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Translational profiling of stress-induced neuroplasticity in the CA3 pyramidal neurons of BDNF Val66Met mice

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Abstract

Genetic susceptibility and environmental factors (such as stress) can interact to affect the likelihood of developing a mood disorder. Stress-induced changes in the hippocampus have been implicated in mood disorders and mutations in several genes have now been associated with increased risk, such as brain-derived neurotrophic factor (BDNF). The hippocampus has important anatomical subdivisions, and pyramidal neurons of the vulnerable CA3 region show significant remodeling after chronic stress, but the mechanisms underlying their unique plasticity remain unknown. This study characterizes stress-induced changes in the *in vivo* translating mRNA of this cell population using a CA3-specific EGFP reporter fused to the L10a large ribosomal subunit (EGFPL10a). RNA-sequencing after isolation of polysome-bound mRNAs allows for cell-type specific, genome-wide characterization of translational changes after stress. The data demonstrated that acute and chronic stress produced unique translational profiles and that the stress history of the animal can alter future reactivity of CA3 neurons. CA3-specific EGFPL10a mice were then crossed to the stress-susceptible BDNF Val66Met mouse line to characterize how a known genetic susceptibility alters both baseline translational profiles and the reactivity of CA3 neurons to stress. Not only did Met allele carriers exhibit distinct levels of baseline translation in genes implicated in ion channel function and cytoskeletal regulation, but also activated a stress response profile that was highly dissimilar from wild type mice. Closer examination of genes implicated in the mechanisms of neuroplasticity, such as the NMDA and AMPA subunits and the BDNF pathway,

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revealed how wild type mice were able to upregulate many of these genes in response to stress, but Met allele carriers failed to do so. These profiles provide a roadmap of stress-induced changes in a genetically homogenous population of hippocampal neurons and illustrate the profound effects of gene-environment interactions on the translational profile of these cells.

Keywords

stress; hippocampus; gene expression; RNA-Seq; BDNF-Val66Met

Introduction

The hippocampus is a brain region essential for learning and memory, and has been implicated in mood disorder etiology[1]. Additionally, the hippocampus is highly responsive to stress, which results in impaired learning and memory and increased anxiety and depressive-like behaviors in rodents[2, 3]. These behavioral outcomes have been linked to stress-induced changes in neuronal structure and function in the hippocampus[4]. Given that environmental stress is known to contribute to the onset and progression of mental illnesses such as bipolar disorder, depression and post-traumatic stress disorder (PTSD) in humans, studying the molecular changes underlying stress-induced plasticity in the hippocampus has important translational relevance for understanding these disorders.

Pyramidal neurons of the CA3 region of hippocampus have a well-established role in spatial memory[5] and are of particular interest for mood disorders and stress susceptibility because they show pronounced dendritic retraction in response to chronic stress[6, 7], as well as decreased spine density[8]. These structural changes correlate with impairments in memory tasks and defects in long term potentiation (LTP)[9]. Stress-induced remodeling of CA3 neurons and effective LTP depends on several signaling pathways including glucocorticoids, glutamate and brain-derived neurotrophic factor (BDNF)[10].

BDNF has been implicated in neuroplasticity and mood disorders[11], where increased levels have been observed in rodent hippocampus after antidepressant treatments[12] and heterozygous knockout mice show attenuation of chronic stress-induced remodeling of CA3 pyramidal neurons[13]. A common human single nucleotide polymorphism (SNP) in the BDNF gene, which results in an amino acid change from a valine to a methionine at position 66 (Val66Met) has been associated with both memory impairments and increased risk of depression and anxiety-related disorders[14–16]. Importantly, human BDNF Met allele carriers exposed to stress were found to have decreased hippocampal volume and an increased likelihood of developing depression[17, 18]. Genetically modified knock-in mice with the Val66Met allele not only recapitulate the anxiety and depression-like phenotypes[19], but also show increased stress reactivity and impaired learning and memory after chronic stress[20], making them an important genetic model of increased stress susceptibility.

This study seeks to evaluate gene-environment interactions between the BDNF Val66Met allele and chronic stress on gene translation in CA3 pyramidal neurons of adult male mice. Several studies have characterized whole hippocampal gene expression in response to stress[21–23] and in BDNF Val66Met mice[24], but no studies have examined the stress by

genotype interaction in a cell type-specific manner using high-throughput techniques. This study uses a transgenic mouse line to isolate *in vivo* translating mRNA from a genetically homogenous population of CA3 pyramidal neurons. These expression profiles provide a refined map of stress-induced changes at the molecular level that are unique to CA3 pyramidal neurons, which are distinctly vulnerable to chronic stressors[4, 25], and will help unravel the mechanisms underlying the plasticity and damage-vulnerability of these cells in response to stress.

Materials and Methods

Animals

Gprin3-BacTRAP transgenic mice were generated in the Heintz Lab as previously described and backcrossed onto the C57/Bl6 background strain[26]. BDNF Val66Met knock-in mice were generated as previously described[19] and were backcrossed more than 12 generations to the C57Bl/6 background prior to starting experiments. To generate mice carrying both the bacTRAP transgene and the BDNF Val66Met knock-in allele, offspring from several mating pairs from each line were crossed together and subsequently maintained as their own independent line within the colony to maintain genetic homogeneity. To control for litter specific effects (such as the quality of maternal care) mice in each group were selected from across multiple litters. Animals were group housed (n=4–5) in standard cages (28.5×17×13cm) and were kept on a 12-h light-dark cycle (lights off 1800h) in a temperature-controlled room maintained at 21±2°C. Food and water were available ad libitum. The Rockefeller University Institutional Animal Care and Use Committee approved all experimental procedures involving animals.

Stress Paradigm & Tissue Collection

Male mice, 7–12 weeks old, were used for all conditions. Acute Forced Swim Test (FST) stress was performed for 6 mins in 2L of RT water in a 4L beaker. Mice were removed and dried and allowed to recover for 1hr in the home cage before sacrificing by cervical dislocation. CRS began at 7–8 weeks of age and was carried out in 50mL conical tubes for 2hr/d starting at 10:00h for 21 consecutive days. Control mice were left undisturbed. On day 22 mice were sacrificed (CRS) or subjected to forced swim (CRS+FST). All groups had unstressed, age-matched controls that were sacrificed concurrently. Mice were immediately decapitated, brains were removed and hippocampus was fresh dissected and prepared for TRAP protocol.

TRAP and RNA-Seq Analyses

Translating Ribosome Affinity Purification (TRAP) was performed as described in Heiman et al., 2014[27]. Briefly, hippocampal tissue from Gprin3–EFGPL10 mice (n=6 mice for each TRAP-seq, n=2 for qRT-PCR) was dissected out and placed in ice-cold dissecting buffer (HBSS, 2.5mM HEPES-KOH, 35mM Glucose, 4mM $NaHCO_3$, 100μg/mL cycloheximide) Tissue was manually homogenized in homogenization buffer (10mM HEPES-KOH, 150mM KCl, 5mM $MgCl_2$, 0.5mM DTT, protease inhibitors, RNasin and Superasin RNAse inhibitors, 100ug/mL cycloheximide) and centrifuged at 2,000×g for 10 min at 4°C, and supernatants were incubated with Nonidet P-40 for 5 min on ice before a 15-min spin at

20,000×g at 4°C. Supernatant was applied to Streptavidin MyOne T1 Dynabeads (Invitrogen no. 65601) that had been incubated with biotinylated protein-L (Pierce no. 29997) and α-EGFP antibodies (19C8 and 19F7 from the MSKCC antibody core facility) overnight at 4°C in 0.15M KCl buffer containing (10mM HEPES, 0.15M KCl, 5mM MgCl, 1% NP40, 0.05mM DTT, RNasin RNAase inhibitor, 100μg/mL cycloheximide). After immunoprecipitation, unbound fractions were saved, and beads were washed five times with 0.35 M KCl wash buffer (as above with 0.35M KCl) before beads were resuspended in lysis buffer (Stratagene Absolutely RNA Nanoprep Kit no. 400753). Unbound fraction was isolated using Qiagen Lipid RNA isolation kit (cat#74804) using a Qiacube (cat#9001292) per the manufacturer's instructions. Quantification and RNA integrity were determined by using a Bioanalyzer (Agilent), and only samples with RNA Integrity Numbers greater than eight (out of 10) were used for sequencing.

Samples were prepared for sequencing by the Rockefeller University Genomics Core Facility using the TrueSeq RNA Sample Preparation Kit v2 (Illumina) and barcoded to allow samples to be multiplexed within a flow cell lane. Barcoded cDNA libraries were sequenced on an Illumina HiSeq 2500 in a single lane to obtain 100-bp single-end reads at an approximate sequencing depth of 25–35 million reads per sample. Raw reads were trimmed to remove sequencing artifacts (10bp from 5′ end) and filtered to remove low quality reads (read with a QC < 20 in more than 10% of bases were discarded) before alignment to mouse genome (mm10) using TopHat2[28]. Differential expression analysis was conducted with Strand NGS software (Agilent), in which DESeq[29] was used to quantify transcript reads and obtain Z scores and fold change values for individual genes. Genes with $p<0.001$, Benjamini-Hochberg corrected and fold change greater than 1.5 were selected for further analysis. Differences in integrated read density were visualized against the mouse genome by using Strand NGS or IGV (Broad Institute). GO categories were manually curated from results of DAVID functional annotation cluster tool[30, 31].

Immunohistochemistry

For immunohistochemistry, animals were killed by deep anesthesia with pentobarbital (100 mg/kg), followed by transcardial perfusion with heparinized saline and then 4% (wt/vol) paraformaldehyde (PFA) using a peristaltic perfusion pump (Fisher Scientific). Brains were postfixed for 24 h in 4% PFA before sinking in 30% (wt/vol) sucrose for 3 d. Finally, brains were flash-frozen on dry ice and stored at −80°C until they were cut using a cryostat into 40μm-thick sections, which were then stored −80°C before being processed for immunohistochemistry. Immunohistochemical staining was carried out on 3–4 serial 40μm coronal sections from the rostrocaudal extent of the hippocampus as follows: sections were washed 3 × 10 min in 0.1 M PBS solution (pH 7.4), blocked in 0.5% BSA in 0.1 M PBS with 0.25% Triton X-100 for 1h before incubation in primary antibody. Antibodies NeuN (1:1,000; MAB377, Chemicon) and GFAP (1:1,000; AB7260 Abcam) were diluted in the blocking solution overnight at 4 °C. On the following day, sections were rinsed in 0.1 M PBS (3 × 10 min) before being incubated with species appropriate secondary antibody (Alexa568 gt@Rb A11011, Alexa568 gt@ms A11004; Life Technologies) diluted 1:1000 in PBS for 1hr at RT. After three washes in 0.1 M PBS (3 × 10 min), slides were coverslipped using Vectashield with DAPI (H-1200, Vector Laboratories). The immunoreactivity for each

cover-slipped section was assessed by a Nikon ECLIPSE 90i microscope, equipped with the digital camera DS-Fi1.

Preamplification and qRT-PCR

A separate cohort of animals was used for qRT-PCR validation of RNA-seq data. TRAP mRNA from two Gprin3-BAC-TRAP mice were pooled per replicate, and a total of four biological replicates were prepared per group (Control and FST). RNA was isolated as described above and reverse transcribed using AB Superscript reverse transcriptase, 100ng of RNA was added to each reaction. Primers were designed and verified for specificity using UCSC genome browser and NCBI Primer blast tools. Primers were purchased from IDT and resuspended to a stock concentration of 100uM in TE. Final primer pool contained all primers at concentration of 50nM in nuclease free H2O. Preamplification was carried out using Taqman preamp master mix as described by the manufacturer instructions (cat#4369016) and final product was diluted 1:5 in H2O for use in qRT-PCR reactions. SYBR green master mix was used with all primers and run on a Quantstudio 12k flex thermocycler. Fold change was calculated using the 2ΔΔCT method and normalized to GAPDH levels[32].

Results

Isolation of in vivo translating mRNA from CA3 pyramidal neurons

Pyramidal neurons of the CA3 region are uniquely vulnerable to the effects of stress[4, 25]. In this study, the response of CA3 neurons to stressors is characterized using the Translating Ribosome Affinity Purification (TRAP) technique, a powerful approach to interrogate translating mRNAs from genetically defined cell populations in vivo[26, 33]. TRAP has been used successfully to identify cell type specific translational changes in a variety of experimental conditions including behavioral and pharmacological manipulations[26, 34, 35]. To study expression changes in CA3 neurons of the hippocampus in response to stress using the TRAP methodology, transgenic mice were generated that expressed an EGFPL10a ribosomal fusion protein under control of the *Gprin3* promoter (Gprin3-BacTRAP). Reference in situ hybridization data (Allen brain atlas, www.brain-map.org) and BAC-EGFP reporter mice (www.gensat.org) demonstrate that *Gprin3* expression is restricted to the CA3 region in the hippocampus. Expression of the EGFPL10a transgene was localized primarily in the cell bodies of CA3 pyramidal neurons, confirming these previous findings (Fig. 1A). Double immunohistochemical labeling for the EGFP and either the neuronal marker NeuN or the glial marker GFAP confirmed that cells expressing the transgene were neuronal (Fig. 1B).

Anti-EGFP immunoprecipitations (IPs) were performed on dissected hippocampi from Gprin3-bacTRAP mice to purify polysomes and isolate polysome-bound mRNA from CA3 neurons. qRT-PCR was performed to quantify *Gprin3* and *Gfap* mRNA levels in either the immunoprecipitated (TRAP) or supernatant (unbound) fractions to validate that the RNA isolated by the TRAP protocol reflected enrichment from the targeted cell population. There was a 7-fold enrichment of *Gprin3* and conversely a nearly 80% depletion of *Gfap*, a glial marker, in the TRAP fraction compared to the unbound sample (Fig. 1D). TRAP and

unbound mRNA was subjected to RNA-sequencing (RNA-Seq), allowing for the characterization of differential expression of all mRNAs undergoing translation. Examination of canonically neuronal (*Tuj1*) or glial (*Aqp4*) genes in the sequencing data similarly confirmed enrichment of neuronal genes in the TRAP fraction (Fig. 1E). Unbiased clustering across all genes and samples showed that the TRAP samples clustered independently from the unbound samples, demonstrating consistency of the method across multiple experiments (Supp. Fig 1). To further confirm that mRNA isolated by TRAP was primarily from CA3 neurons, both a fine structure and differential expression search were conducted using the Allen Brain Atlas to identify genes enriched in either CA3 pyramidal neurons or the dentate gyrus (DG). The top hits from each region were manually curated to confirm the gene expression pattern, and a heatmap illustrating the clustering of RNA-seq results for 20 genes from either CA3 or DG revealed significant enrichment for CA3-specific genes, and depletion of DG-specific genes, in the Gprin3 TRAP IPs (Fig. 1F).

Acute and chronic stress differentially alter the translational profiles of CA3 neurons

Gprin3-BacTRAP mice were subjected to stress conditions identical to those described in Gray et al., (2014), in which the animals received either a naïve forced swim stress (FST), chronic restraint stress for 2h/day for 21 days (CRS), or a combination of CRS followed by a FST on day 22. TRAP mRNA was collected for RNA-seq to characterize patterns of stress-induced differential gene expression. After a naïve FST, 2,440 genes were identified as both significantly differentially expressed ($p<0.001$, FDR corrected) and exhibiting a greater than 1.5 fold change by RNA-Seq. Several immediate early genes identified as significantly increased or decreased after FST in the TRAP-Seq data were validated by qRT-PCR, with fold-change levels showing a significant correlation ($R^2=0.79$; $p<0.01$) between the two methods (Fig. 2A). The identification of numerous immediate early genes, such as *Fos* and *Arc* is consistent with whole hippocampal studies of acute stress-induced neuronal activation. Further, 617 genes identified as changed by FST in either the TRAP or unbound fractions by RNA-Seq were identical to those changed on whole hippocampal microarrays[21]. Studies comparing concordance rates of differential expression between microarray and RNA-Seq technologies have identified rates between 70–75% when using identical mRNA samples[36, 37]. The RNA-Seq data from acute FST has a 60% concordance rate with the previous publication, despite coming from an entirely different cohort of mice, which demonstrates that this is a highly reliable representation of gene expression changes. Importantly, the TRAP-Seq identified genes that were not significant on the microarray platform, demonstrating that the cellular specificity of this technique can improve detection sensitivity.

After CRS, 616 genes were significantly different from unstressed controls using the same criteria ($p<0.001$ FDR; $FC>1.5$). Only 146 of the same genes were identified as changed after either CRS or FST in the TRAP fractions (Fig. 2B), which indicates that acute and chronic stress result in distinct translational changes in CA3 neurons.

To reveal how a history of chronic stress can alter translational reactivity to a novel stressor, mice were subjected to a novel swim stress 24h after the final CRS exposure (CRS+FST). While over 1,000 of the significantly altered genes from CRS+FST mice were also changed

by naïve FST, there were over 500 genes unique to the CRS+FST condition, demonstrating that the stress history of the animals alters the molecular reactivity of CA3 neurons (Fig. 2C). Acute swim stress results in significant elevation of circulating corticosterone and activation of the glucocorticoid receptor (GR) in the hippocampus (reviewed in[38]). Previous studies using genome-wide methods have identified GR-responsive genes in the rodent hippocampus[39]. Using these data, expression levels of known GR-responsive genes were plotted across stress conditions. Consistent with an acute elevation of corticosterone, an unbiased clustering of GR-responsive genes based on expression levels revealed that FST and CRS+FST were the most dissimilar from control and CRS (Fig. 2D). However, numerous differences are still apparent between FST and CRS+FST (Fig. 2D(i) & (ii)), which demonstrates that there are certain known GR-responsive genes that are no longer activated or inactivated after CRS in response to an elevation of corticosterone.

Interestingly, these changes were observed despite the fact that no difference was detected in the levels of GR (*Nr3c1*) itself across stress conditions (Fig. 2E). However, significant differences in MR (*Nr3c2*), as well as three known chaperone proteins *Fkbp5*, Hsp70 (*Hspa1a*), and *Bag1* were observed across the stress conditions (Fig. 2E). Previous studies have shown that changes in the GR-MR balance or differences in the levels of these chaperones are sufficient to alter the levels of GR-dependent transcripts[40–42]. Acute and chronic stress has been associated with changes in BDNF mRNA levels in hippocampus[43, 44], and a non-significant decrease is observed here in the translational fraction of CA3 neurons (Fig. 2F). However, significant changes were observed in the neurotrophin receptors TrkB and TrkC (*Ntrk2*/*Ntrk3*), which were both increased with CRS, as were neurotrophin associated molecules, sortilin1 (*Sort1*) and *Trio*, which will be discussed below.

BDNF Val66Met mice have a unique translational profile and response to stress in CA3 neurons

To identify changes in the translational profiles of CA3 neurons associated with a genetic susceptibility to mood disorders, Gprin3-BacTRAP mice were crossed with BDNF Val66Met mice, a translationally relevant model of a human polymorphism that has been correlated with anxiety and depression[16, 19]. TRAP was performed on the hippocampus of Gprin3-bacTRAP mice and that were either heterozygous ($BDNF^{Met/+}$) or homozygous ($BDNF^{Met/Met}$) for the Met allele, and purified mRNA was used for RNA-Seq. Examination of reads mapped to the *Bdnf* locus confirmed the presence of the point mutation in both genotypes (Supp. Fig. 2) and revealed decreases in levels of *Bdnf* exons 4, 6, and 9 in CA3 neurons of $BDNF^{Met/+}$ and $BDNF^{Met/Met}$ mice (Supp. Fig. 3), which is consistent with previous in situ hybridization results[45]. Differential expression analysis identified 1,420 genes that were different in heterozygous mice and 3,736 differentially expressed genes in homozygous mice for the Met allele compared to WT controls (Fig. 3A; $p<0.001$ FDR corrected; FC > 1.5). Over 60% of the genes identified as differentially expressed in heterozygotes were also present in homozygotes (Fig. 3A). Gene ontology analysis of differentially expressed genes from the $BDNF^{Met/+}$ mice identified enrichment of gene clusters involved in transmembrane structure, cellular homeostasis, and ion transport (Table 1).

To further explore how Met allele carriers respond to chronic stress, double heterozygous Gprin3-bacTRAP/BDNF Val66Met mice were exposed to CRS for translational profiling to identify expression changes in CA3 associated with increased stress susceptibility. $BDNF^{Met/+}$ exhibited greater transcriptional reactivity to CRS than WT mice, with over 1,700 genes identified as changing with stress in $BDNF^{Met/+}$ compared with over 600 in WT mice (Fig. 3B,C). Interestingly, there was very little overlap among genes changed by stress in WT mice and $BDNF^{Met/+}$, suggesting that even one copy of the Met allele was sufficient to substantially alter the translational profile of CA3 neurons after chronic stress. BDNF and GR interact to produce synergistic changes in gene expression[46]. Therefore, levels of the GR-responsive genes from Polman et al. (2013) were investigated in $BDNF^{Met/+}$ mice (Fig. 3D). Examination of the heatmap revealed several important patterns. First, there were GR-responsive genes that were increased by CRS in WT mice, but not in $BDNF^{Met/+}$ (Fig. 3Di). Second, several genes were increased by stress in $BDNF^{Met/+}$ and not in WT mice (Fig. 3Dii). Lastly, numerous GR-responsive genes had a difference in baseline levels by genotype, which were not changed by stress (Fig. 3Diii).

Together these data suggest that the genetic susceptibility to mood disorders conferred by the Met allele results in both an altered baseline state of gene expression, but more importantly the mRNA profile of these cells in response to stress is almost entirely distinct from WT mice. Interestingly, many of the same genes changed by acute stress in wild type mice overlap with genes that show baseline difference in $BDNF^{Met/+}$ mice (Supp. Fig. 4). This further demonstrates how genetic susceptibility and environmental stress can interact to produce a unique cellular response and may share common mechanisms.

Genes implicated in LTP and neuroplasticity are altered in stress and BDNF-Val66Met mice

Pyramidal neurons of the CA3 regions are primarily glutamatergic[47] and stress is known to regulate the expression of glutamate receptor genes in the hippocampus[48]. Glutamatergic signaling is essential for LTP, which is mediated by NMDA and AMPA receptors and necessary for hippocampal spatial memory[49]. Therefore, changes in the levels of genes encoding NMDA and AMPA receptors were investigated.

Both NMDA and AMPA receptors showed significantly increased translation with chronic stress, particularly the AMPA subunits *Gria1*, *Gria2*, and *Gria3*, and the NMDA subunits *Grin1* (Fig. 4A,B). Previous studies have characterized electrophysiological defects in LTP/LTD in BDNF Val66Met mice[50]. These defects suggest that there may be baseline differences in the expression of NMDA and AMPA receptors subunits. In CA3 neurons, both $BDNF^{Met/Met}$ and $BDNF^{+/Met}$ mice showed significant decreases in the NMDA receptor subunit genes *Grin1* and *Grin2a* (NR2a), but increases in *Grin2c* (Fig. 4A,B). Most interestingly, $BDNF^{Met/+}$ mice exposed to CRS failed to show the same increases in AMPA and NMDA subunits observed in WT mice.

BDNF signaling is also known to facilitate LTP[51, 52]. A modest decrease in total BDNF mRNA levels was observed in the TRAP fraction in response to both acute and chronic stress in WT mice (Fig. 2F), and significant decreases in total BDNF mRNA, as well as the coding exon 9 of the *Bdnf* gene, were observed in the TRAP fraction from both the $BDNF^{Met/Met}$ and $BDNF^{Met/+}$ mice (Fig. 4C; Supp. Fig. 3A). The neurotrophin receptors for

BDNF, TrkB (*Ntrk2*) and TrkC (*Ntrk3*), were significantly increased by chronic stress, however, TrkC showed a rapid downregulation in response to acute stress (Fig. 4C). Sortilin1 (*Sort1*), a protein involved in trafficking of BDNF, was similarly decreased in response to acute stress, but increased by chronic stress. Because the expression levels of another neurotrophin receptor, p75 (*Ngfr*), were too low for reliable detection in the CA3 TRAP fractions, it's downstream signaling protein, Trio, was examined. Trio has been linked to the neuroplastic effects of BDNF[53, 54] and its mRNA was significantly increased in response to chronic stress in the TRAP-seq results (Fig. 4C). Carriers of the Met allele showed the most significant decreases in the expression of *Sort1*, and $BDNF^{Met/Met}$ mice also had significantly decreased levels of *Trio* (Fig. 4C).

Hormone receptor levels were also altered by the interaction of the BDNF Val66Met mutation with stress. GR was only increased in the $BDNF^{Met/+}$ mice subjected to CRS (Fig. 4D) compared with unstressed WT levels. Whereas MR was significantly decreased by genotype in $BDNF^{Met/+}$ mice and showed an increase only in $BDNF^{Met/+}$ mice in response to CRS, but did not increase in WT mice exposed to CRS. Unlike observations from the NMDA & AMPA receptors, the GR chaperones, *Fkbp5* and *Bag1* showed similar increases in levels after CRS, irrespective of the Met allele. Hsp70 family gene, *Hspa1a* (Hsp72), was significantly increased by CRS in WT mice, but was unchanged by CRS in Met allele carriers.

These gene expression changes reveal a potential mechanism underlying the impaired hippocampal LTP observed in BDNF Met allele mice[50]. Further, the differences in the translational response between WT and BDNF Met allele carriers to chronic stress provides substantial insight into the increased stress sensitivity of the BDNF Met allele mice.

Discussion

The present study used cell-type specific translational profiling to identify stress-induced changes in genes undergoing active translation in CA3 pyramidal neurons of the hippocampus. This cell population has a specialized role in learning and memory, and is particularly vulnerable to chronic stress and over-stimulation, as in seizures[4, 25, 55]. These results advance our understanding of the molecular changes that underlie the unique structural and electrophysiological plasticity occurring in this especially vulnerable subpopulation of hippocampal neurons in response to stress as well as with genetic manipulation of the BDNF gene.

Analysis of the CA3-specific translational profiles supports several conclusions. First, similar to findings in whole hippocampus, chronic and acute stress result in highly distinct molecular changes within this cell population (Fig. 2B). Second, exposure to a novel stressor after a history of chronic stress results in a different expression profile than observed upon naïve exposure to the same stressor (Fig. 2C). Third, many of the changes occur in known GR responsive genes (Fig. 2E), although we have shown previously that glucocorticoid treatment does not mimic the effect of acute stress or vice versa[21]. Comparisons with previous mRNA studies from whole hippocampus will help identify novel regulatory mechanisms as well as the cellular or regional specificity of the stress response of specific

genes. Lastly, mice with a genetic predisposition to increased stress susceptibility have a unique baseline translational profile in CA3 neurons (Fig. 3A; Table 1) and neurons from BDNF Met allele mice respond to chronic stress by activating a highly distinct set of genes from WT mice (Fig. 3B,C). Many of these differentially responsive genes in pyramidal neurons are known to be implicated in LTP and memory formation (Fig. 4). The patterns of stress-induced gene translation in Met allele mice identified potential mechanisms underlying their increased susceptibility to anxiety-like behavior[19] and stress-induced impairments in learning and memory and depressive-like behavior[20].

TRAP-Seq improves stress-induced expression profiling

Several previous studies have examined gene expression changes in response to stress in either whole hippocampal tissue[21–23] or using laser capture to isolate specific subregions[56]. These techniques both yield a heterogeneous mix of cell types that display highly varied gene expression profiles, a common problem when studying brain tissue. Using the Gprin3 bacTRAP reporter line allows for the interrogation of gene expression specifically in highly vulnerable CA3 pyramidal neurons. The present data also provide a more refined characterization of stress-induced changes than previous microarray studies by selecting for poly-adenylated mRNAs undergoing active translation. TRAP mRNA results have been shown to provide a vastly more accurate molecular profile than whole tissue assays[33]. Further, expression profiling by RNA-seq provides an increased dynamic range over microarrays and allows for the identification of novel transcripts and isoforms[57]. Together, TRAP mRNA purification and RNA-sequencing represent a significant advance over previous studies of stress-induced changes in hippocampal gene expression.

Given both the increased cellular specificity of the TRAP-IP and quantitative resolution of RNA-seq, differences that were previously undetectable in whole hippocampal lysates can be identified. For example, previous reports in $BDNF^{Met/+}$ mice did not show a baseline difference in levels of BDNF exon 9 mRNA in whole hippocampal qRT-PCR experiments[20]. In the TRAP-seq data, a decrease in both total BDNF transcript density (Fig. 4C) and specifically the levels of BDNF exon 9 were detectable in both $BDNF^{Met/+}$ and $BDNF^{Met/Met}$ mice (Supp. Fig3. A). Conversely, the ability to isolate the actively translating fraction of mRNA from a specific cell population has also resulted in subtle differences from previous data that hint at additional cellular mechanisms in the regulation of certain genes. Several studies have reported changes in hippocampal BDNF mRNA with stress[43, 44], but there have also been studies reporting no difference[58, 59]. In the present data, no difference was found after acute or chronic stress in the WT TRAP-Seq fraction (Fig. 2F). One possibility is that previous studies observed changes in transcription of *Bdnf* that is not immediately undergoing translation. Multiple studies of BDNF have shown it to be highly dynamic, with varying results across stress paradigms, timing and species in hippocampus[60]. Future work will be necessary to resolve these questions about BDNF regulation and other stress-induced genes as the field moves forward.

Despite some differences between this data and previous studies, these results largely confirm genes changed by stress in hippocampus, with 617 genes identified as significantly different after FST by both RNA-Seq fractions and microarray as one example. In addition

to the changes observed in CA3, the cell specificity of this data can be further validated against whole hippocampal microarray data by examining changes in the unbound fraction of genes whose expression is restricted to other hippocampal subregions. For example, *Btg1* expression is highly specific to the dentate gyrus (Allen Brain Atlas, www.brain-map.org). This gene was significantly decreased by FST in both the unbound RNA-Seq results and whole hippocampal microarray studies, but showed no difference in the TRAP IPs since it is not expressed in CA3 pyramidal neurons. Importantly, CA3 pyramidal neurons represent a subset of cells in the hippocampus, and changes occurring in this population after stress may be unobservable in whole tissue lysate. Therefore, the TRAP provides a more reliable list of candidate genes for further study. These data will provide the foundation for numerous future experiments interrogating the mechanisms underlying stress-induced neuroplasticity of CA3 pyramidal neurons by allowing us to link molecular changes with those changes already observed in LTP and morphology.

Translational profiling of Gene-Environment interactions

After demonstrating that chronic stress leads to a remodeling of the translational stress response in CA3 neurons, we sought to evaluate the interaction of the BDNF Val66Met SNP with this chronic stress paradigm. This SNP leads to altered trafficking of the BDNF mRNA to dendrites[61] and diminishes the activity dependent release of BDNF protein[16, 19]. BDNF release at the synapse is required for forms of early and late LTP, the former involving post translational modification of downstream signaling targets and the latter involving protein translation[62]. BDNF release can lead to phosphorylation of NMDA receptor subunits as well as insertion of AMPA receptors into the postsynaptic membrane, thus increasing synaptic strength. BDNF signaling through TrkB also leads to phosphorylation of synaptic proteins such as Rims1[63], which are important in neurotransmitter release. BDNF action in hippocampal neurons also leads to elevated protein levels of the NMDA receptor subunits NR1, NR2A and NR2B, and increased local translation of AMPA receptor subunits[64, 65]. Thus, the diminished activity dependent secretion of BDNF, combined with the impaired dendritic localization of BDNF mRNA in the Val66Met animals provides a plausible link between the altered translational profile observed in CA3 neurons in these mice and LTP deficits shown previously[50].

BDNF Val66Met mice also show an exaggerated response to a seven-day chronic stress, with larger CORT elevation, disruptions in HPA axis and greater memory impairment[20]. This suggests that impairments in activity dependent secretion impair the ability of the neurons to undergo stress-induced plasticity that is normally observed in CA3 neurons and is dependent on GR signaling[4]. Recent studies have shown that BDNF activation of the TrkB receptor leads to phosphorylation of GR. When these phosphorylation residues are mutated, mice display a lack of the chronic stress-induced structural plasticity in cortical neurons that is observed in WT[66]. The authors also show that GR signaling without BDNF signaling in vitro activates a different set of genes than when both systems are activated together[46], suggesting that BDNF-GR crosstalk is important in the structural remodeling observed during chronic stress, and could be a mechanism underlying the lack of plasticity in BDNF Val66Met mice. These results point to a model where CA3 neurons in BDNF Met allele carriers lack the necessary plasticity to provide an adaptive hippocampal circuitry, which

leads to impaired negative HPA feedback and memory deficits. A pathway analysis of genes changed in BDNF$^{Met/+}$ compared to wildtype mice demonstrated a preponderance of ion channel and neurotransmission-related genes (Table 1), such as glutamate (*Grin2a, Grin3a, Grm5*) and GABA (*Gabra1*) receptor subunits, as well as the Ca+ channels (*Cacna1b, 1c, 1e*), which are critically involved in LTP and other forms of neuroplasticity. Additionally, in response to chronic stress, WT mice show up regulated translation of *Grin1*, and *Gria1, 2*, and *3*, consistent with previous reports that have shown an upregulation of *Grin1* and *Gria1* after chronic stress[67–69], whereas BDNF$^{Met/+}$ mice not only have lower basal levels of these genes, but the level do not change in response to stress.

Interestingly, the baseline changes in BDNF Val66Met mice showed considerable overlap with genes changed by acute stress in wild type mice (1,066 gene), and far less overlap with genes changed by CRS in wild type mice (121 genes; Supp. Fig. 4). This suggests that even in the absence of stress the Met allele confers many of the same changes that are occurring after acute stress, and therefore the highly divergent response of BDNF$^{Met/+}$ mice to CRS may be accounted for by the fact that they are starting from a baseline similar to a stressed wild type mouse (“pre-stressed”). However, there were over 300 genes changed that were unique to the unstressed BDNF$^{Met/+}$ mice that are equally as likely to account for their altered stress reactivity. Therefore, future experiments will seek to further characterize both of these overlapping and non-overlapping sets to better identify the genes that confer increased risk for mood disorders in this translational model.

In conclusion, these data provide a blueprint of the cell-type specific changes occurring in vivo in CA3 pyramidal neurons in response to stress and in the presence of a genetic mutation that is known to alter stress susceptibility. These findings will support future studies seeking to alter the effects of stress through pharmacological or environmental manipulations, and may help others identify significant gene expressions changes in pathways beyond the glutamatergic and BDNF systems that are involved in remodeling of the neural circuitry underlying stress susceptibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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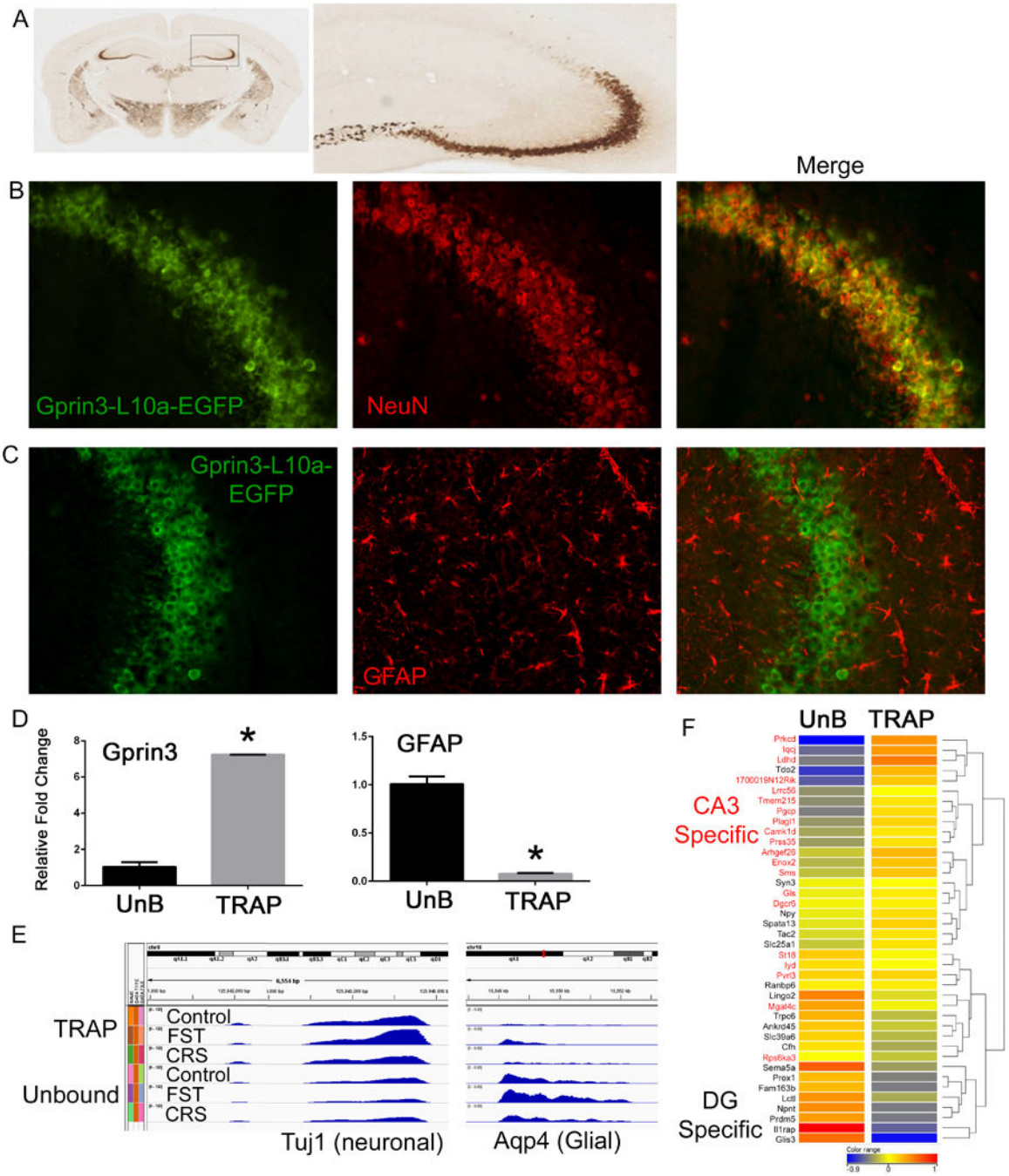

Figure 1. Gprin3-BacTRAP mice allow for the isolation of translating mRNA from CA3 neurons

A) Immunohistochemical labeling for the EGFP fusion proteins shows expression in several brain regions, but in hippocampus the labeling is primarily in the CA3 region. B–C) Double labeling for either NeuN or GFAP confirm that Gprin3-EFGP-L10a positive cells are neuronal. D) qRT-PCR from TRAP-IP and Unbound (UnB) mRNA isolations demonstrates significant enrichment for *Gprin3* in the TRAP fraction and the glial specific marker *Gfap* in the unbound fraction. E) Read density plots from RNA-Seq data of the canonically neuronal gene *Tuj1* and the glial gene Aqp4 demonstrate enrichment of neuronal genes in the TRAP

fraction and glial genes in the unbound fraction across stress conditions (FST= forced swim test; CRS= Chronic Restraint Stress). F) Heatmap of genes expressed in either dentate gyrus (black) or CA3 (red) shows that the TRAP-IP is highly enriched in CA3 specific genes, whereas dentate specific genes are enriched in the unbound fraction. (*$p<0.05$)

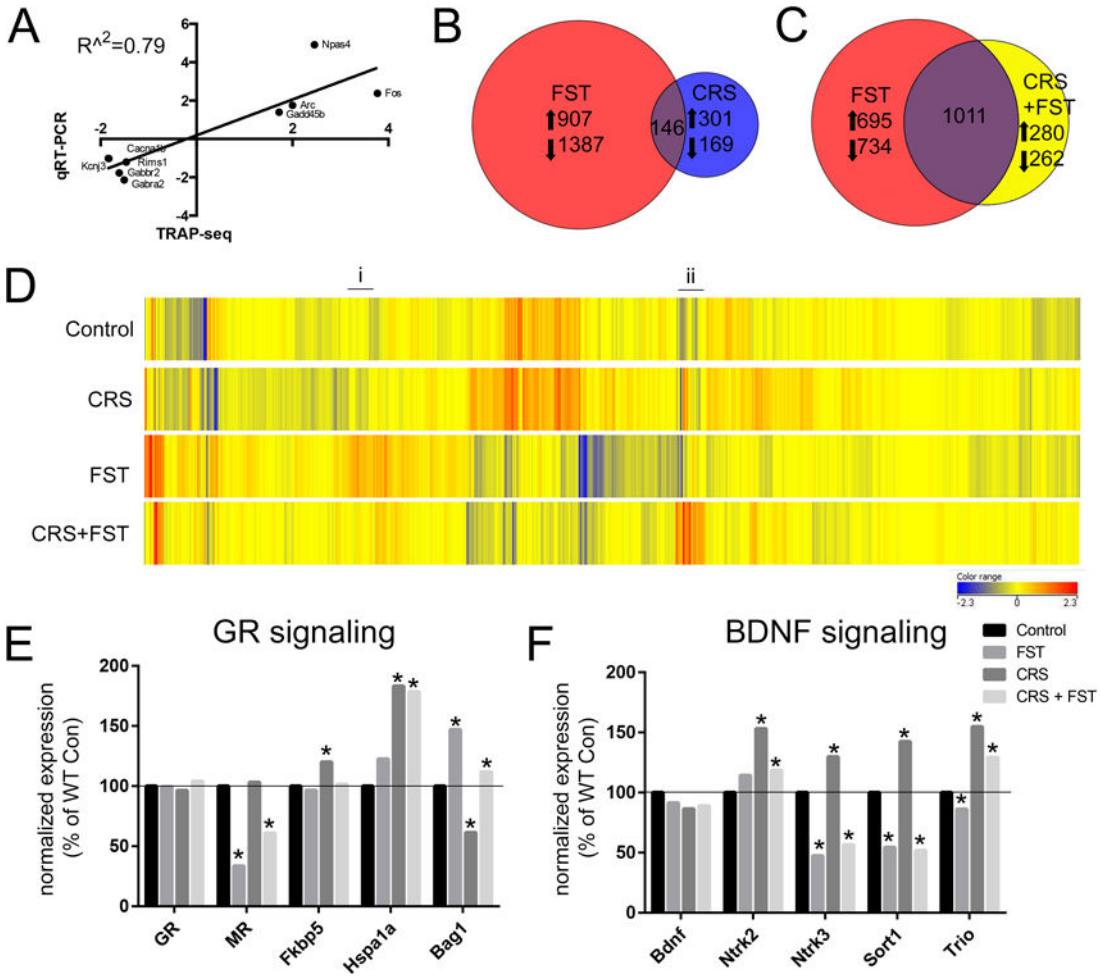

Figure 2. Translational changes in CA3 neurons reveal unique activation profiles in response to stress

A) Scatter plot illustrating the correlation between the fold changes from RNA-seq and qRT-PCR for 9 genes differentially regulated by FST. B) Venn diagram illustrating that the number of genes differentially expressed after an acute stress (FST= forced swim test, red circle) were distinct from the genes that were changed after exposure to chronic stress (CRS= chronic restraint stress, blue circle). C) Venn diagram showing that exposure to a novel acute stressor after three weeks of chronic restraint (CRS+FST, yellow circle) activated significantly different genes than after a naïve exposure to the same FST. D) Heatmap illustrating expression level changes of known GR-regulated transcripts from Polman et al., (2013). Transcripts upregulated only in response to naïve FST are highlight with (i) and transcripts upregulated only when exposed to FST after CRS are highlighted with (ii). E) Levels of the glucocorticoid receptor and selected chaperone genes. F) Levels of *Bdnf* and selected downstream signaling molecules. (*p<0.001; Benjamin-Hochberg corrected for FDR)

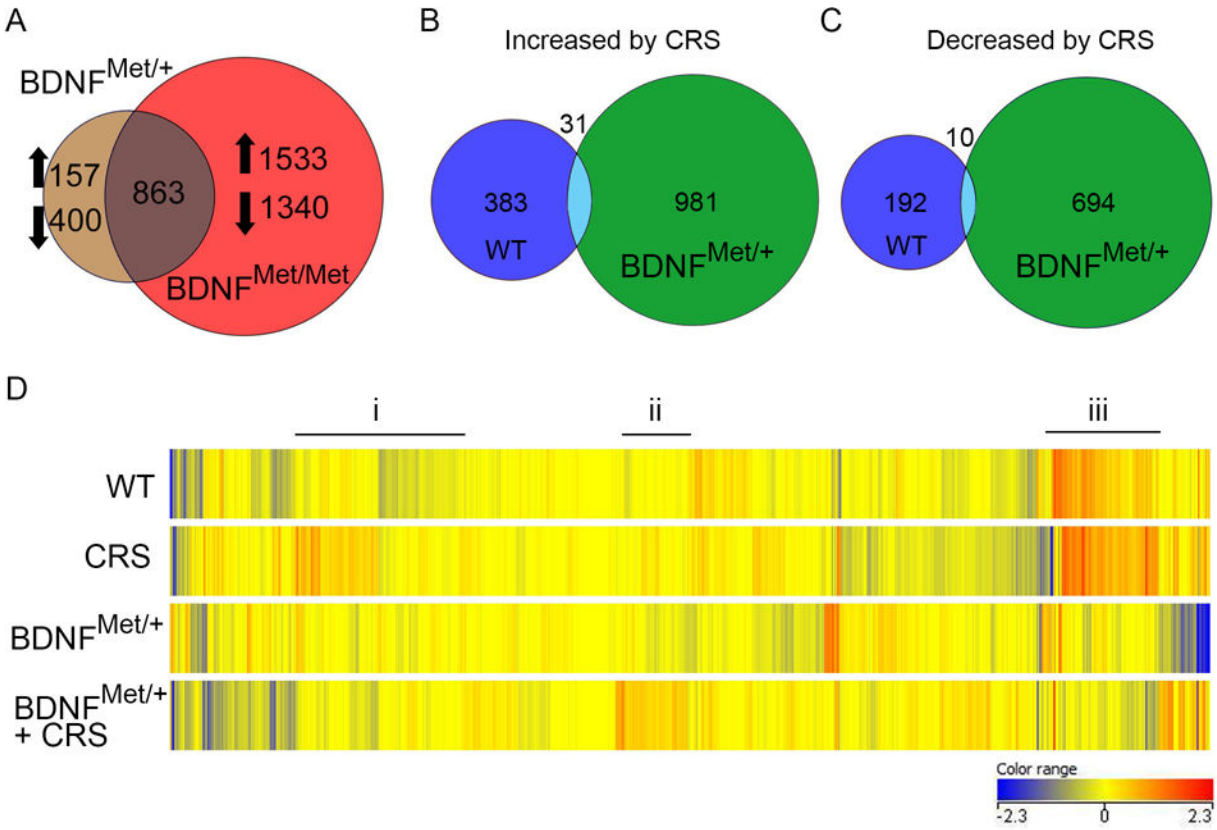

Figure 3. BDNF-Val66Met mice exhibit a unique translational profile in response to stress

A) Venn diagram showing that similar genes were differentially expressed in both the BDNF$^{Met/+}$ and BDNF$^{Met/Met}$ mice compared to WT. B,C) Venn diagram illustrating that BDNF$^{Met/+}$ mice exposed to CRS exhibited numerous unique expression changes that were not present in WT mice after CRS. D) Heatmap of expression level changes in GR-regulated transcripts revealed i) genes increased by CRS in WT mice that did not show the same change with CRS in BDNF$^{Met/+}$, ii) genes increased by CRS in BDNF$^{Met/+}$ that were primarily unchanged with stress in WT mice and iii) genes that were different by genotype at baseline and changed by stress in BDNF$^{Met/+}$.

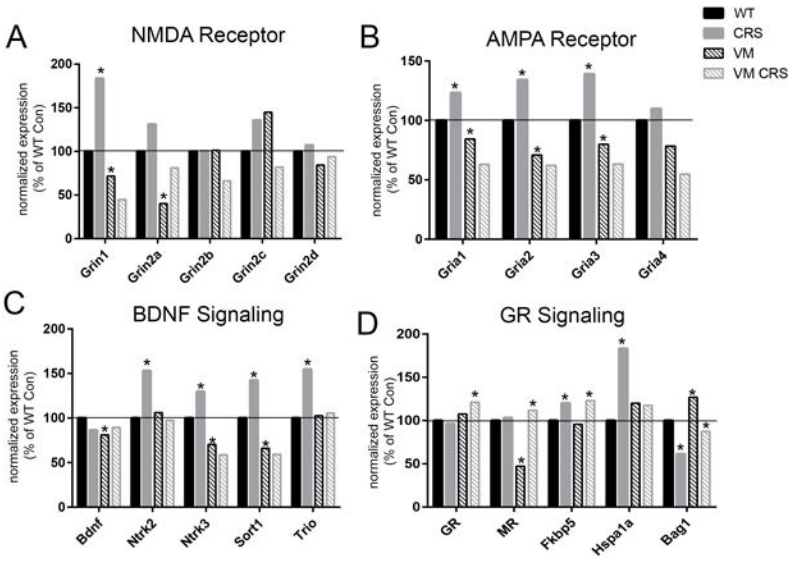

Figure 4. Changes in translation of LTP and BDNF-related genes in CA3 pyramidal neurons

A) Changes in expression of the NMDA receptor subunits from the TRAP fraction in response to acute stress (FST), chronic stress (CRS), or heterotypic stress (CRS+FST) plotted as a percentage change from control levels normalized to 100. B) Levels of AMPA receptor subunit expression as a percent of control in response to stress. C) Changes in the relative expression levels of BDNF receptors and downstream signaling molecules after stress. D) BDNF-related gene expression levels in $BDNF^{+/Met}$ and $BDNF^{Met/Met}$ mice normalized to wildtype levels. (*p<0.001; Benjamin-Hochberg corrected for FDR)

Table 1

Enrichment of GO Pathways in genes changes in $BDNF^{Met/+}$ CA3 Neurons

Rank	Common GO Term for Cluster	Enrichment Score	Selected Example Genes
1	Transmembrane & Membrane	9.66	APP, CREB3, CREB3L1, GRIN2A, GPR56, GRM5, L1CAM, NCAM2, NLGN3, SLC1A2, SLC1A3
2	Glycoproteins & N-linked Glycosylation	5.71	CLCN3, FGFRL1, GABRA1, GRIK2, IL1RAP, NCAM2, NLGN3, NPY2R, ROR1, TGF1B
3	Lysosomes	5.13	CTSB, CLCN7, LAMP1, LAMP2, MT1, NPC1, NOS1, TMEM9, TRIM23
4	Cellular Homeostasis	4.87	ANK1, BCL2, GRIK2, GRIN2a, IGF1, NR3C2, S100B, SGK1, TGFB1
5	Cell-Cell Adhesion	3.56	ARHGAP6, ASTN1, CELSR2, CNTN1, CSTN2, L1CAM, MCAM, NCAM2, NELL1, NELL2, NLGN3
6	Ion Transport	3.51	CACNA1C, CLCN3, GABRA1, GABRA2, KCNQ3, KCNV1, SLC1A2, SLC1A3, TRPC5
7	Neuronal projection	3.12	APC, BACE1, GABBR2, IQGAP1, KIF5A, NCAM2, NOS1, NRCAM, SHANK2, TGFB1
8	Cytoskeletal Components	3.06	ACTN1, ACTN2, ACTN4, ANK1, IQGAP2 MARCKS, MYH10, PARD6B
9	Exocytosis	3.02	CPLX2, EXOC4, EXOC5, EXOC7 NR3C2, RAPGEF4, RIMS1, RIMS3, RIMS4, SYN3
10	Ca+ Ion Transport	2.96	CACNA1B, CACNA1C, CACNA1E, CACNA2D1, GRIN2A, GRIN3A, KCNQ3, SLC9A6, SLC9A7,