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Chronic stress alters milk composition in TPH2 heterozygous mice: implications for neurodevelopment

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Abstract

The relationship between cerebral neuroregulatory factors and breast milk composition remains poorly understood. Prolonged stress adversely affects both the maternal organism and milk quality. This study aimed to investigate changes in the milk chemical composition of mice predisposed to affective disorders (TPH2 heterozygous) under chronic stress conditions, compared to unstressed heterozygous and wild-type (WT) controls. C57BL/6 mice heterozygous for TPH2 (Het, n=5) were subjected to daily isolation stress from postpartum days 2 to 9; unstressed Het (n=5) and WT (n=5) mice served as controls. Milk samples were collected on day 10 and analyzed using Raman spectroscopy (785 nm wavelength). Milk from TPH2 Het mice lacked characteristic peaks of specific proteins and fatty acids but exhibited a higher fatty acid ratio than WT milk, indicating altered milk composition associated with changes in offspring nerve fiber myelination components. Stress exposure in TPH2 Het females increased the intensity of saturated fatty acid peaks in milk. These findings demonstrate altered milk nutritional composition in TPH2 Het females, with stress exposure revealing a compensatory mechanism affecting milk lipid profiles.

Keywords: Tryptophan hydroxylase 2, anxiety, Raman spectroscopy, milk.

1. Introduction

The postpartum period is a critical phase of motherhood, involving profound physiological and psychological adaptations. Maternal mental well-being during this time is crucial not only for the mother but also for the healthy development of the newborn. Notably, up to 20% of women are susceptible to postpartum depression (PPD), which can adversely affect the mother's well-being and the child's cognitive, behavioral, and emotional development with long-term consequences if untreated (Amici *et al.*, 2022).

Motherhood, as a complex socially motivated behavior, involves the plasticity of several key neuromodulatory brain systems, including serotonin (5-HT), dopamine, gamma-aminobutyric acid, and norepinephrine. Serotonin (5-HT) is of particular interest due to its central role in mood regulation. Its synthesis in the brain depends on the enzyme tryptophan hydroxylase 2 (TPH2). Disruption of TPH2 expression is strongly linked to the development of anxiety and depressive disorders (Gorlova et al., 2020; Pratelli & Pasqualetti, 2019). Mice with a knockout of the TPH2 gene represent a well-established biomodel of stress-induced depression. Heterozygosity for TPH2 (TPH2 Het) results in partial TPH2 inactivation and a documented 20-30% reduction in brain serotonin levels, closely mirroring the neurochemical features of human affective disorders (Waider *et al.*, 2011). Under stress, the phenotypic profile of TPH2 Het individuals shifts towards increased anxiety and aggression (Auth *et al.*, 2018; Svirin, 2022).

When PPD is diagnosed and treated pharmacologically, breastfeeding is often discontinued, and mother's milk is replaced with formula. Consequently, there is a significant gap in understanding how neuroregulatory factors, particularly those involved in affective disorders like serotonin dysfunction, influence the composition of breast milk, and thus its potential impact on the infant (Wada and Lönnerdal, 2015; Wang *et al.*, 2017).

To date, little is known about how decreased serotonergic innervation, whether mediated by endogenous (e.g., genetic) or exogenous (e.g., stress) factors, affects breast milk components. Investigating milk composition requires analytical techniques capable of providing detailed biochemical information non-destructively. Raman spectroscopy is particularly suited for this purpose, offering narrow bands, high resolution, and the ability to extract comprehensive chemical and physical information from samples without destruction (Chandra *et al.*, 2024).

This study aimed to identify changes in the chemical composition of milk in heterozygous TPH2 female mice, a model genetically predisposed to affective disorders, following exposure to chronic stress, utilizing the analytical power of Raman spectroscopy.

2. Materials and Methods

2.1. Animals

The experiment was carried out on the basis of the Experimental Clinic-Laboratory of Biologically Active Substances of Animal Origin of the Federal State Budgetary Institution "FSC Food Systems named after. V.M. Gorbatov" RAS. The mouse strain under study is maintained by harem breeding of heterozygous knockout individuals of the TPH2 SPF category on the C57BL/6J genetic background. Animals were housed with free access to food and water in individually ventilated Bio A.S. cages. (Vent II, EHRET, Germany) type 2L (size 350 x 200 x 140 mm). The conditions for keeping the animals were standardized: temperature - 20 ± 3 °C, humidity - 35 ± 2%, input and output air flow - 95 ± 5 m3/h, as well as day/night lighting from 6:00 to 18:00 / from 18:00 to 6:00).

2.2. Stress model and milk collection

The sample size (n=5 per group) was determined based on the expected large effect size of the genetic manipulation and stress intervention on milk composition, as suggested by preliminary data and similar studies utilizing Raman spectroscopy for biomarker detection. While this limits the generalizability of the findings, it provides a basis for initial exploratory investigation. Wild-type (Wt, n=5), heterozygous TPH2 knockout females without stress (Het, n=5), and heterozygous TPH2 knockout females subjected to chronic stress (Het-Stress, n=5) were used. Milk sampling was performed on postpartum day 10 (PND10). A power analysis indicated that detecting large effects in Raman peak intensities (Cohen's d>1,2) at α=0,05 with 80% power requires 4-6 animals per group. Therefore, using n=5 per group affords a high probability of identifying statistically significant differences in milk spectral

parameters. Moreover, Raman spectroscopy averages data across multiple sampling points, thereby reducing within-group variability and enhancing measurement reliability.

To model chronic stress, Het-Stress females were subjected to daily restraint stress from PND2 to PND9. Each day, the dam was immobilized in a restraint device inside her home cage for 1 hour, while the litter remained in the cage but out of her reach. To prevent litter hypothermia, a 100 W infrared lamp was directed at the cage. This protocol is an adaptation of established early postpartum stress models known to induce robust maternal anxiety-like phenotypes (Otayf and Gadallah, 2024).Before milk collection, litters were isolated in a mesh container within the home cage for 3 hours to prevent direct physical contact. Subsequently, mothers and litters were reunited for 3-5 minutes to stimulate milk ejection *(Willingham et al.*, 2014). Females were then anesthetized via inhalation of isoflurane (Laboratorios Karizoo, Spain) using a BrandRWD anesthesia unit (R540, China). Oxytocin (0.1 ml/head) was administered intraperitoneally to facilitate milk letdown. Milk droplets were collected using an automatic pipette (Thermo Fisher Scientific, USA) into Eppendorf tubes (Sarstedt, Germany) for a maximum of 15 minutes. Females were returned to their home cage after full recovery of respiratory and motor functions (approximately 1 hour).

2.3. Raman Spectroscopy

The chemical composition of milk was assessed using InVia Raman spectroscopy (Renishaw, UK) at a wavelength of 785 nm and recording Raman spectra at a focal length of 50x. Milk in a total volume of 25 µl was stabilized in an aluminum foil well. Scanning was carried out at various points of the well to account for sample heterogeneity, with an exposure time of 10 s and 1 accumulation in a general range from 870 to 1800 cm^{-1} and 2690-3040. cm^{-1}. At least three spectra were recorded for one sample; all measurements were carried out at room temperature. The obtained spectra were processed in WiRE 5.5 software by removing cosmic rays; baseline correction; normalization and smoothing of peaks by smoothing using Savitsky-Golay second derivative algorithms. The analysis and identification of Raman spectra were carried out by the position of the peak, determined by its maximum, corresponding to the vibration frequency of the chemical bond, using the Interactive IRUG Spectrum database (De Gelder *et al.*, 2007).

2.4. Statistical Analysis

Statistical analysis was performed using Statistica 10.0 (StatSoft, USA). Given the non-parametric nature of the spectral data and the small sample size, intergroup differences were assessed using the Kruskal-Wallis H-test followed by post-hoc Dunn's test for multiple comparisons. Data are presented as median and interquartile range. The level of statistical significance was set at $p<0,05$. Exact p-values are reported for all significant comparisons.

3. Results

Raman spectroscopic analysis of mouse milk across 870-3040 cm^{-1} revealed that nearly all detected peaks differed between wild-type (Wt), TPH2 heterozygous (Het), and stressed TPH2 heterozygous (Het-Stress) groups. Seventeen peaks showed clear quantitative shifts, and five peaks underwent complete presence/absence changes, underscoring profound alterations in milk biochemistry driven by genotype and stress (Fig. 1).

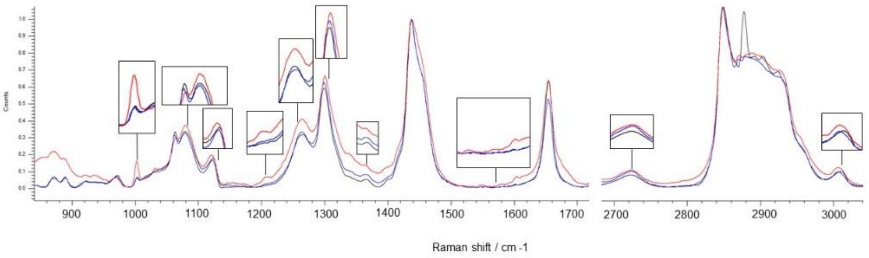

Figure 1. Raman shift peaks in milk simples. Note: red line – TPH2 Het milk, black – TPH2 Het* stress milk and blue line – WT milk in the range 870-3040 cm^{-1}

3.1 Fatty Acid Profile Alterations

The 971 cm^{-1} peak, characteristic of unsaturated fatty acid CH_2 vibrations, remained essentially constant across groups, indicating preservation of unsaturated lipid fractions despite genetic or stress challenges. In contrast, the saturated fatty acid marker at 1441 cm^{-1} increased in Het-Stress samples relative to both Wt and Het, suggesting that chronic isolation stress promotes accumulation of saturated lipids in milk. At 1300 cm^{-1}, reflecting =C–H bending in phospholipids, Het samples displayed greater variability than Wt, implying metabolic heterogeneity in membrane lipid synthesis associated with TPH2 heterozygosity.

The 1063 cm^{-1} peak, indicative of ordered lipid domains, was markedly elevated in both Het and Het-Stress groups compared to Wt. This four-fold increase highlights a fundamental shift in milk fat globule physical organization, which may affect lipid digestion and neonatal energy supply.

High-frequency cholesterol markers at 2849 and 2871 cm^{-1} exhibited contrasting patterns: 2849 cm^{-1} showed pronounced variability in Het, suggesting dysregulated sterol metabolism, while 2871 cm^{-1} remained relatively stable across groups, indicating selective maintenance of certain sterol components under stress (Table 1).

Table 1. Exploratory analysis of Raman peak intensities in mouse milk samples. Note: Qd – Qualitative difference, NA – not available, FAS – saturation of fatty acids, FAS^{un} – unsaturation of fatty acids

Region (cm^{-1})	Interpretation	WT Median (IQR)	HET Median (IQR)	HET+Stress Median (IQR)	p-value	Effect size (η^2)
870	Vibrations CH_2	0.029 (0.012-0.047)	0.064 (0.085-0.184)	0.061 (0.058-0.064)	0.10	0.52
890	C–C skeletal stretching of sugars	0.051 (0.048-0.053)	0.056 (0.055-0.057)	0.056 (0.053-0.057)	0.48	0.14
922	**C-C rings of proline**	**0.056 (0.050-0.058)**	**0.091 (0.060-0.093)**	**absent**	**Qd**	**NA**
971	Vibrations CH_2 FAS^{un}	0.084 (0.077-0.089)	0.088 (0.086-0.137)	0.324 (0.321-0.328)	0.10	0.68
1003	C–C stretching of FAS chains and phospholipid acyl chains	0.084 (0.077-0.084)	0.098 (0.074-0.122)	0.058 (0.057-0.144)	0.89	0.56
1063	C–C stretching of FAS chains and phospholipid acyl chains	0.064 (0.051-0.082)	0.307 (0.304-0.310)	0.323 (0.310-0.355)	0.04	0.99
1079	**C–O–C stretching in metaphosphite anion PO_2^-**	**0.293 (0.276-0.317)**	**absent**	**0.324 (0.320-0.347)**	**Qd**	**NA**
1122	C–C stretching in lipid hydrocarbon backbones	0.176 (0.169-0.177_	0.148 (0.140-0.150)	0.167 (0.151-0.353)	0.26	0.29

1262	Amide III and reflect unsaturation	0.309 (0.298-0.323)	0.350 (0.290-0.351)	0.352 (0.342-0.353)	0.16	0.15
1300	CH_2 twisting mode. indicative of FAS	0.561 (0.546-0.576)	0.593 (0.492-0.598)	0.592 (0.587-0.595)	0.22	0.21
1441	FAS	0.994 (0.977-1.000)	0.985 (0.826-1.003)	1.021 (1.017-1.024)	0.09	0.35
1655	C=C stretching of FAS[m] and overlapping amide I of proteins	0.609 (0.604-0.649)	0.673 (0.580-0.680)	0.672 (0.646-0.701)	0.30	0.22
1744	C=O stretching of ester bonds in triglycerides. a principal lipid marker	0.170 (0.163-0.178)	0.159 (0.146-0.163)	0.166 (0.163-0.178)	0.30	0.30
2723	overtone and combination bands reflecting mixed lipid-protein vibrations	0.095 (0.094-0.096)	0.096 (0.080-0.112)	0.085 (0.073-0.096)	0.74	0.42
2849	CH_2 symmetric stretching of FAS	0.999 (0.992-1.003)	0.992 (0.852-1.250)	0.982 (0.898-0.997)	0.65	0.68
2871	CH_3 asymmetric stretching. reflecting cholesterol and branched-chain amino acids	0.704 (0.675-0.716)	0.712 (0.705-0.719)	0.700 (0.652-0.703)	0.78	0.33
2879	**CH_2 symmetrical stretching vibrations**	**absent**	**0.998 (0.992-1.140)**	**absent**	**Qd**	**NA**
2889	CH_2 asymmetric stretching of lipid acyl chains	0.681 (0675-0.689)	0.705 (0.525-0.709)	0.717 (0.717-0.720)	0.06	0.66
3009	=C–H stretching of FAS[m]	0.090 (0.089-0.091)	0.098 (0.088-0.122)	0.094 (0.091-0.099)	0.46	0.39

3.2 Protein and Amino Acid Components

The amide III band at 1262 cm^{-1}. diagnostic of protein secondary structure. was elevated in both Het and Het-Stress. suggesting maintained or enhanced protein synthesis capacity. potentially as a compensatory response to genetic and environmental stress. The 1122 cm^{-1} peak. representing amino acid backbone vibrations. decreased in Het compared to Wt and partially recovered under stress. This pattern indicates that stress may trigger adaptive changes in milk protein composition to support offspring development (Table 1).

3.3 Qualitative Peak Shifts

Raman spectroscopic profiling revealed distinct qualitative shifts in several peaks that underscore how TPH2 heterozygosity and chronic stress reshape milk biochemistry. Two peaks – at 1079 cm^{-1}. corresponding to metaphosphite anion (PO_2^-) vibrations. and at 2925 cm^{-1} (Table 1). assigned to asymmetric C–H stretching in lipid chains – were completely absent in milk from unstressed Het females but fully restored in stressed Het females. This reversible loss and recovery suggest that TPH2 heterozygosity disrupts mammary phosphate metabolism and lipid organization under baseline conditions. while chronic isolation stress reactivates pathways that re-establish normal phospholipid and lipid membrane synthesis.

In contrast. chronic stress in Het mice induced selective elimination of peaks at 922 cm^{-1} and 1362 cm^{-1}. The 922 cm^{-1} band. linked to proline and hydroxyproline ring deformations. implicates collagen and extracellular matrix remodeling within the mammary gland; its loss under stress indicates altered structural protein processing that could influence milk viscosity or secretion. Likewise. disappearance of the 1362 cm^{-1} CH_2 symmetric bending mode points to stress-dependent perturbations in saturated lipid packing. potentially affecting milk fat globule stability and neonatal lipid uptake.

Remarkably, stress also evoked the emergence of two novel peaks – at 1368 cm^{-1}. reflecting COO^- symmetric stretching in carboxylate groups. and at 2893 cm^{-1}. associated with protein backbone vibrations. The 1368 cm^{-1} signal suggests activation of alternative fatty acid

oxidation or lipid remodeling pathways. possibly generating unusual phospholipid species under chronic stress. The 2893 cm^{-1} band likely arises from newly synthesized or post-translationally modified milk proteins. hinting at a stress-driven shift in mammary protein expression that may serve adaptive functions. such as enhancing immunoprotective or stress-response factors in milk (Table 1).

Together. these qualitative peak shifts illustrate a dynamic interplay between genotype and environment: TPH2 heterozygosity compromises key phosphate- and lipid-related processes in milk synthesis. while chronic stress not only reactivates some disrupted pathways but also promotes novel metabolic adaptations. Biologically. these changes may influence milk's structural properties. nutrient availability. and signaling functions. with downstream consequences for offspring growth. gut maturation. and neurodevelopment.

Peaks at 971. 1262. and 1655 cm^{-1} reflect unsaturation degree. while signals at 1300. 1441. and 1744 cm^{-1} correspond to saturated bonds and ester groups (Pchelkina *et al.*, 2023). All genotypes exhibited intense peaks at 1300 and 1655 cm^{-1}. Stressed Het mice showed 17.3% higher intensity relative to WT (and 1.6% relative to unstressed Het). According to Table 2. WT milk demonstrated lower geometric mean ratios (GeoMean=0.549).

Table 2. Content of unsaturated fatty acids in milk samples

I FAS^{im} / I FAS	WT	TPH2 Het	TPH2 Het *Stress
I 971 / I 1300	0.143	0.229	0.227
I 971 / I 1441	0.081	0.136	0.131
I 971 / I 1744	0.468	0.791	0.766
I 1262 / I 1300	0.557	0.588	0.580
I 1262 / I 1441	0.317	0.349	0.336
I 1262 / I 1744	1.826	2.033	1.960
I 1655 / I 1300	1.116	1.155	1.139
I 1655 / I 1441	0.636	0.686	0.659
I 1655 / I 1744	3.661	3.993	3.846
GeoMean	0.549	0.683	0.664

4. Discussion

The present study demonstrates that TPH2 heterozygosity in mice – an established model of central serotonergic dysfunction – results in measurable alterations in milk biochemical composition. as assessed by Raman spectroscopy. Notably. wild-type mouse milk displayed a consistent pattern of saturated and unsaturated fatty acid markers. as well as the presence of key peaks characteristic of both peptide and lipid molecular structures. In contrast. the milk of TPH2 heterozygous females was marked by the absence or attenuation of specific peaks associated with certain proteins and fatty acids. in accordance with previous findings that link serotonin signaling deficits to altered neuronal and systemic metabolism (Alenina *et al.*, 2009; de Wolf *et al.*, 2021; Thangaraj *et al.*, 2024).

Chronic stress exposure further modulated these milk composition changes. While the absence of key spectral peaks was also observed in the stressed TPH2 heterozygotes. some markers – particularly those associated with phospholipid and protein metabolism – were partially restored. suggesting an activation of compensatory biosynthetic pathways in response to prolonged stress. In line with other reports. stress in postpartum females is known to impact lipid and bioactive metabolite content of milk. with implications for nutrient availability and

biochemical resilience in the offspring (de Wolf *et al.*, 2021; Mohanty *et al.*, 2016a; Otayf and Gadallah. 2024; Thangaraj *et al.*, 2024).

The disrupted presence of phosphate- and lipid-associated peaks (e.g. the metaphosphite anion and long-chain fatty acids) in TPH2-modified and stressed mice aligns with the essential role of phosphorus in neural development and cellular metabolism (Schönfeld and Wojtczak. 2016; Tzvetkov *et al.*, 2020). Moreover. the observed shifts in cholesterol-associated markers highlight the importance of cholesterol and its derivatives in neonatal brain maturation and cell membrane synthesis. as described in both animal and human milk research *(Jensen et al.*, 1978; Thangaraj *et al.*, 2024; Tzvetkov *et al.*, 2020).

Bioactive peptides derived from milk proteins also warrant attention. These molecules. which can be generated by enzymatic digestion or fermentation. contribute to immunomodulatory. opioid-like. and neuroregulatory functions – affecting both the gut and central nervous system. as described in several recent reviews (Mohanty *et al.*, 2016a). Notably. peptides such as casein-derived casomorphins and β-lactorphin have been shown to directly influence the gut-brain axis. with effects potentially mediated by serotonin signaling and microbiota interactions (Mohanty *et al.*, 2016b; Robinson *et al.*, 2025; Singh and Gaur. 2024).
Nevertheless. despite these biochemical differences. it remains unclear to what extent the milk-borne changes observed in this model translate to measurable neurodevelopmental or behavioral outcomes in offspring – a limitation that merits further investigation in longitudinal and interventional studies. The findings herein are constrained by sample size and by the exploratory nature of non-targeted spectroscopic analysis. However. the results are consistent with accumulating evidence that milk composition. shaped by genetics and maternal experience. has the potential to influence early postnatal development through both nutritional and signaling pathways (Mohanty *et al.*, 2016b; Singh and Gaur. 2024; Thangaraj *et al.*, 2024; Trinchese *et al.*, 2024).

In summary. this study provides a detailed. non-destructive characterization of milk composition shifts in a model of serotonergic compromise and chronic stress. linking specific biochemical changes to genetic and environmental factors with possible relevance for offspring neurodevelopment. Further mechanistic studies are necessary to clarify the functional consequences of these molecular differences.

5. Conclusion

This study demonstrates that genetic predisposition to affective disorders. represented by TPH2 heterozygosity. and exposure to chronic stress lead to pronounced alterations in the biochemical composition of mouse milk. The observed changes include diminished intensity or absence of specific Raman peaks corresponding to key proteins and fatty acids. as well as an increased ratio of saturated to unsaturated fatty acids compared to wild-type controls. Importantly. chronic stress in TPH2 heterozygous females was associated with both further disruptions and compensatory restoration of certain lipid and protein markers. highlighting metabolic plasticity of the mammary gland in response to environmental and genetic factors. The findings suggest that both serotonergic imbalance and environmental stressors contribute to the regulation of milk composition. potentially affecting the nutritional and neurodevelopmental support provided to offspring. Although this work does not directly address functional consequences in neonates. the results provide a basis for future studies investigating the mechanistic links between maternal neurobiology. milk composition. and developmental outcomes.

Taken together. these results reinforce the value of non-destructive Raman spectroscopic methods for revealing subtle. physiologically meaningful shifts in milk biochemistry and lay groundwork for further research into the interactions between maternal health. nutrition. and early-life programming.

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Conflict of interests

The authors affirm that they have no competing interests to disclose.

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