'emotion-related' connections) (Hara et al., 2011; Hara et al., 2012; Sood et al., 2014). However, concrete evidence of these effects has yet to be directly uncovered.

Overall, the current study provides many new avenues of inquiry into hippocampal stress processing and highlights *Tac2* as an influential gene beyond the amygdala and hypothalamus. It is likely reasonable to conclude that *Tac2* is involved in stress processing and neuroplasticity based on our current analysis. But, further work must be done to affirm this conclusion and better outline the roles of *Tac2* and its co-expressed genes. An overarching, and lofty, goal of pursuing the avenues outlined in this study is to translate findings into useful interventions for humans. *Tac2*'s human homologue (*TAC3*) and its corresponding receptor (*TACR3*) are well characterized (Fergani and Navarro, 2017 see Table 1) and have been long seen as potential sites for understanding and treating human psychiatric disease (Spooren, Riemer, and Meltzer, 2005). However, clinical trails of Nk3R-antagonist efficacy (e.g. Osanetant, Talnetant) have been equivocal at best, despite being well tolerated (Griebel and Holsboer, 2012). Interestingly, a recent study in C57BL/6N and BALB/c showed upregulation of *Tac2* in response to social isolation stress (SIS), locally orchestrating the behavioral effects of multiple brain regions – an effect that was attenuated by Osanetant (Zelikowski et al., 2018). Zelikowski and colleagues noted that the effect of SIS is well-established in both mice and humans, calling for reexamination of Nk3R antagonists as treatment for mood disorders resulting from social isolation and other stressors. We contend that continued use of bioinformatics techniques combined with well

established protocols will lead to better understanding of the complexities of stress's effect on the hippocampus and neuropsychiatric disease, opening up new avenues of intervention.

Acknowledgments

The authors thank Dr. Robert Williams for his financial support, bioinformatics support, and experiment design.

Funding

This study was supported by National Institutes of Health grants [R01AA021951] (LL, BJ) and [U01AA014425] (LL), and Internal New Grant Support Funding [E073239005] from the University of Tennessee Health Science Center (LL).

Abbreviations:

ANOVA	Analysis of Variance
Avp	arginine vasopressin
BH	Benjamini-Hochberg procedure
BM	black module
B6	C57BL/6J
CeM	central amygdala
CMS	chronic mild stress
D2	DBA/2J
eQTL	expression QTL
GM	GeneMANIA gene list
GR	glucocorticoid receptors
Hcrt	hypocretin/orexin
LRS	likelihood ratio statistic
LTD	long-term depression
LTP	long-term potentiation
MR	mineralocorticoid receptors
NkB	Neurokinin B
Nk3R	Nk3 receptor
NH	normal housing
Npy	neuropeptide Y
QTL	quantitative trait loci

RI	Recombinant inbred
RMA	robust multi-array average
Sst	somatostatin
TBP	TATA box-binding protein
CT	threshold cycle
TOM	Topological Overlap Matrix
WGCNA	Weighted gene co-expression network analysis
SIS	Social Isolation Stress

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Research Highlights

- Hippocampal *Tac2* mRNA expression increases in response to chronic mild stress in mice
- *Tac2* is a cis-regulated gene in the hippocampus
- 69 genes associate with *Tac2*, several of which encode major neuropeptides involved in hippocampal stress signaling and neural plasticity
- 46 stress-related phenotypes, specifically fear conditioning response, are significantly correlated with *Tac2*

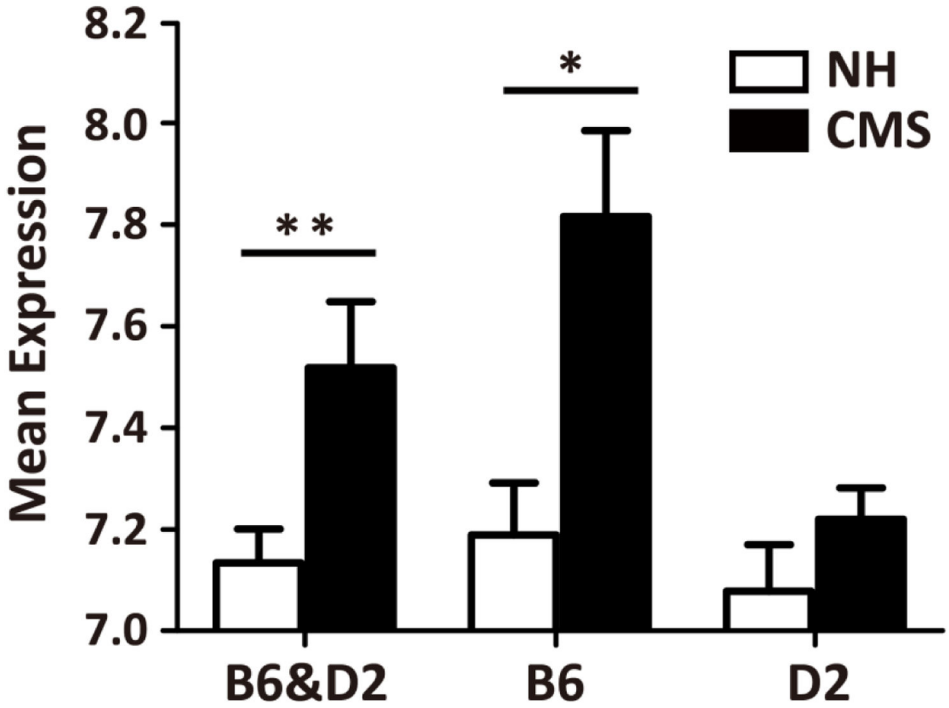

Fig. 1. Gene expressions in a log2 transform for *Tac2* in the NH and CMS treated hippocampus in BXD mouse parental stains (mean±sem). There are 4~5 mice in each group/strain.

* indicates p<0.01 for individual strain; ** indicates p<0.01 for combined.

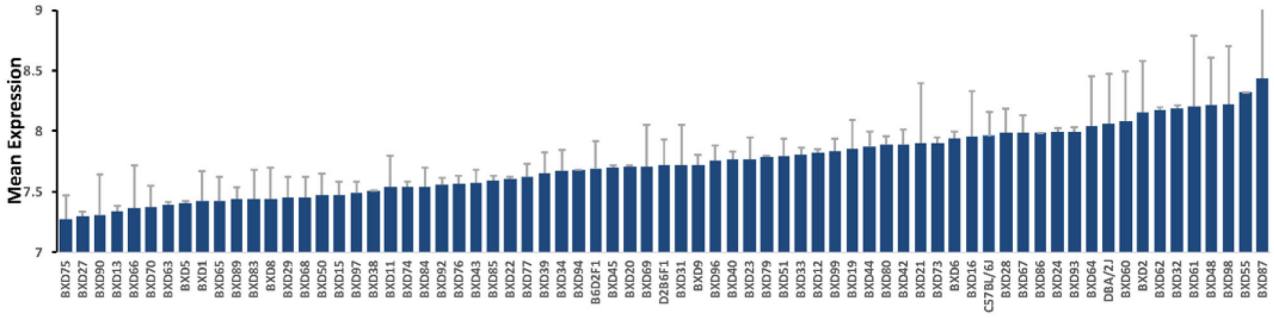

Fig. 2. Variable Expression of *Tac2*.

Expression data shown for 67 BXD strains, two F1 strains (B6D2F1 and D2B6F1), and parental strains (B6 and D2). The x-axis denotes the strain while the y-axis denotes mean expression given in a log2. Each bar shows mean expression values±standard error of the mean (sem)

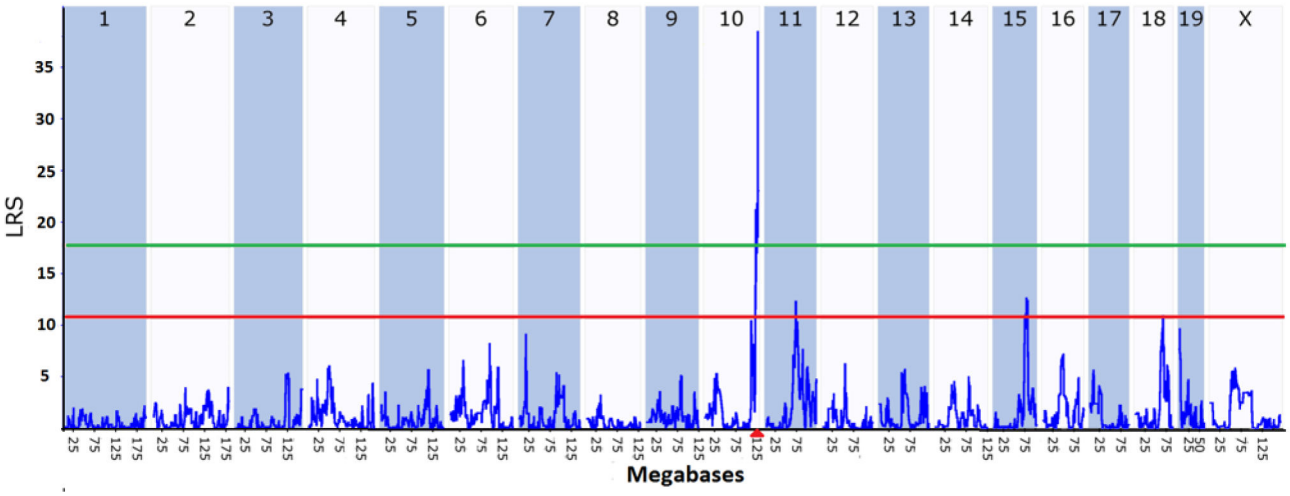

Fig. 3. Interval mapping of *Tac2* in the hippocampus.

Interval mapping indicates a genome-wide significant eQTL on chromosome 10. The x-axis denotes physical position (Mb) and the y-axis provides the likelihood ratio statistics score (LRS). Red line represents genome-wide suggestive (LRS = 10.69) and green represent significant (LRS = 17.73) thresholds. Red triangle represents the physical location of *Tac2*.

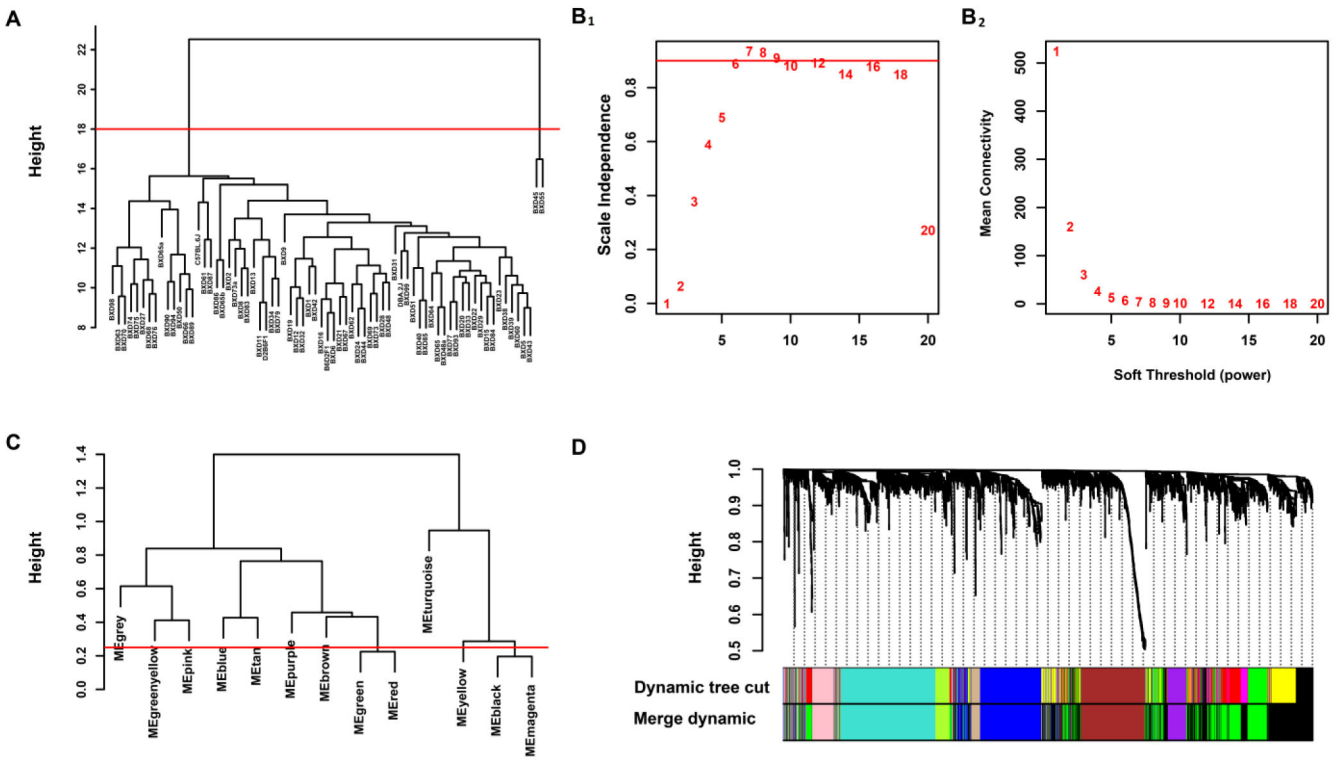

Fig. 4. Gene co-expression analysis with WGCNA.

A. Clustering dendrogram of samples based on hclust function. **B.** Analysis of network topology for various soft-thresholding powers. B_1 displays the scale-free fit index (y -axis) as a function of the soft-thresholding power (x -axis). B_2 displays the mean connectivity (degree, y -axis) as a function of the soft-thresholding power (x -axis). **C.** Clustering dendrogram of module eigengenes. **D.** Cluster dendrogram and module assignment for Dynamic tree and merge dynamic modules.

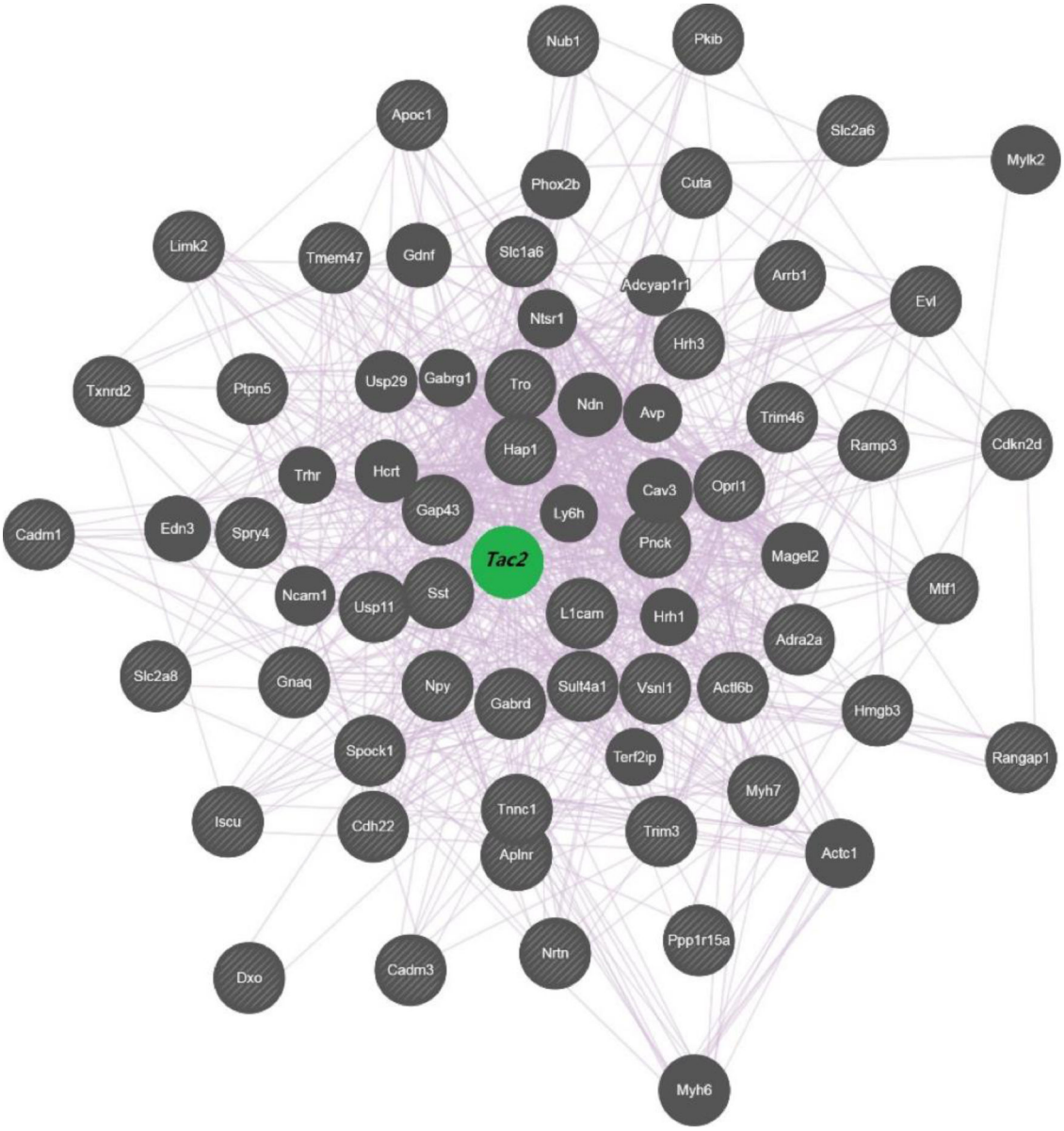

Fig. 5. Gene co-expression network of *Tac2 (green circle)*.

69 genes are involved in total, displayed as solid grey circles (not directly expressed with *Tac2*) or striped grey circles (directly expressed with *Tac2*). 48 genes are directly expressed with *Tac2*. Lines represent co-expression relationship between connected gene pairs.

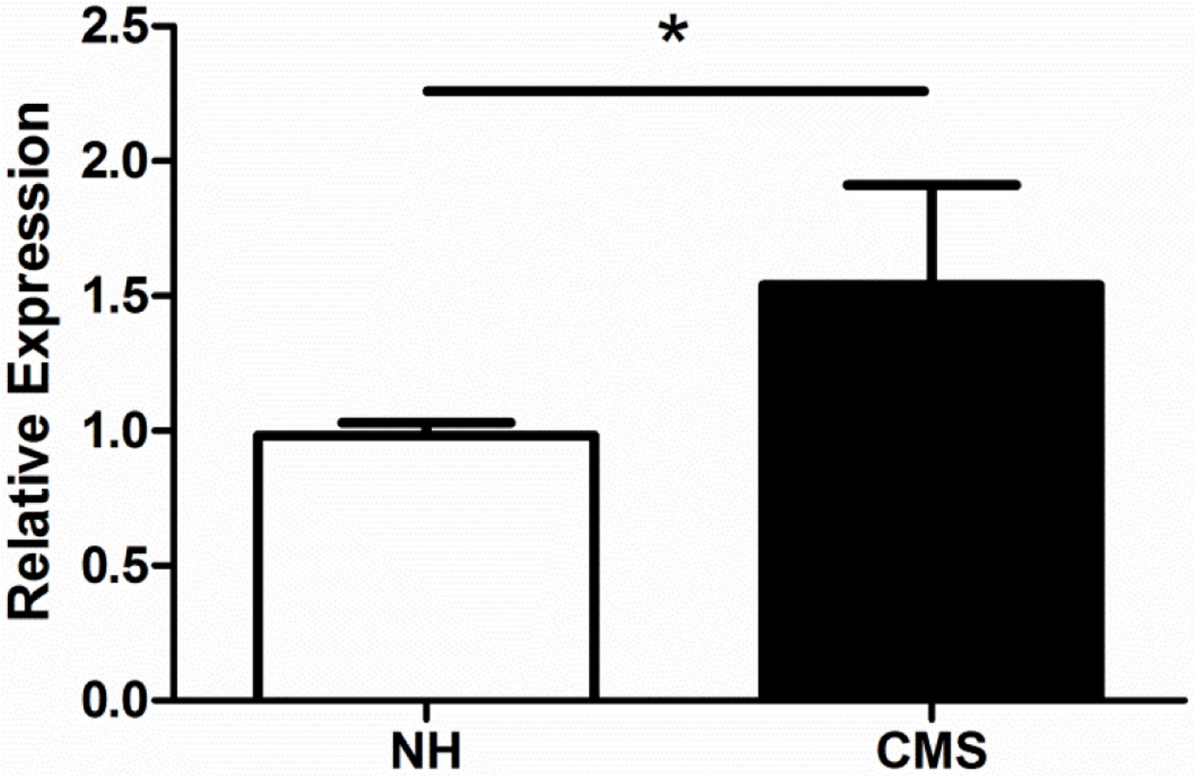

Fig. 6. RT-PCR expression of *Tac2* in the NH and CMS treated mouse hippocampus in combined B6 and D2 strains (mean±sem). (7 mice in each group).

* indicates $p<0.01$.

Table 1

Weekly CMS Protocol

Day	Light Phase Stressor, 9:00 AM	Dark Phase Stressor, 1:00 PM
Monday	Isolation, 15 minutes	Fox odor, 1 hour
Tuesday	Wet bedding, 15 minutes	Tilted cage 45°, 1 hour
Wednesday	Foreign mice odors, 1 hour	New Cage + No Bedding, 1 hour
Thursday	New cage + Wet Bedding, 1 hour	Isolation, 15 minutes
Friday	Fox odor, 1 hour	Tilted cage, 1 hour
Saturday	Light phase, no additional stressor, 12 hours	Light phase instead of dark phase, 12 hours
Sunday	No Stressors, 24 hours	No Stressors, 24 hours

Table 2

Genetic variants of the *Tac2* gene

SNP ID	Chr	Mb	Alleles	Gene	Function	B6	D2
rs229201473	10	127.725179	G/-	*Tac2*	upstream_gene_variant	G	-
rs29368338	10	127.725767	A/G	*Tac2*	Intron	A	G
rs37784442	10	127.726174	A/G	*Tac2*	Intron	A	G
rs29337469	10	127.726294	A/C	*Tac2*	Intron	A	C
rs36898445	10	127.726769	G/A	*Tac2*	Intron	G	A
rs29368760	10	127.727488	G/A	*Tac2*	Intron	G	A
rs29322066	10	127.728196	G/A	*Tac2*	Exon 3, Synonymous	G	A
rs38414531	10	127.728678	G/A	*Tac2*	Intron	G	A
rs37636262	10	127.729019	C/G	*Tac2*	Intron	C	G
rs37159093	10	127.729241	A/G	*Tac2*	Intron	A	G
rs29353763	10	127.729459	A/G	*Tac2*	Intron	A	G
rs36312330	10	127.729539	T/G	*Tac2*	Exon, 3' UTR	T	G
rs29317988	10	127.729603	G/T	*Tac2*	Intron	G	T
rs29375578	10	127.729848	T/C	*Tac2*	Intron	T	C
rs29337515	10	127.730103	C/T	*Tac2*	Intron	C	T

Note: '-' indicates deletion

Table 3

Top 20 Black Module enriched pathways

Pathway Name	Number of Genes	P value	Adjusted P value
Metabolic pathways	47	5.61E-19	6.23E-17
Protein processing in endoplasmic reticulum	12	1.69E-08	0.000000938
Vascular smooth muscle contraction	9	8.14E-07	2.26E-05
Gap junction	8	0.00000064	0.0000226
MAPK signaling pathway	12	2.41E-06	5.35E-05
Glycerolipid metabolism	6	0.00000363	0.0000672
Regulation of actin cytoskeleton	10	1.24E-05	0.0002
Long-term depression	6	0.0000273	0.0003
Long-term potentiation	6	2.14E-05	0.0003
Sphingolipid metabolism	5	0.0000202	0.0003
Amino sugar and nucleotide sugar metabolism	5	4.40E-05	0.0004
Leukocyte transendothelial migration	7	0.0000595	0.0005
Glycerophospholipid metabolism	6	4.97E-05	0.0005
Amyotrophic lateral sclerosis (ALS)	5	0.0000931	0.0006
Neurotrophin signaling pathway	7	0.0001	0.0006
Neuroactive ligand-receptor interaction	10	0.0001	0.0006
Axon guidance	7	0.0001	0.0006
Dilated cardiomyopathy	6	0.0000904	0.0006
Lysosome	7	6.96E-05	0.0006
Pyrimidine metabolism	6	0.0002	0.0011