**Pre-pubertal low intensity exercise mitigates the effects of an early-life stressor in adolescent FSL rats, regardless of prenatal exercise**

***Supplementary data***

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**S1 Methods**

**S1.1 Quantitative analyses of hippocampal monoamines, GSH and GSSG**

After weighing the sample, 250 µL of the internal standard solution (ethyl-4-hydroxy-2-quinolinecarboxylate) was added. The mixtures were homogenized by sonication (twice for 12 s, at an amplitude of 14 µ; MSE ultrasonic disintegrator, Nuaillé, FRA). The mixture was left on ice for 20 min to complete protein precipitation and centrifuged at 20 817 rcf for 20 min at 4 °C. The supernatant was transferred to a HPLC sample vial. Quantification of norepinephrine (NE), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA), GSH and GSSG was determined using liquid chromatography/mass spectrometry (LC-MS). A Venusil ASB C18, 2.1 x 150 mm, 3 µm column (Agela Technologies, Torrance, CA, USA) HPLC column was used for chromatographic separation. The Ultivo® Triple Quadrupole LC/MS System, controlled by MassHunter™ software from Agilent Technologies®, Inc. (Santa Clara, CA 95051 US), consisting of a quaternary pump, column oven, autosampler and a triple quadrupole mass detector, was used. A gradient mobile phase consisting of A: 0.1% formic acid/LC grade water and B: 0.1% formic acid/methanol were prepared. The software was then programmed to inject 1 µL of the prepared sample onto the LC-MS for analysis. Monoaminergic, GSH and GSSG concentrations in the tissue samples were determined by comparing the peak area of each marker to that of the respective standards (range: 31.5 – 500 ng/ml). Linear standard curves (regression coefficient > 0.95) were found in this range for all the analytes. Final concentrations were expressed as ng/g wet weight tissue. All reference and the internal standard were obtained from Merck® (RSA). Chemicals used for the analysis were Milli-Q water, LC grade MeOH, and formic acid (99%). The standards and samples were prepared in LC grade MeOH containing, 0.1% formic acid. Apart from the Milli-Q water, all of these were also obtained from Merck®.

**S1.2 Metabolic profiling**

Briefly, 400 µl methanol, 75 µl water and 50 µl of the internal standard (50 ppm 3-phenylbutyric acid) was added to each hippocampus in safe-lock microcentrifuge tubes, together with a single steel bead (3 mm Ø). Thereafter, the tissue was homogenised for 2 min at 20 Hz using the Retch M400 vibration mill. Following the homogenisation of each tissue sample, 400 µl chloroform, with an additional 200 µl water was added to each sample and vortexed for 30 s. The samples were then centrifuged at 2000 x g for 5 min at 4 °C to induce phase separation. 400 µl of the top polar phase and 300 µl of the bottom apolar phase were transferred to glass vials and dried under nitrogen. The samples were stored at -80 °C until derivatization. In addition to this, quality control samples were also prepared from pooled aliquots of all sample extracts.

Prior to their analysis via GC-TOF-MS, all samples were derivatized via oximation and silylation as previously performed ([Lindeque *et al.*, 2013](#_ENREF_1); [Terburgh *et al.*, 2019](#_ENREF_2)). For oximation, 50 µl methoxyamine solution (200 mg methoxyamine dissolved in 10 ml of pyridine) was added to each glass vial containing the dried sample extract. Thereafter, each sample was vortexed for 30 s to dissolve the dried extract and incubated for 1 h at 60 °C. After allowing the samples to cool to room temperature, silylation was performed by adding 50 O-Bis(trimethylsilyl)-trifluoro-acetamide, containing 1% trimethylchlorosilane to the oximated samples and vortexed for 30 s. The samples were then incubated for 40 min at 40 °C. All liquids from each sample were individually transferred into a flat bottom insert and placed inside the same vial that was used for derivatization. Each vial was then loaded onto an Agilent 7693 auto sampler for GC-TOF-MS analysis.

**S1.3 GC-TOF-MS analysis**

The GC-TOF-MS system used comprises of an Agilent 7890A series gas chromatograph with Agilent 7693 auto sampler coupled to a LECO Pegasus HT time-of-flight mass analyser with an electron impact ionization source. One µL sample was injected (using a 1:10 split ratio) into the front inlet which remained at a constant temperature of 250 °C. Helium was used as the carrier gas at constant flow of 1.4 mL/min. Metabolites were separate in a Restek RXi-1MS column (30 m x 0.18 mm x 0.18 µm) using the following oven temperature gradient: the oven remained at 50 °C for 1 min after injection and then increased 5 °C/min until it reached 100 °C. The temperature ramp then increased to 10 °C/min until 160 °C, whereafter the temperature increased further until 230 °C at a rate of 13 °C/min. During the final phase, the oven temperature increased 20 °C/min until it reached 300 °C, where it remained for 2 min before cooling befor the next run. The transfer line and ion source temperature were kept constant at 225 °C and 200 °C, respectively. The data was obtained at an acquisition rate of 20 spectra/s (50 - 800 m/z).

**S2 Results**

As discussed elsewhere, the findings of themanuscript were interpreted and discussed in terms of the largest and statistically non-zero effect size. For transparency, the data used to create *Figure 3*, is presented in *Table S1*, below, together with all other significant findings.

**Table S1: Summary of effect magnitude influences on behaviour**

*Values are presented as partial eta squared.* ***ELA:*** *Early-life adversity.* ***JUV:*** *Juvenile activity.* ***PRE:*** *Prenatal activity.*

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **PRE** | **ELA** | **JUV** | **PRE\*ELA** | **PRE\*JUV** | **ELA\*JUV** | **PRE\*ELA\*JUV** |
| **Open field test** |
| *Distance* | 0 | 0.019 | 0.032 | 0.056 | 0.001 | 0.007 | 0.057 |
| **Forced swim test** |
| *Immobility* | 0.028 | 0.038 | 0.050 | 0.041 | 0.001 | 0.010 | 0.002 |
| *Swimming* | 0.092 | 0.006 | 0.019 | 0.142 | 0.090 | 0.042 | 0.001 |
| *Struggling* | 0.001 | 0.030 | 0.036 | 0 | 0.016 | 0 | 0.005 |
| **Elevated plus maze** |
| *Open arms* | 0.040 | 0.005 | 0.054 | 0 | 0 | 0.007 | 0.008 |
| ***Average*** | ***0.032*** | ***0.020*** | ***0.038*** | ***0.048*** | ***0.022*** | ***0.013*** | ***0.015*** |
| **95% Confidence interval limits** |
| ***Lower*** | ***-0.010*** | ***0.001*** | ***0.021*** | ***-0.025*** | ***-0.027*** | ***-0.007*** | ***-0.015*** |
| ***Upper*** | ***0.080*** | ***0.038*** | ***0.056*** | ***0.120*** | ***0.070*** | ***0.034*** | ***0.044*** |

**S2 Results**

**S2.1 Open field test**

Based on the significant three-way interaction, no specific intergroup differences were identified by the Bonferroni multiple comparison test (*p* > 0.05 in all instances) for distance moved in the open field on PND36 (*Figure 4A*). As discussed in the manuscript, distance moved in the open field test had no statistically significant effect on any of the forced swim test behavioural parameters (*p* > 0.05 in all instances; *Table 2*).

**A2.2 Forced swim test**

Based on the significant PRE\*ELA interaction (*Figure S1-A*; *F*1, 113 = 4.85, *p* = 0.03, *ηp2* = 0.04), both EXE/non-MSEW (*p* = 0.004, *dunb* = 0.7 [0.2; 1.2]) and SED/MSEW (*p* = 0.002, *dunb* = 0.7 [0.2; 1.2]) spent more time immobile in the FST, compared to SED/non-MSEW controls.



Figure S1: Significant two-way interaction for forced swim test behaviour (supplementary)

***(A)*** *Time spent immobile and* ***(B)*** *swimming in the FST. Data points represent the mean ± 95% CI, with male and female indicated in blue and pink respectively. Statistical analyses are reported in the text.* ***FST:*** *Forced swim test.* ***EXE:*** *Low intensity exercise.* ***SED:*** *Sedentary.*

For time spent swimming (*Figure S1-B*), the interactions between ELA\*JUV (*F*1, 113 = 5.02, *p* = 0.03, *ηp2* = 0.04), PRE\*JUV (*F*1, 113 = 11.21, *p* = 0.001, *ηp2* = 0.09) and PRE\*ELA (*F*1, 113 = 18.64, *p* ≤ 0.0005, *ηp2* = 0.14) were all significant after correcting for distance moved in the open field test. However, based on effect magnitudes, the latter was considered the most robust and used for multiple comparison reporting. In this regard, EXE/non-MSEW pups (irrespective of JUV) swam 21 s [13; 28 s] less than SED/non-MSEW controls (*p* ≤ 0.0005, *dunb* = 1.5 [0.9; 2.0]). Of note, MSEW (irrespective of JUV) also reduced swimming in pups born to a SED dam by 14 s [7; 21 s], in relation to SED/non-MSEW controls (*p* ≤ 0.0005, *dunb* = 0.8 [0.3; 1.4]). In contrast, when combined with PRE, MSEW (again irrespective of JUV) increased swimming behaviour by 10 s [2; 17 s], when compared to EXE/non-MSEW controls (*p* ≤ 0.0005, *dunb* = 0.6 [0.1; 1.2]).

**A2.3 Anatomical markers**

In addition to the significant influence of JUV on whole brain weight, both PRE (*F*1, 107 = 6.43, *p* = 0.013, *ηp2* = 0.06) and ELA (*Figure S2*; *F*1, 107 = 15.83, *p* ≤ 0.005, *ηp2* = 0.13) significantly influenced the brain weight on PND38, with the latter inducing a larger effect. Still, the brain weight of pups born to a dam that exercised (irrespective of ELA and JUV) were 0.08% [0.02; 0.14%] heavier than those born to a sedentary dam. In contrast, the brain weight of MSEW pups (irrespective of PRE and JUV) weighed 0.12% [0.06; 0.18% less than those of non-MSEW controls. No other significant contributing factor than described in *the manuscript* was identified for heart weights.



Figure S2: Significant effect of an early-life stressor on whole brain weight of MSEW and non-MSEW FSL rats, irrespective of PRE and JUV (supplementary)

*Data points represent the mean ± 95% CI, with male and female indicated in blue and pink respectively. Statistical analyses are reported in the text. EXE: Low intensity exercise. JUV: Juvenile exercise. MSEW: Maternal separation and early weaning. PRE: Prenatal exercise. SED: Sedentary.*

**A2.4 Hippocampal monoamine levels and redox state**

For hippocampal norepinephrine (*Figure S3-A*), a significant three-way interaction (*F*1, 102 = 18.033, *p* ≤ 0.0005, *ηp2* = 0.150) was identified, together with two-way interactions for PRE\*ELA (*F*1, 102 = 24.51, *p* ≤ 0.0005, *ηp2* = 0.194) and ELA\*JUV (*F*1, 102 = 17.17, *p* ≤ 0.0005, *ηp2* = 0.144) but not PRE\*JUV (*F*1, 102 = 3.54, *p* = 0.063, *ηp2* = 0.034). Noteworthy differences that were identified by the Bonferroni multiple comparison test for the three-way interaction, included that between EXE/MSEW/EXE, SED/non-MSEW/SED (*p* ≤ 0.0005, *dunb* = 2.7 [1.7; 4.0]), EXE/non-MSEW/EXE (*p* ≤ 0.0005, *dunb* = 2.6 [1.6; 3.7]), and SED/MSEW/SED (*p* ≤ 0.0005, *dunb* = 2.3 [1.3; 3.5]), respectively.

Similarly, hippocampal serotonin levels (*Figure S3-B*) were also influenced by all three factors (*F*1, 106 = 13.05, *p* ≤ 0.0005, *ηp2* = 0.11), with similar differences identified for norepinephrine levels. Compared to EXE/MSEW/EXE animals, SED/non-MSEW/SED (*p* ≤ 0.0005, *dunb* = 1.4 [0.6; 2.3]), non-EXE/MSEW/EXE (*p* ≤ 0.0005, *dunb* = 1.7 [0.8; 2.6]), and SED/MSEW/SED (*p* ≤ 0.0005, *dunb* = 1.4 [0.6; 2.3]) differed significantly.

Hippocampal serotonin turnover (*Figure S4-A*) was only influenced by PRE (*F*1, 106 = 6.44, *p* = 0.013, *ηp2* = 0.06), so that pups born to a dam that exercised during the prenatal period (regardless of ELA and JUV), had lower 5-HIAA/5-HT values, when compared to those born to a sedentary dam (*dunb* = 0.5 [0.1; 0.9]).



Figure S3: Significant two-way interaction for hippocampal monoamine levels (supplementary)

***(A)*** Hippocampal norepinephrine *and* ***(B)*** *serotonin levels of the FSL rats. Data points represent the mean ± 95 % CI, with male and female indicated in blue and pink respectively. Statistical analyses are reported in the text.* ***EXE:*** *Low intensity exercise.* ***JUV:*** *Juvenile exercise.* ***MSEW:*** *Maternal separation and early weaning.* ***PRE:*** *Prenatal exercise.* ***SED:*** *Sedentary.*

As for hippocampal redox state (*Figure S4-B*), PRE (*F*1, 106 = 41.30, *p* ≤ 0.0005, *ηp2* = 0.28), independently influenced GSH/GSSG ratio, in addition to the mentioned JUV (*F*1, 106 = 35.25, *p* ≤ 0.0005, *ηp2* = 0.25) influence. In this regard, PRE (irrespective of ELA and JUV) increased hippocampal GSH/GSSG ratio, when compared to their respective control groups (*dunb* = 1.03 [0.7; 1.4].



Figure S4: Significant two-way interaction for hippocampal serotonin turnover and redox state (supplementary)

***(A)*** *Hippocampal serotonin turnover**(5HIAA/5HT) and (B) redox state (GSH/GSSG) of FSL rats on PND38, regardless of juvenile (prepubertal) exercise. Data points represent the mean ± 95 % CI, with male and female indicated in blue and pink respectively.Statistical analyses are reported in the text.* ***EXE:*** *Low-intensity exercise.* ***SED:*** *sedentary.*

References

Lindeque, J.Z., Hidalgo, J., Louw, R. & van der Westhuizen, F.H. 2013. Systemic and organ specific metabolic variation in metallothionein knockout mice challenged with swimming exercise. *Metabolomics*, 9(2):418-432. <https://doi.org/10.1007/s11306-012-0459-8>

Terburgh, K., Lindeque, Z., Mason, S., Van der Westhuizen, F. & Louw, R. 2019. Metabolomics of Ndufs4−/− skeletal muscle: adaptive mechanisms converge at the ubiquinone-cycle. *Biochimica et biophysica acta (BBA)-molecular basis of disease*, 1865(1):98-106. <https://doi.org/10.1016/j.bbadis.2018.10.034>