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Full Length Article

Cerebellar mitochondrial dysfunction and concomitant multi-system fatty acid oxidation defects are sufficient to discriminate PTSD-like and resilient male mice

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ABSTRACT

The impact of trauma on mental health is complex with poorly understood underlying mechanisms. Mitochondrial dysfunction is increasingly implicated in psychopathologies and mood disorders, including post-traumatic stress disorder (PTSD). We hypothesized that defects in mitochondrial energy metabolism in the cerebellum, an emerging region of interest in the pathobiology of mood disorders, would be associated with PTSD-like symptomatology, and that PTSD-like symptomatology would correlate with the activities of the mitochondrial electron transport chain (mtETC) and fatty acid oxidation (FAO) pathways. We assayed mitochondrial energy metabolism and fatty acid profiling using targeted metabolomics in mice exposed to a recently developed paradigm for PTSD-induction. 48 wild type male FVB.129P2 mice were exposed to a trauma, and PTSD-like and resilient animals were identified using behavioral profiling. Mice displaying PTSD-like symptomatology displayed reduced mtETC complex activities in the cerebellum, and cerebellar mtETC complex activity negatively correlated with PTSD-like symptomatology. PTSD-like animals also displayed fatty acid profiles consistent with FAO dysfunction in both cerebellum and plasma. Machine learning analysis of all biochemical measures in this cohort of animals also identified plasma acetylcarnitine, along with reduced activity of cerebellar complex I and IV as well as succinate:cytochrome c oxidoreductase as state predictive discriminators of PTSD-symptomatology. Our data also suggest that trauma-induced impaired mtETC function in the cerebellum and concomitant impaired multi-system fatty acid oxidation are candidate drivers of PTSD-like behavior in mice. These bioenergetic and metabolic changes may offer an informative window into the underlying biology and highlight novel potential targets for diagnostics and therapeutic interventions in PTSD.

1. Introduction

Post-traumatic stress disorder (PTSD) is a debilitating psychiatric condition that precipitates following exposure to a traumatic event. Symptoms include recurrent and intrusive recollections, dreams or flashbacks; avoidance or isolation from thoughts, feelings and persons; memory problems; persistent negative emotions and distorted cognition; sleep disturbance; irritability; hypervigilance; exaggerated startle; reckless or destructive behavior, and difficulty concentrating (Flory and Yehuda, 2015). While the lifetime prevalence of traumatic event exposure is high (64–70%) (Benjet et al., 2016), the lifetime prevalence of PTSD is relatively low (1.3–12.2%) (Karam et al., 2014), suggesting the possibility of an underlying biological factor driving PTSD-vulnerability or -resilience.

Mitochondrial dysfunction has been increasingly implicated in several psychiatric disorders including anxiety, depression, schizophrenia, bipolar disorder, and PTSD (Shao et al., 2008; Hollis et al., 2015; Pei and Wallace, 2018; Morava and Kozicz, 2013). Mitochondria are

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integral to several pathophysiologic features of PTSD, including regulation of stress response and fear memory, neuroinflammation, synaptic plasticity, apoptosis, and hippocampus-pituitary-adrenal axis regulation (Preston et al., 2018; Picard et al., 2015). Recent studies have identified altered expression of genes involved in mitochondrial energy metabolism in the dorsolateral pre-frontal cortex of patients with PTSD (Su et al., 2008). Reduced mtDNA copy number has been identified in peripheral blood cells of combat veterans with PTSD (Bersani et al., 2016), and mutations in mitochondrial genes of the mtETC were recently identified as being associated with PTSD-vulnerability in humans (Flaquer et al., 2015). Exposure to stress, independent of vulnerability to PTSD-like symptomatology, has been shown to induce dysregulation of genes associated with mitochondrial energy metabolism in rats, including the fatty acid oxidation (FAO) pathway (Su et al., 2008), and altered brain mitochondrial energy metabolism in several brain areas in rats, including the hippocampus (Han et al., 2013). Similar effects were observed in stressed humans (Zhang et al., 2015). Stress exposure has been shown to increase mitochondrial membrane permeability, Complex IV release, mtETC dysfunction, and mitochondrial Ca2+ buffering demand, and subsequently disrupts adenosine triphosphate production (Han et al., 2013; Xing et al., 2013; Wan et al., 2016; Xiao et al., 2009). The mtETC chain and fatty acid oxidation pathways are closely linked (Wajner and Amaral, 2015)and while altered fatty acid profiles have been implicated in psychopathology (Berger et al., 2019)and PTSD (Mellon et al., 2019), the role of the fatty acid oxidation pathway itself in the pathophysiology of PTSD has not been investigated.

PTSD research has traditionally focused on brain areas directly involved in fear learning, memory, and executive function, such as the amygdala, hippocampus, and pre-frontal cortex (Shalev et al., 2017). While not part of the canonical stress-response or PTSD neural pathways, the cerebellum is an emerging region of interest in susceptibility to psychopathology, including altered cognition, mood disorders, and PTSD (Holmes et al., 2018; Rabellino et al., 2018; Baldaçara et al., 2012), and has recently been implicated in vulnerability to psychopathology generally (Hariri, 2019). The cerebellum's best known function is its coordination of motor function, though recent evidence suggests that the cerebellum may coordinate a wide range of cortical functions, including emotion, fear response, and cognition, making it an area of particular interest in PTSD.

We hypothesized that mice displaying PTSD-like behavior would also display a reduced mitochondrial electron transport chain (mtETC) complex activity and impaired mitochondrial fatty acid oxidation (FAO) in the cerebellum, and that PTSD-like symptomatology would directly correlate with the function of the mtETC and FAO pathways. Using a recently developed preclinical model of PTSD-induction capable of resolving PTSD-like and resilient animals in mice (Lebow et al., 2012), we assayed for evidence of reduced mtETC complex activities and FAO dysfunction in cerebellum. To discover potential peripheral biomarkers for cerebellar bioenergetics potentially discriminating PTSD-like and resilient mice, we also assayed fatty acid profiles in circulating plasma. We report a stress-associated reduction in cerebellar mtETC complex activities in animals displaying PTSD-like symptomatology, which correlated with PTSD-like symptom score. Using multi-platform targeted metabolomics, we also show that this mtETC dysfunction results in characteristic biosignatures consistent with impaired fatty acid oxidation in both cerebellum in plasma, discriminating PTSD-like and resilient mice.

2. Methods and materials

2.1. PTSD induction and behavioral assessment

48 male FVB.129P2 mice, 10 weeks of age, housed 4-to-a-cage under standard laboratory conditions, were exposed to the PTSD induction paradigm published by Lebow et al. (2012). See Supplementary Methods for a detailed protocol. Open field testing was performed prior to PTSD-induction. All experiments were conducted in accordance with the Tulane University Institutional Animal Care and Use Committee (IACUC) and reported in accordance with the ARRIVE reporting guidelines for animal studies.

2.2. Sacrifice and necropsy

All animals were sacrificed via live decapitation without the use of anesthetic. Tissue samples were collected and flash frozen in liquid nitrogen. See Supplementary Methods for additional information.

2.3. Blinding and randomization

All samples were blinded and randomized for post-hoc analysis. Researchers were not aware of the group allocations during analysis.

2.4. Excluded animals

One animal identified as PTSD-like was excluded from the analysis as it was a significant outlier in both PTSD symptom score as well as post-hoc analysis.

2.5. Corticosterone ELISA

ELISA for plasma corticosterone was performed using the Mouse Corticosterone (ab108821) and ELISA Kit from Abcam.

2.6. Mitochondrial electron transport chain complex activity measurements

Activities of Complex I (CI), Complex II (CII), Complex III (CIII), Complex IV (CIV), succinate:cytochrome c oxidoreductase (SCC), and citrate synthase (CS) isolated from cerebellum and muscle mitochondria were analyzed with the Konelab 20XT Analyzer (Thermo Scientific) (Rodenburg, 2011). Protein concentration was measured by BCA-based assay and complex activities were normalized to both citrate synthase and protein concentration.

2.7. DNA isolation and mitochondrial DNA copy number RT-qPCR

Genomic DNA was isolated from cerebellum and blood cell fraction using the QIAamp DNA Mini Kit (Qiagen). RT-qPCR was performed with PowerUp SYBR Green Master Mix (Thermo Fisher) (Table S1). The MT-ND1 locus was amplified and normalized to the ACTB locus using the 2(-Delta DeltaC(T)) method (Schmittgen and Livak, 2008).

2.8. Mitochondrial genome long-range PCR and next-generation sequencing

Genomic DNA isolated from cerebellum was amplified using the SequalPrep Long Range PCR Kit (Thermo Fisher) according to manufacturer instructions (For primers see Table S1). Next-generation sequencing of the mtDNA amplicon was performed by the Genome Analysis Core at Mayo Clinic in Rochester MN on the Illumina MiSeq platform.

2.9. Metabolomics

Concentrations of free (C0), acetyl- (C2), propionyl- (C3), butyryl- (C4), isovaleryl- (C5), octanoyl- (C8), lauroyl- (C12), myristoyl- (C14), palmitoyl- (C16), oleoyl- (C18:1), and stearoyl- (C18) carnitine were measured in cerebellum and plasma using liquid chromatography/mass spectrometry by the Metabolomics Core at Mayo Clinic in Rochester MN.

2.10. Statistical analyses

All statistical analyses were performed using GraphPad Prism or

SPSS. Shapiro-Wilk test was used to determine normality of each data set. Unpaired Student's T-test was used to compare two groups with normal distribution. One-way ANOVA was used to compare three or more groups with normal distribution. In the event that one or more groups were non-Gaussian, non-parametric tests were used (Mann Whitney for two groups; Kruskal Wallis for three or more groups). Bartlett's test for variance was run to determine if standard deviations varied significantly. If standard deviations varied significantly, a Browne-Forsythe or Welch's ANOVA was performed. For multiple t-tests, a 10% False Discovery Rate threshold was applied. Data are expressed as averages ± standard deviation. Linear discriminant analysis (LDA) was performed using SPSS. Least Absolute Shrinkage and Selection Operator (LASSO) analysis was performed by the Mayo Clinic Bioinformatics Core. Throughout the study, PTSD-like animals identified through the PTSD-induction paradigm are compared to resilient animals (trauma-exposed controls). Twelve stress-naïve animals are compared to a combined cohort of "stressed" animals comprising both PTSD-like and resilient animals To calculate the linear correlation between mtETC complex activity and PTSD symptom score, additional randomly selected stressed animals which were neither PTSD-like nor resilient were included in the analysis. For acylcarnitine analysis, seven randomly selected naïve animals were analyzed.

3. Results

3.1. Behavioral characterization of PTSD-Like animals

48 wildtype male FVB129.P2 mice were exposed to the PTSD-induction paradigm established by Lebow et al. (2012). Six animals (14.6%) were identified as PTSD-like and seven animals were identified as resilient. Because trait anxiety may modulate the effects of stress and PTSD (Jaksic et al., 2012; Kok et al., 2016), animals performed an open field test prior to PTSD-induction. No significant variation in motility or anxiety-like behavior was observed (Fig. 1A), and LDA was unable to discriminate between PTSD-like and resilient animals based on performance in the pre-trauma open field (Seibenhener and Wooten, 2015)).

As reported by Lebow et al., PTSD-like animals showed significantly more PTSD-like marble burying (%MB), pre-pulse inhibition (%PPI), latency to peak startle amplitude (LPSA), and light phase activity (%LPA) behavior compared to resilient animals (Fig. 1B). While no significant difference in risk assessment (%RA) behavior was observed, Z scores in % RA was significantly correlated with PTSD symptom score among all stressed animals. PTSD-like animals displayed a significantly more PTSD-like average Z value for performance in all PTSD-like behavioral tests (with positive Z values indicating more PTSD-like behavior, and negative

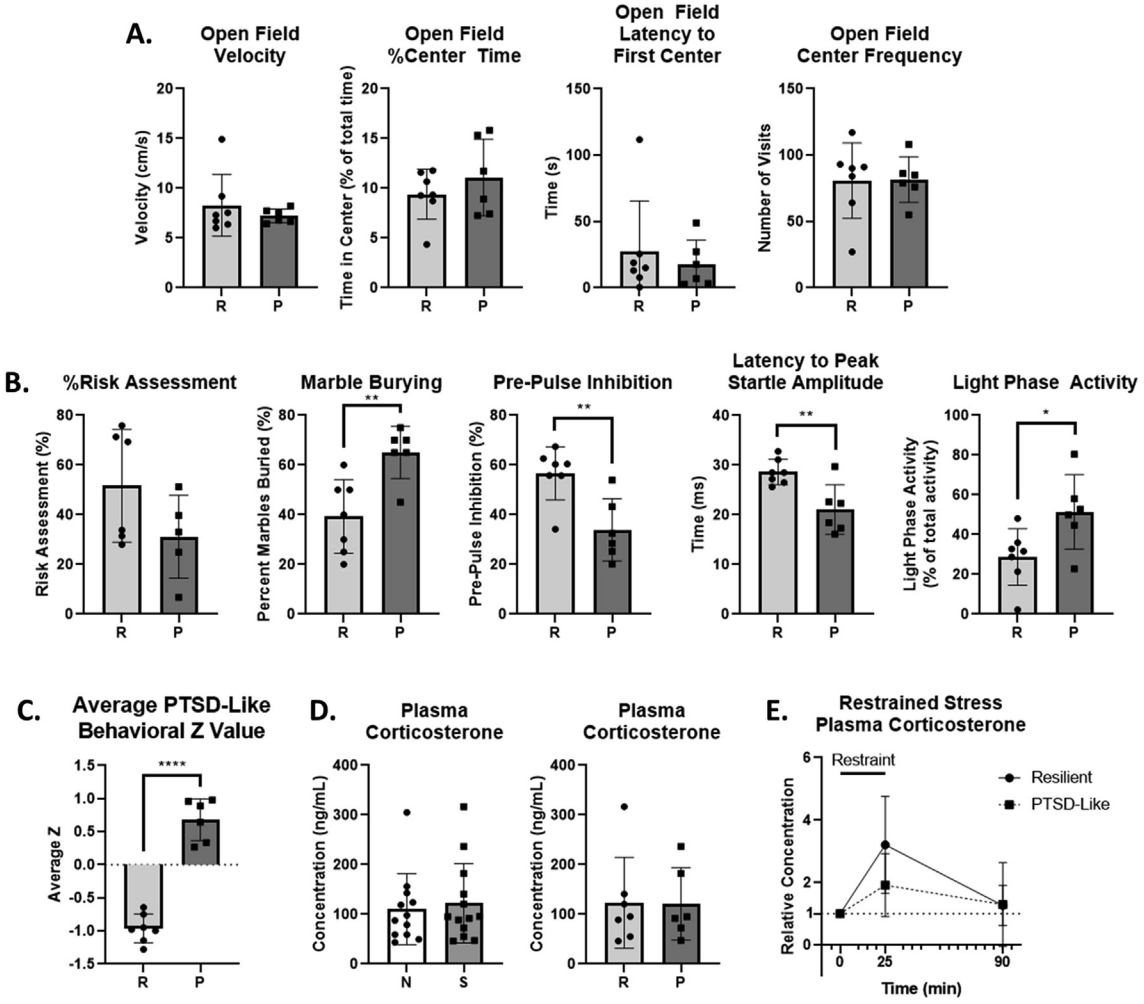

Fig. 1. Characterization of the PTSD-like phenotype. A. PTSD-like (P) animals (n = 6) showed no evidence of altered motility or anxiety like behaviors in a pre-stress open field relative to resilient (R) animals (n = 7). B. PTSD-like animals displayed significantly more PTSD-like behavior in marble burying (%MB), latency to peak startle amplitude (LPSA), pre-pulse inhibition (%PPI), and light phase activity (%LPA), as well as average behavioral Z. C,D. Both stress-naïve (n = 12) and stressed animals (n = 13), as well as PTSD-like (n = 6) and resilient (n = 7) animals showed normal circulating corticosterone at necropsy. E. No difference in restraint stress CORT response was observed between PTSD-like (n = 6) and resilient animals (n = 7). $p < 0.05$(*), $p < 0.01$(**), $p < 0.001$(***), $p < 0.0001$(****).

Z values indicating less PTSD-like behavior) (Fig. 1C) and average behavioral Z was highly correlated with PTSD symptom score suggesting significant synergy between the PTSD-like behaviors in PTSD-like animals. LDA identified LPSA and %PPI as the primary behavioral measures discriminating PTSD-like and resilient animals.

Cortisol and corticosterone are the primary stress hormones in humans and mice, respectively. Reduced circulating cortisol (hypocortisolemia) and cortisol-response dysregulation are commonly associated with PTSD in humans (Wichmann et al., 2017; Deussing and Chen, 2018). Stress--naïve, resilient, and PTSD-like animals all displayed normal plasma corticosterone concentrations at necropsy, and no significant variation between stress-naïve and stressed animals, nor between resilient and PTSD-like animals was observed in plasma corticosterone concentrations at necropsy (Fig. 1D). While plasma corticosterone concentrations varied significantly over the time course of the restraint stress and subsequent recovery ($p = 0.0013$), resilient and PTSD-like animals did not vary significantly in plasma corticosterone concentrations in response to restraint stress. (Fig. 1E).

3.2. Mitochondrial ETC dysfunction associated with PTSD-Vulnerability

Previous studies have identified altered expression of genes of the mtETC in the brains of animals exposed to PTSD-induction stress (Su et al., 2008; Zhang et al., 2015; Xing et al., 2013). As hypothesized, stressed animals displaying reduced CI, CII, CIII, SCC, and average complex activity in the cerebellum relative to stress-naïve animals (Fig. 2A). PTSD-like animals displayed reduced cerebellar CI, CII, CIV, and average complex activity relative to resilient animals (Fig. 2B).

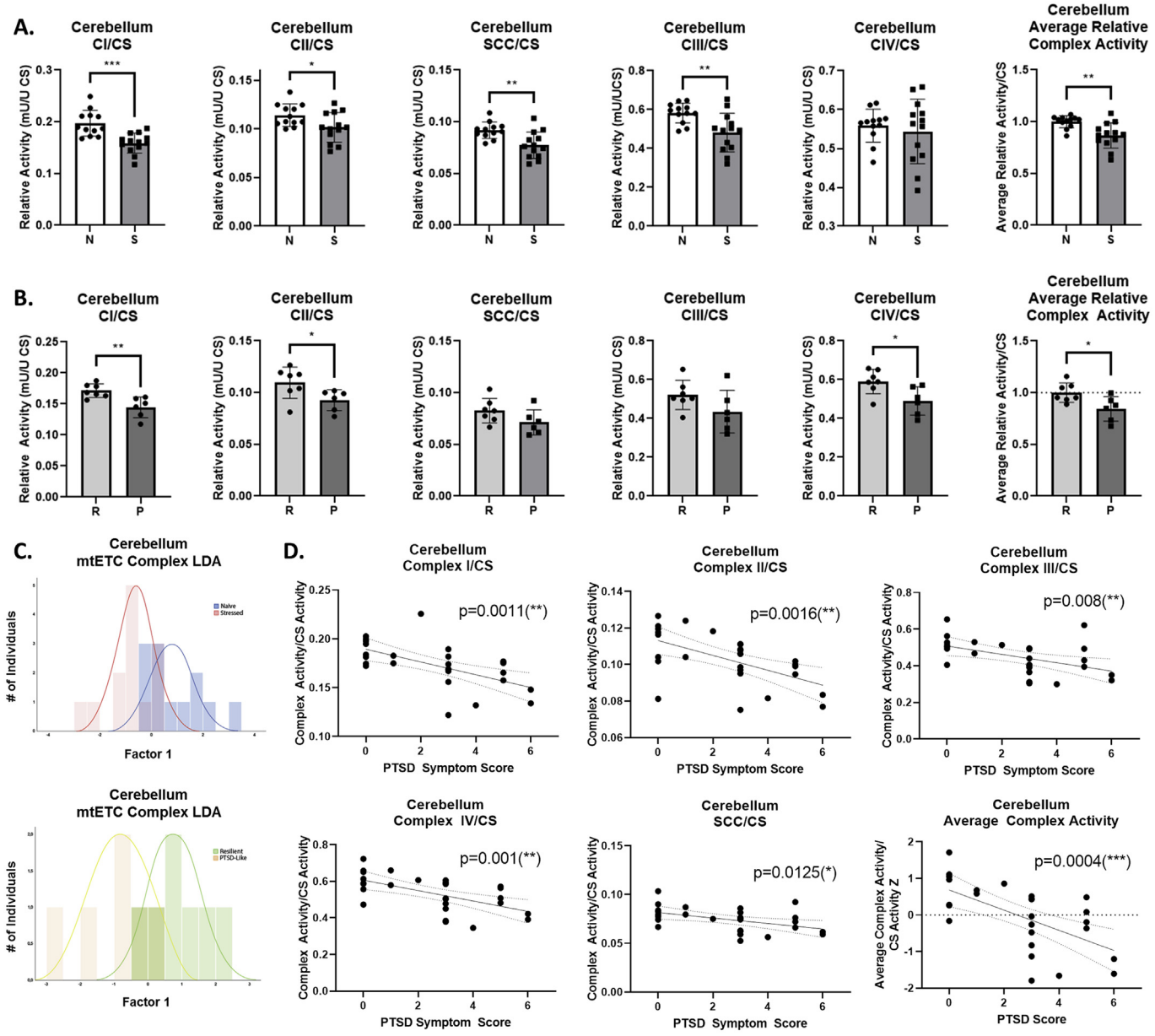

Fig. 2. PTSD-like behavior is directly correlated with stress-induced mitochondrial electron transport chain (mtETC) dysfunction in the cerebellum. A,B. Stressed (S) animals ($n = 13$) displayed reduced complex I (CI), complex II (CII), succinate:cytochrome c oxidoreductase (SCC), and complex III (CIII) (a) and reduced average relative complex activity relative to naive (N) animals ($n = 12$) (b). C,D. PTSD-like (P) ($n = 6$) animals displayed reduced CI, CII, and complex IV (CIV) activity (c) and reduced average complex activity (d) relative to resilient (R) animals ($n = 7$). E. Linear discriminant analysis (LDA) identified CI activity as the primary discriminator of both stress-naïve and stressed animals, as well as PTSD-like and resilient animals. F. CI, CII, CIII, CIV, and SCC activity, as well as average complex activity Z were negatively correlated with PTSD-symptom score in stressed animals ($n = 27$). $p < 0.05$(*), $p < 0.01$(**), $p < 0.001$(***), $p < 0.0001$(****).

mtETC complex activities were normalized to citrate synthase activity, an enzymatic readout for mitochondrial number (Lanza and Nair, 2009).

Among stressed animals, activity of cerebellar CI, CII, CIII, CIV, SCC, and average complex activity Z score, was each inversely correlated with PTSD symptom score (Fig. 2D). However, no cerebellar mtETC complex activity was significantly correlated with performance in any behavioral test nor with the average behavioral Z score, suggesting that reduced cerebellar mtETC complex activity does not affect performance in any PTSD-like behavior, but is associated with an underlying PTSD-like psychopathology.

Linear discriminate analysis (LDA) of cerebellar mtETC complex activities identified CI as the primary function resolving both stress-naïve and stressed animals, as well as resilient and PTSD-like animals (Fig. 2C).

Stressed animals also showed reduced CIII, CIV, SCC, and average complex activity in hindlimb muscle relative to stress-naïve animals (Fig. S1A). However, no difference between PTSD-like and resilient animals was observed in mtETC complex activity (Fig. S1B) and no muscle mtETC complex activity was correlated with PTSD symptom score (Fig. S1C). LDA identified SCC and CII as factors capable of discriminating stress-naïve and stressed animals, however PTSD-like and resilient animals could not be discriminated based on muscle mtETC complex activity. These data suggest that while exposure to stress may have effects on muscle mtETC complex activity, including in muscle, it is the reduction in mtETC complex in the brain specifically which influences vulnerability to PTSD-like symptomatology.

3.3. Mitochondrial DNA alterations associated with trauma exposure

Maintenance of mitochondrial DNA copy number (mtDNAcn) is a tightly regulated physiologic process, and reduced mtDNAcn is a commonly-used indicator of mitochondrial dysfunction and dysbiogenesis (Clay Montier et al., 2009). Reduced mtDNAcn has been reported in brains of patients with Parkinson's disease (Pyle et al., 2016) and in peripheral blood cells (PBC's) of patients with both Parkinson's disease

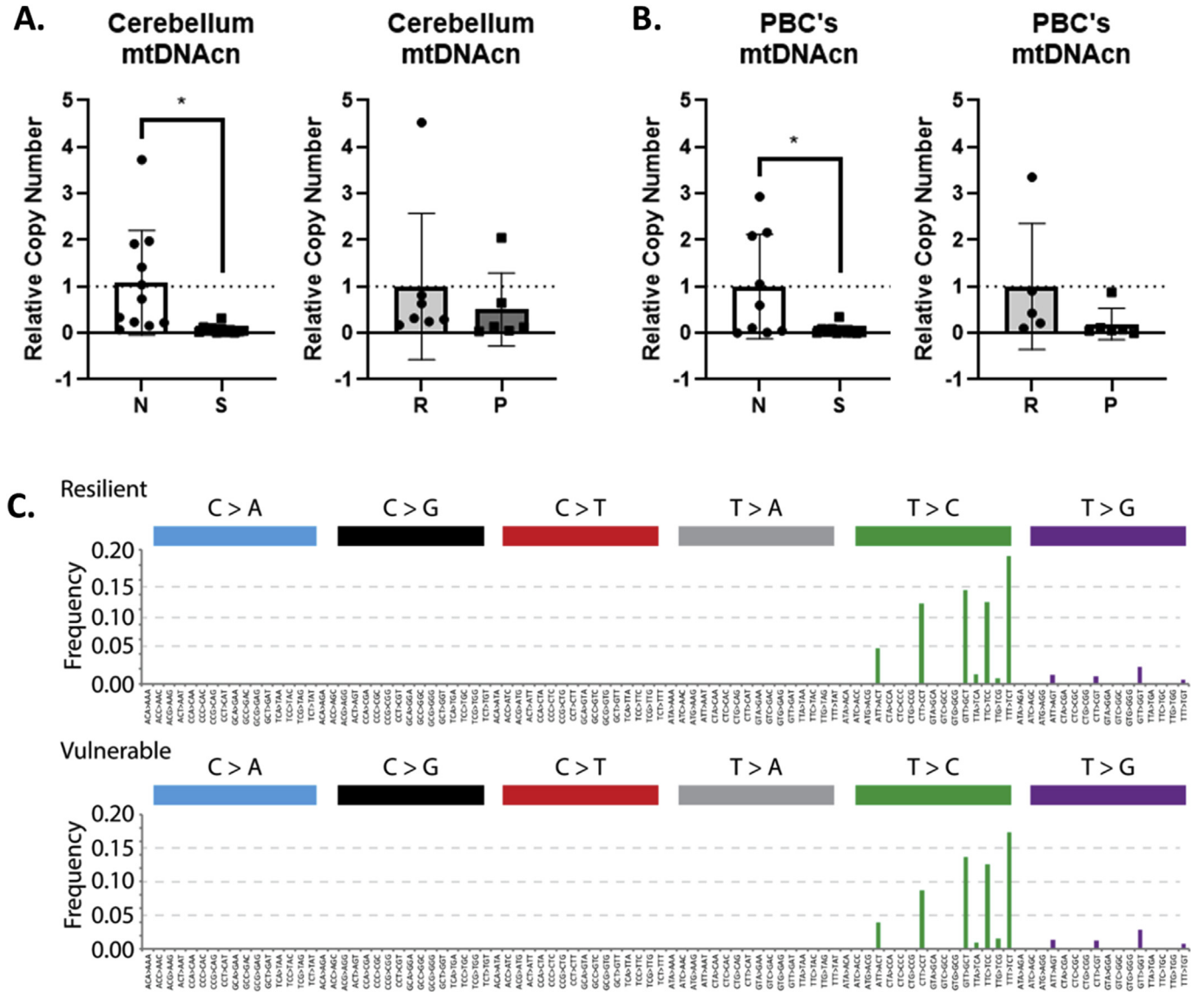

Fig. 3. Mitochondrial DNA (mtDNA) alterations associated with stress exposure and PTSD-like behavior and resilience. A,B. Stressed (S) animals (n = 13) displayed reduced mtDNA copy number (mtDNAcn) relative to naïve (N) animals (n = 12) in both cerebellum (a) and peripheral blood cells (PBC's) (b). PTSD-like (P) animals (n = 6) displayed no difference in mtDNAcn relative to resilient (R) animals (n = 7) in either cerebellum (a) or PBC's (b). C. mtDNA mutation profile of cerebellar mtDNA in PTSD-like (n = 6) and resilient (n = 7) animals displays increased numbers in T > C transition mutations. $p < 0.05$(*), $p < 0.01$(**), $p < 0.001$(***), $p < 0.0001$(****).

and PTSD (Bersani et al., 2016; Pyle et al., 2016). Stressed animals displayed reduced mtDNAcn in both cerebellum and PBC's (Fig. 3A and B). However, no difference between PTSD-like and resilient animals was observed in mtDNAcn in either cerebellum or PBC's.

mtDNA sequencing identified 1039 mtDNA variants among the PTSD-like and resilient animals, but identified no variant present at a heteroplasmy level sufficient to be of pathogenic significance (Schon et al., 2012), and no significant difference in the number of variants between PTSD-like and resilient animals. Assessment of the individual codon alterations shows similar mutation profiles between PTSD-like and resilient animals, with an apparent increased frequency of T > C inversions in both groups (Fig. 3C). T > C transitions can be induced by oxidative deamination of adenine by the reactive nitrogen species N_2O_3 (Ridnour et al., 2004), however, oxidative deamination of guanine, and subsequent G > T and A > C transversion is the most commonly used readout for mitochondrial oxidative damage (Cheng et al., 1992).

3.4. Disruptions in brain FAO associated with trauma exposure and PTSD-Vulnerability

Fatty acid oxidation (FAO) is an important mitochondrial energetic metabolic pathway, especially in response to stress, when increased energy is required, and FAO disruption has been implicated in PTSD pathophysiology (Su et al., 2008; Zhang et al., 2015). As we hypothesized, stress and PTSD-like behavior were associated with FAO disruption in both the cerebellum and plasma.

Stressed animals displayed reduced concentrations of C2, C3, C4, C5 and C8 (Fig. 4A) in the cerebellum. Stressed animals also displayed elevated ratios of the medium (MC) and long chain (LC) acylcarnitines C8, C12, C14, C16, C18:1 and C18, and reduced proportions of the even-chained short-chain (SC) acylcarnitines C2 and C4 as proportions of total acylated carnitines (AC) (Fig. 4B). No difference in the proportions of the odd chain acylcarnitines C3 and C5 were observed. This decreased proportion of even-chained SC:AC and increased proportion of MC:AC and LC:AC is consistent with impaired metabolism of long-chain fatty acids through the fatty acid oxidation pathway. LDA identified C4:AC as the primary function discriminating stress-naïve and stressed animals but was unable to discriminate PTSD-like and resilient animals based on acylcarnitines in the cerebellum (Fig. 4C).

Stressed animals also displayed altered plasma acylcarnitine profiles, including reduced concentrations C2, C3, C4, C5, C8, and C12 relative to naïve animals (Fig. 4E). Reduced plasma C2 and C4 are biomarkers of reduced fatty acid oxidation efficiency, and is consistent with incomplete FAO in the liver (Ribel-Madsen et al., 2016). Stressed animals also showed reduced plasma C0 and elevated AC:C0 relative to stress-naïve animals, suggesting an increased recruitment of acylcarnitines in these stressed animals. Stressed animals also showed increased ratios of C14, C16, C18:1 and C18 to AC relative to stress-naïve animals (Fig. 4F). Elevated circulating C18:1:AC was identified by LDA as the primary function discriminating stress-naïve and stressed animals and was sufficient to discriminate the two groups (Fig. 4G). PTSD-like animals displayed reduced plasma C2:AC. Reduced C2:AC was identified as the primary function discriminating PTSD-like and resilient animals and was sufficient to resolve the two groups (Fig. 4H). These data both corroborate the FAO defect in the brain, implicate multisystem FAO dysfunction in the pathophysiology of PTSD-like behavior, and provide potential biomarkers in blood to identify individuals both exposed to trauma and experiencing PTSD-like symptomatology.

3.5. Biological features most predictive of PTSD-Vulnerability

LASSO analysis of PTSD-like and resilient animals identified 5 variables as highly likely (lambda<0.55) to be predictive of PTSD-vulnerability in stressed animals: reduced activity of cerebellar CI, CIV and SCC relative to protein concentration, reduced average cerebellar mtETC complex activity relative to protein concentration, and reduced

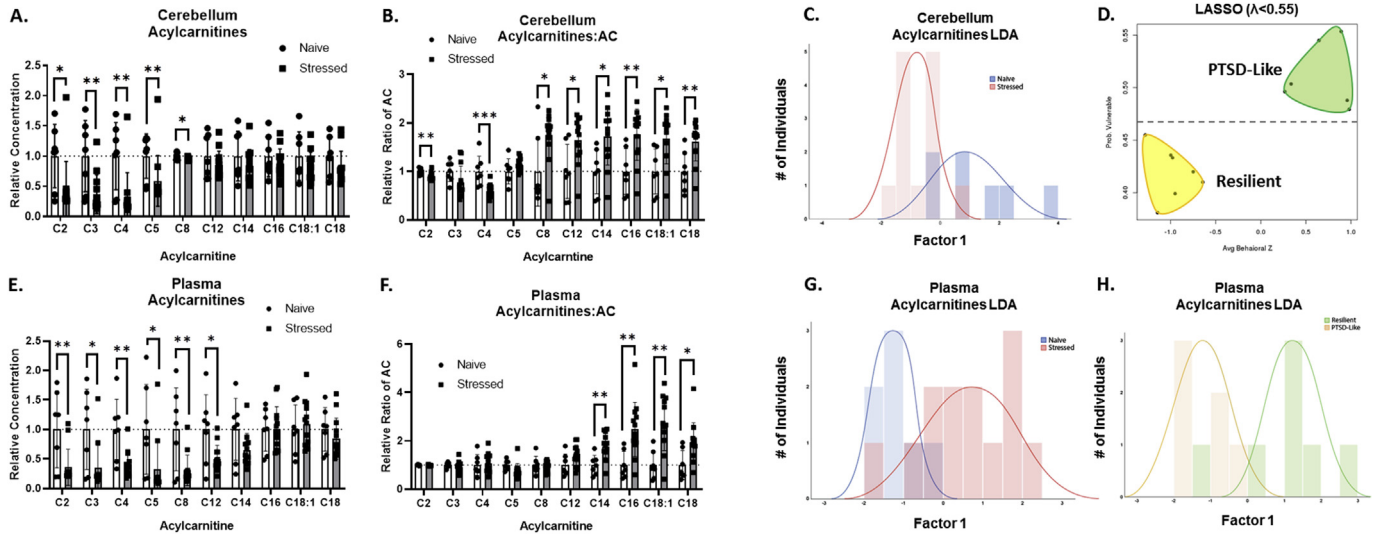

Fig. 4. Altered cerebellar and multisystem fatty acid oxidation in associated with stress and PTSD-vulnerability. Concentration of each acylcarnitine species in stressed or PTSD-like animals is expressed as a proportion of the average concentration of that species in naïve or resilient controls, respectively (for unscaled acylcarnitine concentrations see Fig. S3). A,B. Stressed animals (n = 13) displayed reduced concentrations of C2, C3, C4, C5, and C8 relative to naive animals (n = 7). Stressed animals (n = 13) displayed reduced ratios of C2 and C4, and elevated ratios of C8, C12, C14, C16, C18:1, and C18 to total acylated carnitines (AC) relative to naïve animals (n = 7). C. LDA of cerebellar acylcarnitines identified reduced C4: AC as sufficient to discriminate stress-naïve (n = 7) and stressed (n = 13) animals, but was unable to discriminate PTSD-like (n = 6) and resilient (n = 7) animals. E. Stressed animals (n = 13) displayed reduced concentrations of circulating C2, C3, C4, C5, C8 and C12 relative to naïve animals (n = 7). F. Stressed animals (n = 13) displayed increased ratios of circulating C14, C16, C18:1, and C18 to AC relative to naïve animals (n = 7) G. LDA of circulating acylcarnitines identified elevated C18:1AC as sufficient to discriminate stress-naïve (n = 7) and stressed (n = 13) animals. H. LDA of circulating acylcarnitines identified C2:AC as sufficient to discriminate PTSD-like (n = 6) and resilient animals (n = 7). D. LASSO analysis identified reduced cerebellar complex I (CI), complex IV (CIV), succinate:cytochrome c oxidoreductase (SCC), average cerebellar complex activity, and circulating C2:AC as highly predictive of PTSD-vulnerability, and these factors were sufficient to completely discriminate the two groups. $p < 0.05$(*), $p < 0.01$(**), $p < 0.001$(***), $p < 0.0001$(****).

C2:AC (Table S2). Notably, these five factors were sufficient to completely discriminate PTSD-like from resilient animals, with no overlap between the two groups (Fig. 4D).

4. Discussion

Here we report on cerebellar mitochondrial dysfunction and concomitant multi-system fatty acid oxidation dysfunction as candidate drivers of PTSD-like symptomatology in male mice. Resiliency is an important epidemiologic feature of PTSD, and behavioral paradigms of PTSD that delineate affected and resilient animals are increasingly favored, as are paradigms utilizing multiple behavioral readouts (Deslauriers et al., 2018). The PTSD paradigm used in this study identified induction of PTSD-like symptomatology in ~15% of exposed animals, a rate comparable to that of PTSD precipitation in humans exposed to a traumatic event (Karam et al., 2014). PTSD-like animals displayed multiple PTSD-like behaviors with high synergy, supporting a PTSD-like phenotype, rather than a similar stress-induced psychopathology, e.g. anxiety or depression (Strekalova and Steinbusch, 2010). LDA identified LPSA, which directly correlated with %PPI, as the primary behavior resolving PTSD-like and resilient animals. Hyperarousal has been self-reported as the most significant symptom associated with subjective quality of life in patients with PTSD (Giacco et al., 2013), corroborating the utility of this paradigm as a meaningful preclinical model of PTSD.

We observed numerous multi-system biochemical and physiologic changes in this cohort of animals associated with exposure to trauma, independent of PTSD-like symptomatology or PTSD resilience. Animals exposed to the PTSD-induction paradigm displayed evidence of reduced mtETC complex activity in both cerebellum and muscle, reduced mitochondrial DNA copy number in the cerebellum and PBC's, reduced flux through the fatty acid oxidation pathway, and reduced circulating carnitine and circulating short chain fatty acids. Notably, patterns of stress-induced mtETC complex dysfunction differed between the cerebellum and muscle, with CI being primarily affected in the cerebellum, and SCC and CII being primarily affected in the muscle.

We did not observe the reduced restraint stress CORT response reported by Lebow et al. (2012), nor did we observe evidence of hypocortisolemia in PTSD-like animals. While hypocortisolemia and reduced CORT response have been reported in some patients with PTSD, hypocortisolemia is not universally reported in patients with PTSD. The absence of this endocrine phenotype may reflect strain differences between the FVB animals used in this study and the C57black/6J animals used in the Lebow et al. study. Further investigations will be needed better understand the role of CORT in PTSD symptomatology.

Among the numerous stress effects observed in this cohort, we successfully identified a subset of biochemical changes that either discriminate PTSD-like and resilient animals, or directly correlate with PTSD symptom score. As we hypothesized, PTSD-like animals displayed reduced mtETC complex activity in the cerebellum relative to resilient animals, and the activities of all mtETC complexes, as well as average complex activity Z score, were inversely correlated with PTSD-symptom score. LDA identified CI as the primary complex measure resolving both stress-naïve and stressed animals, as well as PTSD-like and resilient animals. mtDNA sequencing identified 1039 mtDNA SNP's within this cohort, however, no SNP was detected at a heteroplasmy level sufficient to be clinically significant (Schon et al., 2012) and the mutation load did not vary significantly between PTSD-like and resilient animals. These data indicate that the reductions in mtETC complex activity associated with both stress exposure and PTSD-like symptomatology were not associated with alterations in the mtDNA sequence. Citrate synthase (CS) activity is a widely used readout of mitochondrial number and normalizing complex activities to CS activity normalizes complex activity to mitochondrial number (Lanza and Nair, 2009). mtDNAcn is a commonly used measure of mitochondrial number (Lanza and Nair, 2009) and reduced mtDNAcn has been reported in combat veterans with PTSD (Bersani et al., 2016). We observed a stress-induced reduction in mtDNAcn in both cerebellum and PBC's, though mtDNAcn was not significantly reduced in PTSD-like animals relative to resilient animals. However, the reduction of cerebellar mtETC complex activities in PTSD-like animals relative to resilient animals when mtETC complex activities are normalized to protein concentration rather than CS activity (Fig. S2) may indicate that a reduction in both mitochondrial number and mtETC complex activities contributes to reduced mtETC capacity in these animals. Muscle mtETC complex activity was also reduced in stressed animals relative to stress-naïve animals, though there was no difference in muscle mtETC complex activity observed between PTSD-like and resilient animals, and no muscle mtETC complex activity correlated with PTSD symptom score. This suggests that while muscle mtETC dysfunction may be an effect of stress exposure, it is not a reliable biomarker for PTSD-vulnerability.

Both stress exposure and PTSD-like symptomatology were associated with alterations in fatty acid oxidation in both cerebellum and plasma. Stressed animals displayed reduced concentrations of SC acylcarnitines in the cerebellum, consistent with reduced FAO defects in the liver (Ribel-Madsen et al., 2016), as well as reduced concentrations of both SC acylcarnitines and free carnitine, which often occurs secondary to FAO defects (Pons and De Vivo, 1995). Carnitine is an important moderator of short chain fatty acid toxicity in the mitochondria, converting accumulating short chain fatty acyl-CoA species to fatty acylcarnitines, which can then be extruded from the cell and excreted (Hokland, 1988). When normalized to total acylated carnitine (AC), stressed animals displayed elevated proportions of LC and MC acylcarnitines and decreased proportions of even chained SC acylcarnitines in the cerebellum, suggesting decreased oxidation of very long chain fatty acids through the FAO pathway. Consistent with this data, reduced C4:AC was sufficient to discriminate stress-naïve and stressed animals in the cerebellum, and concomitantly elevated C18:1:AC and reduced C2:AC were sufficient to discriminate stress-naïve and stressed animals in plasma.

While no significant difference in acylcarnitine profiles in the cerebellum between PTSD-like and resilient animals were observed, consistent with the FAO defects observed in stressed animals, PTSD-like animals displayed reduced C2:AC relative to resilient animals in plasma, which LDA identified as sufficient to discriminate the two groups. Altered carnitine and fatty acid metabolism have recently been identified in combat veterans with PTSD (Mellon et al., 2019).

The mtETC and FAO pathways are intimately associated, and defects in either are commonly associated with deficiencies in the other (Wajner and Amaral, 2015). Recent investigations have demonstrated a physical association between CI, the trifunctional protein, and the very long chain acyl-CoA dehydrogenase (Wang et al., 2019). The direct correlation of brain mtETC complex activities with PTSD-like behavior implicates mtETC complex dysfunction as the primary driver of this metabolic maladaptation.

LASSO analysis of all biochemical measures in this cohort of animals also identified plasma C2:AC, along with reduced activity of cerebellar CI, SCC, and CIV, as well as reduced average mtETC complex activity, as highly predictive of PTSD-like behavior (Fig. 5). These five state predictive biomarkers for PTSD-like symptomatology were sufficient to completely discriminate PTSD-like and resilient animals with no overlap between the two groups, and could indicate a bioenergetic and metabolic underpinning for the effects of trauma in PTSD.

Acetyl-L-carnitine (LAC) supplementation is an emerging new antidepressant therapy which has been recently shown to display antidepressant effects in both rodents and humans (Wang et al., 2014). PTSD displays high comorbidity with major depressive disorder (MDD) and mitochondrial dysfunction has been implicated in both psychopathologies (Preston et al., 2018). LAC has been hypothesized to improve neuroplasticity and brain energy metabolism (Wang et al., 2014), two putative mechanisms of PTSD-vulnerability related to suboptimal mitochondrial function (Preston et al., 2018). LAC has also been shown to improve both liver function and depressive symptoms in patients with minimal hepatic encephalopathy (Malaguarnera et al., 2011), making

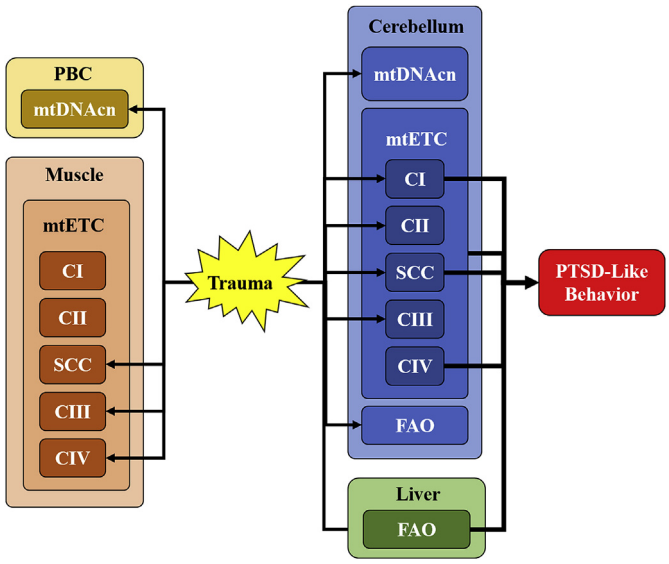

Fig. 5. Multisystem effects of trauma exposure and predictors of PTSD-like behavior in mice. Exposure to trauma was associated with decreased mitochondrial DNA copy number (mtDNAcn) in both cerebellum and peripheral blood cells (PBC's), decreased mitochondrial electron transport chain (mtETC) complex activities in both muscle and cerebellum, and impaired fatty acid oxidation (FAO) in both cerebellum and liver. Of these, reduced mtETC complex activity in the cerebellum and multisystem fatty acid oxidation dysfunction were sufficient to discriminate PTSD-like and resilient animals.

LAC a particularly interesting potential intervention given the liver FAO dysfunction observed in this animal model of PTSD. Future investigations will focus on whether this novel antidepressant also mediates PTSD-like symptomatology.

The cerebellum has long been implicated in executive control function, and defects in executive function have been hypothesized to underlie the emotional dysregulation and hyperarousal symptoms that characterize PTSD (Shalev et al., 2017). Reduced executive-network connectivity has been observed in patients with PTSD (Cisler et al., 2013; St Jacques et al., 2013) and impaired executive control function has been implicated in PTSD-vulnerability (Polak et al., 2012). Given the critical role that mitochondria play in the brain (Picard and McEwen, 2014), it is not surprising that mitochondrial disease patients also experience cognitive defects including reduced executive function (Finsterer, 2012). We have previously hypothesized that suboptimal mitochondrial function may reduce large scale brain network switching and increase vulnerability to PTSD (Preston et al., 2018).

One major strength of the PTSD induction paradigm used in this study is its stringency, *i.e.* only 15% of animals exposed to the paradigm were identified as PTSD-like, which is comparable to the prevalence of PTSD in humans exposed to trauma. Several biochemical features associated with PTSD-like behavior where identified, it is not clear whether these features increase vulnerability to PTSD-like symptomatology (trait) or are the result of PTSD-like symptomatology (state). Several features identified in PTSD-like animals, such as reduced cerebellar CI and SCC activity, appear to be induced by exposure to the trauma, as these features vary significantly between stress naïve and stressed animals. Other features however, such as reduced cerebellar CIV and plasma C2:AC, do not differ significantly between naïve and stressed animals and may be traits that predict vulnerability. Resolving traits that predict PTSD-vulnerability, and states resulting from PTSD-like symptomatology, remains a challenge, not only due to the unique nature of PTSD, a psychopathology defined by its environmental component (precipitation of PTSD canonically requires exposure to a traumatic event), but also by the difficulty in acquiring sufficient diagnostic samples, viz brain tissue, without introducing trauma in the process.

The impact of trauma on mental health is complex with poorly understood underlying mechanisms. The biological fingerprint of trauma exposure may be key to understanding PTSD vulnerability *vs.* resilience, as it encompasses the damage induced as well as the compensatory reactions of the organism. Here, we report integrated analysis of PTSD-like behavior, mitochondrial function and metabolomics data from male mice exposed to a unique PTSD induction paradigm to gain insights into mechanisms associated with PTSD-like symptomatology *vs.* PTSD-resilience. The findings reported here provide new insights into our understanding of cerebellar bioenergetics and concomitant multi-system fatty acid oxidation dysfunction as candidate drivers of PTSD-like symptomatology. We also identified reduced plasma acetylcarnitine discriminating PTSD-like and resilient animals. These bioenergetic and metabolic signatures of trauma exposure could yield leads for improved diagnosis and possible new drug candidates and natural compounds, such as acylcarnitine supplementation.

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Author contributions

G.P. and T.K. conceived the project and designed the research. G.P. performed behavioral and RT-qPCR experiments, data analysis and prepared the manuscript. T.E. assisted in behavioral data analysis and performed mitochondrial electron transport chain complex activities. F.K. assisted in behavioral data analysis and tissue processing. L.S. and M.H. consulted on behavioral experiments. E.M. and T.K. consulted on metabolomics and mitochondrial data analysis.

Declaration of competing interest

No Authors have any conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2020.100104.

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