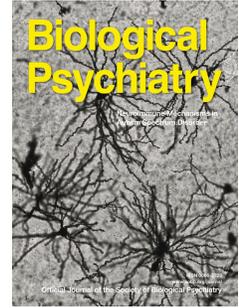


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Transthyretin orchestrates vitamin B12-induced stress resilience

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Abstract

Background: Chronic stress significantly contributes to mood- and anxiety disorders. Previous data suggest a correlative connection between vitamin B12 supplementation, depression, and stress resilience. However, the underlying mechanisms are still poorly understood.

Methods: Using the chronic variable stress mouse model coupled with RNA-sequencing, we determined vitamin B12-induced transcriptional changes related to stress resilience. By viral-mediated gene transfer and *in vivo* epigenome editing, we reveal a functional pathway linking vitamin B12, DNA methylation, and depressive-like symptoms.

Results: We identified Transthyretin (*Ttr*) as a sex-specific key target of vitamin B12 in chronic stress. Accordingly, *TTR* expression was increased postmortem in the prefrontal cortex of male, but not female, depressed patients. Virally altered *Ttr* in the prefrontal cortex functionally contributed to stress- and depression-related behaviors, changes in dendritic spine morphology, and gene expression. In stressed mice, vitamin B12 reduced DNAm in the *Ttr* promoter region. Importantly, using *in vivo* epigenome editing to alter DNAm in the brains of living mice for the first time, we establish a direct causal link between DNAm on *Ttr* and stress-associated behaviors.

Discussion: Using state-of-the-art techniques, this study uncovers a mechanistic link between vitamin B12 supplementation, *Ttr*, and markers of chronic stress and depression, encouraging further studies into dietary interventions for mood disorders.

Introduction

Chronic stress is a major risk factor for mental illnesses, such as major depressive disorder (depression, MDD), which ranks among the leading causes of disability and suicide (1; 2). However, the mechanisms by which chronic stress impacts mental health, and their mitigation by rapid environmental interventions such as dietary factors, remain poorly understood (3).

Vitamin B12 (cobalamin) is an essential nutrient synthesized by bacteria. It serves as a cofactor in the one-carbon metabolism, which provides methyl donors for the methylation of DNA and proteins, including histones. Notably, a common symptom of vitamin B12 deficiency is a depressive phenotype (4) and individuals deficient in vitamin B12 have an elevated risk of developing depression (5–9). Moreover, there is evidence that vitamin B12 supplementation in non-deficient populations may lower the risk of depression (10–12), and help to reverse the pro-depressive effects of early life stress in animal models (13). Previous research in our team demonstrated that a single acute dose of vitamin B12 reduced depressive-like behaviors in mice in the chronic mild stress (CMS)-model (14). Additionally, vitamin B12 rescued stress-induced alterations in plasma mannose and Gmpgb (15), a key enzyme of glycosylation metabolism associated with depression (16). Despite these correlative findings, it is unclear how vitamin B12 mediates stress resilience and improves markers of depression on a molecular level.

Here we explored the effects of an acute dose of vitamin B12 on stress response using a multilevel approach. To that end the chronic variable stress (CVS) mouse model allowed a sex-specific analysis that closely mirrors the molecular aspects of human depression (17). Through next-generation RNA sequencing, we identified Transthyretin (*Ttr*) as a pivotal stress-regulated gene product, which was specifically rescued by vitamin B12 in the prefrontal cortex (PFC) of male mice. Accordingly, we observed altered *TTR* levels in the postmortem PFC of male depressed patients. *Ttr*, a clinically relevant carrier protein for thyroid hormones and retinol, has been previously found to be altered in rodent models of stress (18; 19) and is increasingly recognized for its contribution to various brain functions (20). However, modes of reversing *Ttr* levels remain elusive. *TTR* missense mutations are linked to hereditary amyloidosis, a rare and lethal disease. In this context, *TTR* stands as the first gene edited by CRISPR technology *in vivo* in humans (21). Yet, despite its pivotal clinical role, surprisingly little is known about the chromatin regulation of the *Ttr* gene.

By using a viral approach to alter *Ttr* levels in the PFC, we establish a causal connection between *Ttr*, dendritic spine morphology, and stress-associated behaviors, and we determine transcriptional pathways downstream of *Ttr*. Furthermore, by pioneering the combined use of DNAm editing and jetPEI *in vivo* transfection strategies, we were able to alter the *Ttr* chromatin state in the brains of living mice. We observed that DNAm editing on distinct loci in the *Ttr* promoter region caused behavioral changes associated with stress and mood. In summary, these data unveil a sex-specific molecular pathway with the potential to rapidly ameliorate depression-associated phenotypes and increase stress resilience.

Methods and Materials

Extended methods are found in the supplementary material.

Animal housing and experimental approval. Mice were accommodated in accordance with ethical guidelines set by the Thüringer Landesamt für Verbraucherschutz (TLV). All experiments were carried out under the approved animal license UKJ-18-037 (Germany), aligning with the guidelines of EU Directive 2010/63/EU for animal experimentation. The studies involving genetically modified organisms adhered to S1 regulations as outlined in the GenTAufzV. C57Bl/6J mice were bred at the animal facilities of the University Hospital Jena, Germany, and Friedrich-Schiller-University, Jena, Germany. The mice were provided with standard chow (LASQCdiet Rod16-R, LASvendi GMBH, Soest, Germany), by default containing 50 mg/kg of vitamin B12. The mice used in the experiments were a minimum of 10 weeks old, with both sexes utilized as specified. Housing conditions involved a 14L:10D light cycle.

CRISPR-dCas9 epigenome editing. Vectors coding the dCas9-DNMT3ACD-DNMT3LCD-3xFLAG fusion gene (contains the enzymatic group of the DNA methyltransferase DNMT3) and the control plasmid pCMV-dCas9-mD3A (#78257, Addgene, Watertown, MA, USA) were used as described in (14; 22). Guide-RNAs against the promoter region of *Ttr* were designed via a published protocol (available at www.addgene.org/crispr/church/). Scrambled controls were used as described (14; 22).

Cell culture and transfection. Neuro2A-cells (ATCC #CCL-131) were cultured in six-well plates (10^6 per well) in DMEM + GlutaMAX-1 (25300-054/096, GIBCO by Thermo Fisher Scientific, Waltham, MA, USA) with 10 % fetal bovine serum (South American Origin, #51810-500, Biowest) and 1 % Penicillin-Streptomycin (#15070-063, GIBCO). For CRISPR epigenome editing *in vitro*, vectors were co-transfected using Lipofectamine 2000 transfection reagent (#11668019, ThermoFisher Scientific) in OptiMEM (#31985070, GIBCO). The medium was replaced after 4 h with DMEM + GlutaMAX-1 medium. 48 h after transfection, cells were collected for DNA or RNA extraction.

JetPEI *in vivo* transfection. The procedure was essentially performed as described (23). 12 µg of plasmid DNA (ratio 1:10 for guide-RNA and dCas9-construct containing plasmids) was diluted into 5 µl of sterile 10 % glucose and added to diluted *In vivo*-jetPEI® reagent (#101000040 VWR, Polyplus-transfection). The solution was mixed by pipetting and incubated at RT for 15 min. The jetPEI/Plasmid mix was delivered into the PFC at a rate of 0.2 µl per min, followed by 5 min of rest. Mice were tested 2 days later.

Drugs and chemicals. Mice were intraperitoneally (i.p.) injected with 2.7 mg/kg vitamin B12 (cyanocobalamin, #V6629, Sigma) or saline at an injection volume of 10ml/kg body weight and tested 24 h later.

RNA purification and quantification. RNA extraction involved resuspension in Trizol and chloroform precipitation, followed by washing with isopropanol and 75% ethanol. Subsequently, cDNA conversion was performed using the GoScript™ Reverse Transcriptase kit (#A5001, Promega). Quantitative real-time PCR was conducted on a Bio-rad CFX96 Real-time system. The primer sequences are provided in the supplementary material. The quantitative PCR (qPCR) results were analyzed as described (14).

AAVs & stereotaxic surgery. Bilateral stereotaxic surgery targeting the medial PFC was conducted following established procedures (17). The following three viruses were employed: pAAV.1-CAG-GFP (#37825, Addgene), OE-Ttr-GFP: pAAV-CAG-GFP-P2A-Ttr-WPRE.bgH, KD-Ttr-GFP: pAAV-U6-Ttr-shRNA-2-CAG-GFP-WPRE3 (Ttr-AAVs custom-made by Charité viral vector core, Berlin, Germany).

Behavioral tests and CVS. The acute behavioral tests tail suspension test, forced swim test, sucrose preference, splash test, and novelty-suppressed feeding as well as CVS were conducted as outlined in previous studies and are explained in more detail in the supplementary methods (17; 24).

Dendritic spine analysis. The analysis relied on the detection of AAVs' GFP-fluorophores. Images of 40 μm PFA-fixed brain sections were captured using a Zeiss LSM 880 confocal microscope employing the AiryScan method. Maximum intensity projections were generated using Zen Black and Zen Blue software and subsequently analyzed in NeuronStudio (CNIC, Mount Sinai School of Medicine). Analysis occurred on secondary dendrites of pyramidal neurons (layers 2/3 of the PFC). The overall density of spines, proportions of thin, mushroom, and stubby spines, along with cumulative neck length, were determined using Graph Prism. The density of neck-containing spines refers to the sum of thin and mushroom spine densities.

Next-generation RNA-sequencing. Data tables and extended methods are provided in the supplementary material and via the Gene Expression Omnibus (GEO) database together with raw sequencing data under accession numbers GSE267339 (Table S1, vitamin B12) and GSE267047 (Table S2, OE-Ttr).

DNAme analysis. DNA was purified and bisulfite converted as described in (14) using the DNeasy Blood & Tissue kit (#69506, Qiagen) and the EZ DNA Methylation-Gold kit (#D5006, Zymo, Irvine, CA, USA). Pyrosequencing was conducted on a Pyromark Q96 Sequencer using matching reagents (#97204, #978703 Qiagen).

Postmortem samples. Samples were generously provided by the Douglas-Bell Canada Brain Bank. Experiments were conducted in agreement with Douglas-Bell Canada Brain Bank (REB Approval #04/21) and the Ethics Committee of Jena University Hospital, Germany (Reg.-Nr. 2020-1862-Material). Groups were balanced for age and postmortem interval. Samples with postmortem interval > 100 h were excluded. All donors self-identified as Caucasian with the exception of one control self-identifying as Haitian (4%) and one depressed patient self-identifying as Hispanic (3%). The RNA IQ score was measured using the Qubit RNA IQ Assay-kit (#Q33221, Thermo Fisher Scientific).

Statistics. Statistical analyses were performed utilizing GraphPrism. The comparison of two groups with equally distributed variances employed a two-tailed Student's t-test. In case of unequal variances, Welch's t-test was used. One-way ANOVA and Tukey *post hoc* test were applied when varying a single factor across multiple groups, while Two-way ANOVA and

Bonferroni *post hoc* test were employed when two factors were varied. The cumulative head diameter of dendritic spines was analyzed using the Gehan-Breslow-Wilcoxon test (25). Data points more than two standard deviations away from the average were considered outliers and subsequently removed. During behavioral tests and dendritic spine analysis, experimenters were blind to group assignments to minimize bias.

Results

Vitamin B12 reverses stress-induced changes in *Ttr*-expression. We hypothesized that the vitamin B12-induced reversal of stress effects may be, at least in part, mediated by transcriptional alterations given its' putative influence on DNAm and histone methylation. To investigate this, we conducted RNA sequencing on the PFC using the CVS mouse model (**Fig. 1A-G**). None of the mice were deficient in vitamin B12, which occurred within the regular chow. Mice of the vitamin B12 group additionally received an acute i.p.-injection of 2.7 mg/kg vitamin B12 approximately 24 h before testing (14).

Pathways that were abundant in differentially expressed genes within the CVS group included chromatin regulation, synaptic and intracellular signaling, metabolism, RNA regulation, and protein processing, indicating a widespread restructuring of the PFC in response to stress (**Table S1**). Well-established depression-associated gene products, such as *Fkbp5*, *Fosb*, and *Per1*, were among the affected genes in the CVS group. Additionally, CVS altered the expression of the folate receptor *Folr1*, the methyl transferases *Kmt5a*, *Tmt1A*, *Hnmt*, *Lcmt2*, the methyl transferase reductase *Mtrr*, and the demethylase *Alkbh5*, indicating stress effects on methyl group metabolism (**Table S1**).

Vitamin B12 induced minimal transcriptional changes in the PFC of naïve mice. However, it partially reversed the transcriptional changes induced by CVS (**Fig. 1B, Table S1**).

Ttr was the gene product showing consistently the highest log₂ fold change between experimental groups (**Fig. 1C-F**). *Ttr* expression was increased by CVS and rescued by vitamin B12 within the CVS group. Other genes exhibiting a similar pattern included *p35* (*Cdk5r1*, a Cdk5-activator implicated in learning, memory, schizophrenia, and neurodegeneration (26; 27)), the RNA binding proteins *Hnrnpdl* and *Hnrnp2*, the tyrosine kinase *Src*, the cytoskeletal regulator *Trak1* and the E3 ubiquitin-protein ligase *Znrf1* (**Table S1**). Among those genes differentially expressed by vitamin B12 within the CVS group were also several key synaptic proteins including *Camk2a* and the autism-associated genes *Shank1*, *Shank3*, and *Nlgn2* (**Fig. 1G**). However, their log₂ fold change was considerably lower than that of *Ttr* and they could not be validated by qPCR (data not shown).

The recurrent association of *Ttr* levels with stress in mouse PFC indicates biological robustness in the association between *Ttr* and chronic stress (18; 19). However, as there is no known mechanism of reversal, we chose to focus our subsequent investigations on *Ttr* to characterize modes of regulating this pivotal gene and, in consequence, to reverse states that contribute to pathology.

The CVS-induced increase in *Ttr* and its reversal by vitamin B12 were confirmed by qPCR in an independent cohort (**Fig. 1H**). In female mice, neither CVS nor vitamin B12 exerted an effect on *Ttr* levels in the PFC (**Fig. 1I**).

Next, we examined whether *TTR* levels were affected postmortem in the PFC of depressed patients and suicide victims. As expected, obtainable postmortem samples varied in PMI and age. However, the RNA IQ score was suitable for testing (**Fig. S1**). We did not observe correlations between *TTR* and PMI ($R^2 < 0.01$, $P = 0.97$), *TTR* and RNA IQ Score ($R^2 = 0.05$, $P = 0.50$), or *TTR* and age ($R^2 < 0.01$, $P = 0.82$) in brains from healthy controls.

In male depressed patients, but not in females, *TTR* levels were elevated (**Fig. 1J, K**). *TTR* was particularly increased in male depressed patients who died from accidents or natural causes and less increased in suicide victims (**Fig. S1A, B**). The blunted effect in suicide victims may arise due to the acute drug intake often accompanying suicide, the acute stress, anxiety, or relief of conducting the act, or other mental states associated with it. In females, neither cause of death nor mental health status significantly affected *TTR* levels (**Fig. S1C**).

TTR functions as a carrier protein freely circulating in the cerebrospinal fluid of the brain and the bloodstream (20). Therefore, we assessed *Ttr* levels in other tissues. The liver has a strong baseline expression of *Ttr*. However, neither CVS nor vitamin B12 affected *Ttr* levels in the liver of male mice (**Fig. 1L**). In the brain, *Ttr* is secreted into the cerebrospinal fluid, which enters the systemic blood circulation by intracranial venous sinuses or cervical lymphatics. Moreover, *Ttr* can pass the blood-brain barrier (28). Nevertheless, neither CVS nor vitamin B12 had an impact on *Ttr* levels in plasma (**Fig. 1M**). Finally, we assessed testes tissue. Chronic stress can affect certain health measures in the offspring through the patriline (male germline) (29). The molecular mechanisms that transfer stress, a subjective effect originating in the brain, towards the germline are still unclear. Since *Ttr* can pass the blood-brain barrier, it is conceivable that it may enter the testes as well. However, *Ttr* levels were not increased in the testes in our data set (**Fig. 1N**). In summary, these data suggest that the observed CVS-induced increase in *Ttr* is specific to the brain, limited to the male sex, and can be rapidly reversed by vitamin B12.

Viral-mediated alterations in PFC-*Ttr* alter depressive-like behaviors and dendritic spine morphology.

Next, we investigated whether *Ttr* plays a causal role in the behavioral and morphological changes associated with chronic stress, given that previous associations between *Ttr* and stress had been largely correlative (18; 19). To address this, we stereotaxically injected AAVs to overexpress (OE) or knockdown (KD) *Ttr* in neurons within the PFC of male mice (**Fig. S3**). The AAVs independently expressed GFP, facilitating dendritic spine analysis. Analysis was conducted not earlier than 4 weeks post-injection to ensure stable expression of the constructs. The AAVs had no discernable impact on body weight or behavior in the open field (**Fig. S2**).

OE-*Ttr*, which increased *Ttr* in the PFC (**Fig. 2A-C**), elicited a robust increase in depressive-like behaviors across a variety of tests linked to escape behavior (tail suspension test), grooming (splash test), and feeding in a novel environment (novelty-suppressed feeding) (**Fig. 2D-G**). Additionally, OE-*Ttr* led to a reduction in the density of stubby and neck-containing spines, mirroring the observed effects of CVS (**Fig. 2H-K, Fig. S3**). However, in contrast to CVS, OE-*Ttr* resulted in a decrease in the cumulative head diameter.

RNA-sequencing revealed that OE-*Ttr* altered transcripts with a variety of functions, including immune response (*Cd74*, *H2-Aa*, *H2-Ab1*, *H2-Eb1*, *Cd8a*, *Ifitm1*, *Igha*, *Ighg2b*, *Igkc*, *Tgtp1*), carbohydrate metabolism (*Mrc1*, *Igfbp2*), and signal transduction (*Serping1*, *Slc6a13*, *Slc6a20a*, *Slc6a20a*) (**Fig. 2L-N, Table S2**). Several gene products were linked with either the cytoskeleton or the extracellular matrix (*Tagln*, *Bgn*, *Cnn2*, *Myh11*, *Acta2*, *Myl9*, *Lama1*, *Cilp*), suggesting an

interplay of intra- and extracellular mechanisms that could influence dendritic spine outgrowth and synaptic plasticity, and being generally in line with an extracellular role of *Ttr*. Taken together, OE-*Ttr* mimics specific behavioral and morphological aspects of chronic stress, suggesting that *Ttr* functionally contributes to stress symptoms.

Next, we asked whether a reduction of *Ttr* could counteract specific CVS effects as was observed with vitamin B12 (**Fig. S4**). Consequently, KD-*Ttr* effects were assessed under stress conditions. KD-*Ttr* blocked CVS effects on escape behavior (forced swim test; the tail suspension was not applicable due to the CVS protocol), grooming (splash test), and novelty-suppressed feeding (**Fig. 3A-G**). This behavioral profile was consistent with OE-*Ttr*. In line with the preceding data, KD-*Ttr* rescued CVS effects on stubby and neck-containing spines and may have partially prevented CVS effects on the cumulative head diameter (**Fig. 3 H-N**). In summary, these findings suggest that increased *Ttr* contributes to behavioral and spine-morphological changes associated with chronic stress, while decreased *Ttr* can, at least in part, mitigate the effects of stress.

Altered DNAm on the *Ttr* promoter mediates gene expression changes and behavior.

Despite its clinical relevance, *Ttr* gene regulation remains poorly understood. We investigated a 438 bp region proximal to the transcription start site (**Fig. 4A**), focusing on DNA methylation, which is directly regulated by the one-carbon metabolism or indirectly affected via histone methylation.

We utilized pyrosequencing with primers spanning three adjacent regions to analyze *Ttr* DNAm after CVS and vitamin B12 treatment (**Fig. 4B-D**). While the baseline DNAm was high in this region, we detected a vitamin B12-induced reduction in DNAm within the stressed group. To evaluate the functional significance of these observed DNAm changes, we employed epigenome editing with a dCas9-DNMT3ACD-DNMT3LCD-3xFLAG fusion construct (“dCas9-Dnmt3”) along with guide-RNAs for *Ttr* (14; 22). Twelve gRNAs spanning the three regions were tested in Neuro2A-cells for their impact on *Ttr* gene expression and DNAm (data not shown). A scrambled, non-binding guide-RNA (“gCtrl”) was used as a control. The guide-RNA that most reliably induced DNAm changes while also affecting *Ttr* expression (“gTtr”) was chosen for further analysis (**Fig. 4A, Fig. 4E-I**). gTtr increased DNAm in multiple assessed CG sites (**Fig. 4F, G**), while reducing DNAm in its binding region (**Fig. 4H**), underscoring the importance of including controls for sterical hindrance during binding. Accordingly, while the combination of gTtr with dCas9-Dnmt3 elevated *Ttr* levels, this effect was not observed with a construct expressing an inactive enzymatic group (“dCas9-dDnmt3”) (**Fig. 4I**). These findings suggest that DNAm near the *Ttr* promoter can impact *Ttr* expression *in vitro*, consistent with vitamin B12-mediated effects in stressed mice, although additional factors likely contribute to mediate stress-induced *Ttr* changes in mice that did not receive vitamin B12 (**Fig. 1,2**).

To evaluate whether DNAm plays a functional role in stress-associated behavioral changes, we combined epigenome editing with *in vivo* jetPEI transfection using constructs labeled with fluorophores for confocal tracking (“gTtr-mCherry” and “dCas9-Dnmt3-GFP”, **Fig. 4J-M**). While we obtained a fluorescent signal in the PFC, only a limited number of cells near the injection site were transfected (**Fig. 4K, Fig. S5**, compared with viral approaches in **Fig. 2C, Fig. 3C**). Consequently, we observed a modest yet significant effect of CRISPR treatment on DNAm across CG sites that were not fully methylated within controls (**Fig. 4L**). The effect was absent

with the mute enzymatic construct. Moreover, we noted a significant impact on the immobility time specifically in the forced swim test (**Fig. 4M, Fig. S5**), indicating that increased DNAm on the *Ttr* promoter causally contributes to mood- and stress-associated behavior.

Discussion

In this study, we unveil a tissue- and sex-specific pathway that mediates the acute effects of vitamin B12 supplementation (**Fig. S6**). By employing the chronic variable stress (CVS) mouse model, which exhibits the highest transcriptional-level compliance with depressed patients, we identified *Ttr* as a target affected by vitamin B12. We observed altered *TTR* in the postmortem PFC of male depressed patients. *In vivo* epigenome editing of DNAm on *Ttr* affected immobility time in the forced swim test, demonstrating for the first time that dCas9-editing this chromatin mark can alter behavior in living mice. Moreover, given the pivotal role of *TTR* in other human illnesses such as hereditary amyloidosis, and related CRISPR efforts in humans, a less permanent modification of this gene, as well as increased knowledge on the epigenetic regulation of *Ttr*, will be relevant in a wider context.

We observed an effect of vitamin B12 on *Ttr* levels in stressed mice after one acute i.p.-injection within 24 h. It currently remains unclear whether vitamin B12 supplementation exerts rapid effects on stress response in humans as well and we encourage further investigation in this regard. Lifestyle interventions, particularly those involving dietary factors, are likely to play a more substantial role in personalized medicine. Micronutrients such as vitamins are well-characterized, and well-tolerated molecules with clear biological functions, making them easy to study and administer. In parallel with pre-clinical research, assessment of dietary states and deficiencies in patients may facilitate the treatment of stress-related disorders and guide future clinical studies on the effects of vitamin B12. Given the current research efforts to find novel and rapid-acting antidepressant compounds, vitamin B12-regulated pathways should be investigated further – not only on an RNA level but also in the context of proteomics and metabolomics.

The observation that vitamin B12 exerts effects in female mice without altering *Ttr* suggests the involvement of additional mechanisms. In previous studies, we identified other vitamin B12-mediated changes associated with stress, including alterations in *Ntrk-2* and GMPPB signaling (15). These changes were not detected by RNA-sequencing performed here, suggesting a regulation on protein levels (e.g. GMPPB methylation). Alternatively, this may be attributed to modified protocols (such as the combination of CMS and acute stress for *Ntrk-2*, which was rescued by vitamin B12) (14). Our cumulative findings point towards a multi-level effect of vitamin B12 and emphasize the need for further research, especially in female cohorts.

We did not see a stress effect on *Ttr* expression in females, possibly due to interference of other sex-specific, stress-related pathways or sex hormones (17; 30). Therefore, we did not investigate the outcome of modified *Ttr* levels in females any further.

While vitamin B12 supplementation is typically associated with an increase in methyl donors, our observation of reduced DNAm within the stress group suggests an indirect effect, possibly through histone methylation. Through using the innovative combination of epigenome editing and jetPEI *in vivo* transfection, we achieved *in vivo* DNAm editing for the first time, enabling direct causal examination of behavioral outcomes. We observed significant alterations in DNAm and behavior.

Through RNA-sequencing following viral overexpression of *Ttr*, we identified a diverse array of transcripts downstream of *Ttr*. Several of those transcriptional effects concern pathways that are near the cell membrane, within the extracellular matrix, or regulate the immune response as expected for an extracellular protein. However, the mechanisms through which it regulates the expression of those genes remain enigmatic. It is conceivable that the extracellular Ttr protein binds to receptors that initiate transcription cascades, or regulates neurotransmitter levels. For instance, Ttr alters Igf-signaling, which may be relevant to depression (32; 33), affects SUMOylation (34), and regulates the cleavage of neuropeptide Y (35). Ttr is also associated with oxidative stress and can mediate cytotoxicity through Ca^{2+} efflux from the endoplasmic reticulum (31). Thereby, enhanced Ttr levels could affect neurons within the PFC on multiple levels that may in turn alter gene expression as observed by RNA-sequencing after viral OE of *Ttr*. Alternatively, *Ttr* RNA may interact with intracellular targets. In this respect, many future studies are conceivable, e.g. investigating *Ttr* action on chromatin regulation (transcription factor activity of *Ttr*, regulation of chromatin modifiers by Ttr or *Ttr*, *Ttr* affecting the stability of other RNAs). Different genes were regulated by vitamin B12 and OE-*Ttr*. This may be in part due to the different time courses of acute vitamin B12 injection, daily limited stress induction, and long-term expression of OE-*Ttr* AAVs, as well as PFC-specific versus systemic interventions. In the future, further tests should be conducted regarding whether OE-*Ttr* can block the acute effects of vitamin B12 and whether *Ttr*-regulated genes may also be linked to chronic stress. The utilized AAVs were neuron-specific. In the future, cell-type specific investigations of *Ttr* should be performed, e.g. using FACS-sorting of reporter mouse lines or RNA-scope. This would also address whether Ttr is altered by CVS in glial cells as well, which were not targeted by the AAVs used in this study. The mechanisms, including immune response, signal transduction, and structural gene products, as well as their impact on depressive-like behavior and neural morphology, should be investigated further, especially given the pivotal function of Ttr, not just in stress but also in other conditions.

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Contributions

G.S., J.S.A., L.L., I.H. and O.E. performed mouse experiments and analysis. G.S., L.L. and O.E. planned experiments and handled paperwork involving animal experiments. G.S. double-checked data and statistics. S.C. and O.E. conducted cell culture experiments. J.S.A., O.E. and A.M. analyzed dendritic spines. E.A.H. provided input on *in vivo* transfection and epigenome editing. K.R. and S.H. analyzed the RNA-sequencing data. The team of T.E. provided help with pyrosequencing. G.T. provided human postmortem samples. C.A.H. provided infrastructure and conceptual advice and edited the manuscript. O.E. designed and planned the study, obtained funding, and wrote the article.

Disclosures.

The authors report no biomedical financial interests or potential conflicts of interest.

Literature

1. Bebbington P (2001): The World Health Report 2001 - Mental Health: New Understanding, New Hope. *Soc Psychiatry Psychiatr Epidemiol.* 36: 473–4.
2. Vos T, Abajobir AA, Abbafati C, Abbas KM, Abate KH, Abd-Allah F, *et al.* (2017): Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: A systematic analysis for the Global Burden of Disease Study 2016. *Lancet.* 390: 1211–59.
3. Aly J, Engmann O (2020): The Way to a Human's Brain Goes Through Their Stomach: Dietary Factors in Major Depressive Disorder. *Front Neurosci.* 14: 582853.
4. Wolffenbuttel B, Wouters H, Heiner-Fokkema M, van der Klauw M (2019): The Many Faces of Cobalamin (Vitamin B12) Deficiency. *Mayo Clin Proc Inn Qual Out.* 3: 200–214.
5. Penninx BW, Guralnik JM, Ferrucci L, Fried LP, Allen RH, Stabler SP (2000): Vitamin B(12) deficiency and depression in physically disabled older women: epidemiologic evidence from the Women's Health and Aging Study. *Am J Psychiatry.* 157: 715–721.
6. Tiemeier H, Ruud van Tuijl H, Hofman A, Meijer J, Kiliaan AJ, Breteler MMB (2002): Vitamin B12, folate, and homocysteine in depression: The Rotterdam study. *Am J Psychiatry.* 159: 2099–2101.
7. Moorthy D, Peter I, Scott TM, Parnell LD, Lai C-Q, Crott JW, *et al.* (2012): Status of Vitamins B-12 and B-6 but Not of Folate, Homocysteine, and the Methylenetetrahydrofolate Reductase C677T Polymorphism Are Associated with Impaired Cognition and Depression in Adults. *J Nutr.* 142: 1554–1560.
8. Kim J, Stewart R, Kim S, Yang S, Shin I, Yoon J (2008): Predictive value of folate, vitamin B12 and homocysteine levels in late-life depression. *Br J Psychiatry.* 192: 268–74.
9. Copen A, Bolander-Gouaille C (2005): Treatment of depression: Time to consider folic acid and vitamin B 12. *J Psychopharmacol.* . doi: 10.1177/0269881105048899.
10. Almeida OP, Marsh K, Alfonso H, Flicker L, Davis TME, Hankey GJ (2010): B-vitamins reduce the long-term risk of depression after stroke: The VITATOPS-DEP trial. *Ann Neurol.* 68: 503–510.
11. Syed E, Wasay M, Awan S (2013): Vitamin B12 supplementation in treating major depressive disorder: a randomized controlled trial. *Open Neurol J.* 7: 44–8.
12. Sangle P, Sandhu O, Aftab Z, Anthony A, Khan S (2020): Vitamin B12 Supplementation: Preventing Onset and Improving Prognosis of Depression. *Cureus.* 12: e11169.
13. Paternain L, Martisova E, Campi J, Martnez JA, Ramirez MJ, Milagro FI (2016): Methyl donor supplementation in rats reverses the deleterious effect of maternal separation on depression-like behaviour. *Behav Brain Res.* 299: 51–58.
14. Trautmann C, Bock A, Urbach A, Hübner A, Engmann O (2020): Acute vitamin B12 supplementation evokes antidepressant response and alters Ntrk-2. *Neuropharmacology.* 171: 108112.
15. Franzka P, Turecki G, Cubillos S, Kentache T, Steiner J, Walter M, *et al.* (2022): Altered mannose metabolism in chronic stress and depression is rapidly reversed by vitamin B12. *Front Nutr.* 9. doi: 10.3389/FNUT.2022.981511.
16. Wingo TS, Liu Y, Gerasimov ES, Gockley J, Logsdon BA, Duong DM, *et al.* (2021): Brain proteome-wide association study implicates novel proteins in depression pathogenesis. *Nat Neurosci.* 24: 810–7.
17. Labonte B, Engmann O, Purushothaman I, Hodes G, Lorsch ZS, Hamilton PJ (2017): Sex-Specific Transcriptional Signatures in Human Depression. *Nat Med.* 23: 1102–1111.
18. Lisowski P, Wiczorek M, Goscik J, Juszczak GR, Stankiewicz AM, Zwierzchowski L, Swiergiel AH (2013): Effects of chronic stress on prefrontal cortex transcriptome in mice displaying different genetic backgrounds. *J Mol Neurosci.* .
19. Ikubo K, Yamanishi K, Doe N, Hashimoto T, Sumida M, Watanabe Y, *et al.* (2017): Molecular analysis of the mouse brain exposed to chronic mild stress: The influence of hepatocyte

- nuclear factor 4a on physiological homeostasis. *Mol Med Rep.* 16: 301–9.
20. Alshehri B, D'Souza DG, Lee JY, Petratos S, Richardson SJ (2015): The diversity of mechanisms influenced by transthyretin in neurobiology: Development, disease and endocrine disruption. *J Neuroendocrinol.* .
 21. Gillmore JD, Gane E, Taubel J, Kao J, Fontana M, Maitland ML, *et al.* (2021): CRISPR-Cas9 In Vivo Gene Editing for Transthyretin Amyloidosis. *N Engl J Med.* 365: 493–502.
 22. Engmann O, Labonte B, Mitchell A, Bashtrykov P, Calipari E, Rosenbluh C, Loh Y (2017): Cocaine-induced chromatin modifications associate with increased expression and 3D looping of *Auts2*. *Biol Psychiatry.* 82: 794–805.
 23. Xu SJ, Lombroso SI, Fischer DK, Carpenter MD, Marchione DM, Hamilton PJ, *et al.* (2021): Chromatin-mediated alternative splicing regulates cocaine-reward behavior. *Neuron.* 109: 2943–66.
 24. Scott MM, Marcus JN, Pettersen A, Birnbaum SG, Mochizuki T, Scammell TE, *et al.* (2011): *Hcrtr1* and 2 signaling differentially regulates depression-like behaviors. *Behav Brain Res.* 222: 289–94.
 25. Engmann O, Giralt A, Gervasi N, Marion-Poll L, Gasmi L, Filhol O, *et al.* (2015): DARPP-32 interaction with adducin may mediate rapid environmental effects on striatal neurons. *Nat Commun.* 6: 10099.
 26. Engmann O, Hortobagyi T, Thompson A, Guadagno J, Troakes C, Soriano S, *et al.* (2011): Cyclin-dependent kinase 5 activator p25 is generated during memory formation and is reduced at an early stage in Alzheimer's disease. *Biol Psychiatry.* 70: 159–168.
 27. Engmann O, Hortobagyi T, Pidsley R, Troakes C, Bernstein H, Kreutz M, *et al.* (2011): Schizophrenia is associated with dysregulation of a Cdk5 activator that regulates synaptic protein expression and cognition. *Brain.* 134: 2408–2421.
 28. Rios X, Gomez-Vallejo V, Martin A, Cossio U, Mocillo M, Alemi M, *et al.* (2019): Radiochemical examination of transthyretin (TTR) brain penetration assisted by iododiflunisal, a TTR tetramer stabilizer and a new candidate drug for AD. *Sci Rep.* 9: 13672.
 29. Engmann O, Mansuy IM (2019): Stress and its effects across generations. *Stress Resil Mol Behav Asp.* .
 30. Tang Y, Haslam S, Conrad S, Sisk C (2004): Estrogen increases brain expression of the mRNA encoding transthyretin, an amyloid beta scavenger protein. *J Alzheimers Dis.* 6: 413–20.
 31. Teixeira P, Cerca F, Santos S, Saraiva M (2006): Endoplasmic reticulum stress associated with extracellular aggregates. Evidence from transthyretin deposition in familial amyloid polyneuropathy. *J Biol Chem.* 281: 21998–22003.
 32. Vieira M, Gomes J, Saraiva M (2015): Transthyretin Induces Insulin-like Growth Factor I Nuclear Translocation Regulating Its Levels in the Hippocampus. *Mol Neurobiol.* 51: 1468–79.
 33. Kopczak A, Stalla G, Uhr M, Lucae S, Hennings J, Holsboer F, Kloiber S (2013): IGF-1 is elevated in patients with depression and non-remitters to antidepressant treatment. *Exp Clin Endocrinol Diabetes.* 121: 11.
 34. Wieczorek E, Kedracka-Krok S, Soltys K, Jankowska U, Holubowicz R, Seliga J, Ozyhar A (2016): Is Transthyretin a Regulator of Ubc9 SUMOylation? *PLoS One.* 11: e0160536.
 35. Nunes AF, Saraiva MJ, Sousa MM (2006): Transthyretin knockouts are a new mouse model for increased neuropeptide Y. *FASEB J.* 20: 166–8.
 36. Bolger A, Lohse M, Usadel B (2014): Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics.* 30: 2114–20.
 37. Martin M (2011): Cutadapt Removes Adapter Sequences From High-Throughput Sequencing Reads. *EMBnet.journal.* 17.
 38. Song L, Florea L (2015): Rcorrector: efficient and accurate error correction for Illumina RNA-

- seq reads. *Gigascience*. 4.
39. Kopylova E, Noé L, Touzet H (2012): SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics*. 28: 3211–7.
 40. Hoffmann S, Otto C, Doose G, Tanzer A, Langenberger D, Christ S, *et al.* (2014): A multi-split mapping algorithm for circular RNA, splicing, trans-splicing and fusion detection. *Genome Biol*. 15: R34.
 41. Hoffmann S, Otto C, Kurtz S, Sharma C, Khaitovich P, Vogel J, *et al.* (2009): Fast mapping of short sequences with mismatches, insertions and deletions using index structures. *Plos Comput Biol*. 5: e1000502.
 42. Smith T, Heger A, Sudbery I (2017): UMI-tools: modeling sequencing errors in Unique Molecular Identifiers to improve quantification accuracy. *Genome Res*. 27: 491–9.
 43. Liao Y, Smyth G, Shi W (2014): featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. 30: 923–30.
 44. Wand L, Wang S, Li W (2012): RSeQC: quality control of RNA-seq experiments. *Bioinformatics*. 28: 2184–5.
 45. Love M, Huber W, Anders S (2014): Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 15: 550.
 46. Yu G, Wang L, Han Y, He Q (2012): clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS*. 16: 284–7.
 47. Korotkevich G, Sukhov V, Budin N, Shpak B, Artyomov M, Sergushichev A (2021): Fast gene set enrichment analysis. *BioRxiv*. .
 48. Sorge RE, Martin LJ, Isbester KA, Sotocinal SG, Rosen S, Tuttle AH, *et al.* (2014): Olfactory exposure to males, including men, causes stress and related analgesia in rodents. *Nat Methods*. 11: 629–32.

Figures and figure legends

Fig. 1: CVS and depression affect transthyretin in a sex- and tissue-specific manner and vitamin B12 reverses stress effects in mice. **A)** Experimental plan. **B)** Venn diagram of RNA-sequencing (males). $n = 4$ per group. **C-F)** Volcano plots depicting differentially expressed genes. Note that *Ttr* is among the highest stress- and vitamin B12-induced changes. **G)** Gene expression clusters that were differentially expressed by VB12 within the CVS group (string-db network). **H-N)** qPCR on PFC. **H, I)** Validation of RNA-sequencing data in an independent cohort of mice confirms reversal of stress-induced *Ttr*-changes by vitamin B12 (VB12) in males. **H)** Males: $n = 17-18$; stress effect: $F(1,66) = 6.88$, $P < 0.05$; drug effect: $F(1,66) = 10.62$, $P < 0.01$; *post hoc* test: stress within sal: $*P < 0.05$; drug within VB12: $**P < 0.01$. **I)** Females: $n = 10-13$; stress effect: $F(1,41) = 1.35$, $P = 0.25$; drug effect: $F(1,14) = 1.25$, $P = 0.29$. **J, K)** *TTR* is increased in postmortem samples of male but not female depressed patients (Depr.) vs. controls (Nil). **J)** Males: $n = 15-16$; Welch's t-test: $t_{18,74} = 2.47$, $*P < 0.05$. **K)** Females: $n = 14-16$; $t_{28} = 0.36$, $P = 0.72$. **L-N)** qPCR on other mouse tissues reveals no effect of CVS or VB12. **L)** Liver: $n = 8-9$; stress effect: $F(1,29) = 0.32$, $P = 0.58$; drug effect: $F(1,29) = 0.23$, $P = 0.64$; interaction: $F(1,29) < 0.001$, $P = 0.98$. **M)** Plasma: $n = 7-8$; stress effect: $F(1,26) = 0.53$, $P = 0.47$; drug effect: $F(1,26) = 2.81$, $P = 0.11$; interaction: $F(1,26) = 0.26$, $P = 0.61$. **N)** Testes: $n = 7-8$; stress effect: $F(1,26) = 2.73$, $P = 0.11$; drug effect: $F(1,26) = 0.02$, $P = 0.97$; interaction: $F(1,26) = 2.38$, $P = 0.14$. **C-F)** Individual data points are plotted, **H-N)** with additional means \pm s.e.m. are shown. Non-significant comparisons not unless specified. Sketches were generated with biorender.com.

Fig. 2: Overexpression of *Ttr* mimics effects of chronic stress and alters gene products for synaptic outgrowth. **A)** Schematic of the OE-*Ttr*-GFP AAV. **B)** Experimental plan. **C)** *Ttr* levels are increased in the dorsomedial PFC by the OE-AAV. $n = 4$; $t_6 = 5.02$, $**P < 0.01$. **D-H)** Overexpression (OE) of *Ttr* evokes depressive-like behaviors. **D)** Forced swim test: $n = 9-10$; $t_{17} = 0.93$, $P = 0.37$. **E)** Tail suspension test: $n = 10$; $t_{18} = 3.44$, $**P < 0.01$. **F)** Splash test: $n = 10$; Welch's t-test: $t_{11,31} = 4.14$, $**P < 0.01$. **G)** Novelty-suppressed feeding (NSF): $n = 10$; $t_{18} = 2.26$, $*P < 0.05$. **H-K)** OE-*Ttr* mimics chronic stress effects on dendritic spines. **H)** Overview of OE-AAV infection in the PFC and representative images of dendrites (right hemisphere). AAV with co-expressing GFP fluorophore is depicted green in the overview image and white in the dendritic images. Scale bar for overview: $400 \mu\text{m}$. Scale bar for dendrites: $10 \mu\text{m}$. **I)** Stubby spines: $n = 43-46$ dendrites from 5-6 mice; $t_{87} = 2.01$; $*P < 0.05$. **J)** Neck-containing spines: $n = 43-47$ dendrites from 5-6 mice; $t_{88} = 5.94$; $***P < 0.0001$. **K)** Cumulative head diameter: $\chi^2 = 7.37$, $df = 1$, $**P < 0.01$. To increase resolution, the graph was cropped at the x-axis (from $4 \mu\text{m}$ to $3 \mu\text{m}$ head diameter, therefore not showing 3 of 6.326 spines). All spines were kept in the analysis so that the statistics were not affected. **L-O)** RNA-sequencing in the PFC after viral OE of *Ttr*. $n = 4$ per group. **L)** Heatmap of differentially expressed genes. **M)** Pie chart of assigned pathways. **N)** String-db analysis of shared pathways (RNAs with at least 1 connection shown). The cluster in the upper part is linked to cytoskeleton and synaptic growth. **C-G, I, K)** Individual data points are plotted and means \pm s.e.m. are shown. Sketches were generated with biorender.com.

Fig. 3: Knockdown of *Ttr* blocks the effects of chronic stress. **A)** Schematic of the KD-*Ttr*-GFP AAV. **B)** Experimental plan. **C)** Overview of KD-AAV infection in the PFC. Scale bar 400 μm . **D)** qPCR: $n = 6$; $t_{10} = 2.52$, $*P < 0.05$. **E-H)** Behavioral tests show a reversal of stress-induced behavioral changes by KD of *Ttr*. **E)** Forced swim test: $n = 9$; stress effect: $F(1,32) = 14.60$, $P < 0.001$; *post hoc* test: stress effect within GFP: $**P < 0.01$; AAV within CVS: $*P < 0.05$. **F)** Novelty suppressed feeding: $n = 9-10$; stress effect: $F(1,33) = 4.38$, $P < 0.05$; AAV effect: $F(1,33) = 6.36$, $P < 0.05$; interaction: $F(1,33) = 6.43$, $P < 0.05$; *post hoc* test: stress within GFP: $**P < 0.01$; AAV within CVS: $**P < 0.01$. **G)** Splash test: $n = 8-10$; stress effect: $F(1,33) = 39.55$, $P < 0.0001$; AAV effect: $F(1,33) = 21.44$, $P < 0.0001$; *post hoc* test: stress within GFP: $***P < 0.001$, within KD: $**P < 0.01$; AAV effect within CVS: $***P < 0.001$. **H-N)** CVS-induced dendritic spine changes are reversed by KD of *Ttr*. **H)** Representative images of dendrites. Scale bar 10 μm . **I)** Stubby spines: $n = 29-41$ from 3-4 mice; AAV effect: $F(1,138) = 4.84$, $P < 0.05$; interaction: $F(1,138) = 18.64$, $P < 0.0001$; *post hoc* test: stress within GFP: $***P < 0.001$, AAV within naïve: $***P < 0.001$. **J)** Neck-containing spines: $n = 30-40$ from 3-4 mice; stress effect: $F(1,139) = 14.28$, $P < 0.001$; AAV effect: $F(1,139) = 7.86$, $P < 0.01$; interaction: $F(1,139) = 92.21$, $P < 0.0001$; *post hoc* test: stress within GFP: $***P < 0.001$, within KD: $***P < 0.001$; AAV within naïve: $***P < 0.001$, within CVS: $***P < 0.001$. **K-N)** Cumulative head diameter. To increase the resolution, graphs showing the cumulative head diameter were cropped at the x-axis (from 4 μm to 3 μm head diameter, affecting in total 8 of 11.558 spines. All spines were kept in the analysis so that the statistics were not affected. **K)** CVS within GFP: $\chi^2 = 8.30$, $df = 1$, $**P < 0.01$. **L)** CVS within KD: $\chi^2 = 2.96$, $df = 1$, $P = 0.09$. **M)** AAV within naïve: $\chi^2 = 0.02$, $df = 1$, $P = 0.90$. **N)** AAV within CVS: $\chi^2 = 1.56$, $df = 1$, $P = 0.21$. **D-G, I, J)** Individual data points are plotted and means \pm s.e.m. are shown. Non-significant comparison not listed. Sketches were generated with biorender.com.

Fig. 4: DNAm near the *Ttr* promoter is functionally linked to stress-associated behavior.

A) Overview of the investigated region. Covered CGs are labeled in pink and refer to listed CGs per region. Regions 1-3 were covered separately by pyrosequencing. **B-D)** Pyrosequencing on PFC from mice that had undergone CVS (vs. naïve) and acute vitamin B12 treatment ("VB12", vs. saline). $n = 8-10$. **B)** Region 1. CG4: interaction CVS & vit. B12: $F(1,32) = 4.17$, $P < 0.05$; drug within CVS: $*P < 0.05$; stress within VB12: $*P < 0.05$. **C)** Region 2. CG1: stress effect: $F(1,35) = 7.16$, $P < 0.05$; drug effect: $F(1,35) = 5.05$, $P < 0.05$; interaction: $F(1,35) = 4.75$, $P < 0.05$; drug within CVS: $**P < 0.01$; stress within VB12: $**P < 0.01$; CG2: drug effect: $F(1,34) = 4.35$, $P < 0.05$; drug within CVS: $*P < 0.05$; CG3: stress effect: $F(1,36) = 7.37$, $P < 0.05$; drug effect: $F(1,36) = 6.08$, $P < 0.05$; drug within CVS: $*P < 0.05$; stress within VB12: $*P < 0.05$. **D)** Region 3. CG3: all comparisons n.s. **E-M)** Epigenome editing of DNAm on the *Ttr* promoter using a Dnmt3A/L-dCas9 construct (v.s. dDnmt3A/L-dCas9). **E)** Overview. **F-I)** Editing in Neuro2A-cells. **F-H)** Pyrosequencing. $n = 6$. **F)** Region 1. gRNA effect: $F(1,30) = 110.0$, $P < 0.001$; position effect: $F(2,30) = 168.8$, $P < 0.001$; interaction: $F(2,30) = 4.01$, $P < 0.05$; *post hoc* test: gRNA effect for all CGs: $***P < 0.001$. **G)** Region 2. gRNA effect: $F(1,30) = 15.89$, $P < 0.001$; position effect: $F(2,30) = 21.7$, $P < 0.0001$; interaction: $F(2,30) = 4.96$, $P < 0.05$; *post hoc* test: gRNA effect for CGs 2, 3: $**P < 0.01$. **H)** Region 3. Interaction gRNA & CG: $F(2,30) = 18.44$, $P < 0.001$; *post hoc* test: gRNA effect for CG1: $*P < 0.05$, for CG3: $***P < 0.001$. **I)** qPCR shows a selective increase in *Ttr* expression after editing. $n = 7-13$. results. Enzyme

effect: $F(1,37) = 4.33$, $P < 0.05$; interaction: $F(1,37) = 4.33$, $P < 0.05$. *Post hoc* test: Enzyme effect within gTtr: $*P < 0.05$; gRNA effect within Dnmt3-dCas9: $*P < 0.05$; **J-M**) *In vivo* epigenome editing using jetPEI-transfection. **J**) Overview. **K**) Confocal image of injection site (10x magnification). **L**) Editing alters DNAm across CGs that were not at 100% methylation in the gCtrl-group. $n = 8-10$; treatment effect: $F(2,123) = 6.64$, $P < 0.01$; position effect: $F(4,123) = 353.6$, $P < 0.0001$; *post hoc* test: CG2 on region 3: treatment: $*P < 0.05$. Scale bar 400 μm . **M**) Increased immobility time in the forced swim test after editing *Ttr*: $n = 9-10$. One-way ANOVA: $F = 5.75$, $P < 0.01$; Tukey' *post hoc* test: gCtrl guideRNA within Dnmt3-dCas9: $**P < 0.01$; Enzyme-group within *Ttr*: $*P < 0.05$. Purple: predicted binding site of gTtr. Individual data points are plotted and means \pm s.e.m. are shown. Non-significant comparison not listed. Sketches were generated with biorender.com.

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