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Genetically predisposed and resilient animal models of depression reveal divergent responses to early-life adversity

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Original Article

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Abstract

Objective: Early-life adversity (ELA) is one of the strongest predictors of childhood depression that may be exacerbated by a genetic predisposition to develop depression. We therefore investigated the bio-behavioural effects of an early-life stressor in an accepted rodent model of depression. ***Methods:*** The Flinders sensitive line (FSL) and resistant line (FRL) rats were subjected to an early-life stressor, whereafter their bio-behavioural response during pubertal onset was evaluated. Male and female pups were maternally separated for 3 h per day from postnatal day 02 (PND02) to 17, when they were also weaned. Control animals were left undisturbed, until weaning on PND21. Depressive-like behaviour was analysed on PND21 and reassessed on PND36. Hippocampal monoamine levels, markers of oxidative stress and metabolic markers implicating mitochondrial function were also measured. ***Results:*** On PND21, the non-maternal separation and early weaning (non-MSEW) FSL rats spent 10% more time mobile than their FRL controls in the tail suspension test (TST) yet displayed increased depressive-like behaviour in the forced swim test (FST) on PND36. This depressive-like behaviour coincided with increased hippocampal norepinephrine levels, serotonin turnover and a dysfunctional redox state. Maternal separation and early weaning (MSEW) appeared to initially reduce early-life (PND21) depressive-like behaviour in the TST but then induced depressive-like behaviour on PND36 and increased norepinephrine levels more profoundly in the FRL rats. ***Conclusion:*** These findings highlight the need to further investigate the stress response pathway in these animals and that the absence or presence of genetic susceptibility may influence the presentation of ELA effects.

Significant outcomes

- Juvenile FSL rats (irrespective of sex) may be a useful model to further investigate adolescent depression constructs.
- MSEW is effective in inducing depressogenic bio-behavioural effects.
- The bio-behavioural response of FRL offspring to MSEW was more significant.

Limitations

- No neurochemical analyses were performed on PND21, limiting the understanding of the observed behaviour at this age.
- Secondly, the current study did not measure any HPA-related markers, such as corticosterone, and therefore it remains unclear if the observed differences between the FSL and FRL rats are indeed related to two different stress responses.
- Finally, because of the link between cardiovascular disease and depression, prospective studies should also consider measuring blood pressure or heart rate variability in the Flinders line rats to build on the anatomical differences observed and reported here.

Introduction

Childhood is a developmental period characterised by extensive neuroplasticity that renders the developing brain vulnerable to environmental insults and experiences (Heim *et al.*, 2010). Such a disruption may contribute to the aetiology of various psychiatric disorders, including major

depressive disorder (Andersen & Teicher, 2008; Marco *et al.*, 2011), which is one of the most commonly diagnosed mental disorders in school-aged children (Centers for disease control and prevention, 2020). In fact, it is the fourth leading cause of global illness and disability amongst adolescents (15-19 years) and the fifteenth in those younger than 14 years of age (World Health Organization, 2022). Although mental health statistics in the South African paediatric population is limited, an estimated 13–21% of adolescents (14–26 years) experience depressive symptoms (Nduna *et al.*, 2013; Barhafumwa *et al.*, 2016). In the USA, almost two million children (3-17 years) have been diagnosed with depression (Centers for disease control and prevention, 2020; Ghandour *et al.*, 2019), of which 17% of children (2–8 years), 5% of 12-year-olds and 17% of 17-year-olds have at least experienced a major depressive episode in the year before (Centers for disease control and prevention, 2020; Cree *et al.*, 2018; Selph & McDonagh, 2019). In 2016, 62 000 adolescents died because of self-harm (World Health Organization, 2022), making it the third leading global cause of death in this age group (World Health Organization, 2022).

Early-life adversity (ELA) refers to traumatic experiences, such as loss of a parent, abuse or neglect that can cause detrimental long-term effects. In this regard, a survey of 21 countries found that childhood adversities (i.e. neglect, abuse and parental mental illness) were accountable for 29.8% of debilitating juvenile disorders, including but not limited to mood, anxiety, behavioural and substance disorders (Kessler *et al.*, 2010), making early-life adversities one of the strongest contributing factors (LeMoult *et al.*, 2020) and important antecedent to depression (Dube *et al.*, 2001; Heim & Nemeroff, 2001; Vibhakar *et al.*, 2019). Adding to this, being born into a family with a history of depression can increase a child's risk of developing depression (Fihrer *et al.*, 2009; Thompson *et al.*, 2018; Tirumalaraju *et al.*, 2020). In addition, perinatal and postnatal depression increases the offspring's risk of developing depression later in life by as much as 70% (Tirumalaraju *et al.*, 2020). Although the exact mechanism for this increase remains unclear, persistent maternal depression could lead to neglected parental engagement and an impaired ability to meet the child's social and developmental needs (Dietz *et al.*, 2008). Pathways that have been implicated in the bio-behavioural alterations associated with ELA include the redox, bio-energetic (i.e. mitochondrial function) (de Souza *et al.*, 2020), serotonergic (Lee *et al.*, 2007; de Souza *et al.*, 2020) and inflammatory (Wang *et al.*, 2017) pathways. Maternal separation is therefore a useful intervention, often used in animal studies to model maternal neglect (de Kloet *et al.*, 2005) and investigate the neurodevelopmental aspects of neuropsychiatric conditions (Tractenberg *et al.*, 2016; Alves *et al.*, 2020).

The Flinders sensitive line (FSL) rat is an accepted animal model of depression (Overstreet & Wegener, 2013), sensitive to the lasting effects of maternal separation (El Khoury *et al.*, 2006; Wörtwein *et al.*, 2006; Ellenbroek *et al.*, 2016) and social isolation (Mncube *et al.*, 2021). Importantly, the effects of ELA or neglect are generally limited to long-lasting effects (i.e. into adulthood). For instance, male adult FSL rats, maternally separated for 3 h per day between postnatal day 02 (PND02) and 14, display increased depressive-like behaviour (El Khoury *et al.*, 2006), altered inflammatory markers (Carboni *et al.*, 2010) and levels of neuropeptides associated with depression (Wörtwein *et al.*, 2006; Ellenbroek *et al.*, 2016). Expanding on the maternal separation model, George *et al.* (2010) developed an early-life neglect model, adding early weaning to the maternal separation protocol to exacerbate the lasting effects. Early weaning has been shown to alter hippocampal glucocorticoid receptor levels (Kikusui *et al.*, 2006) and induce anxiogenic and aggressive behaviour (Kikusui *et al.*, 2004). Based on the findings of George et al. (2010), maternal separation and early weaning (MSEW) induced long-lasting behavioural changes akin to anxiety, despair and hyper-activity, without adversely impacting metabolic functions. In addition to the lack of short-term (i.e. pubertal) bio-behavioural data of maternal separation in the Flinders line rats, this MSEW protocol has to the best of our knowledge not been investigated in the offspring of this specific strain. Therefore, considering that the FSL rat is accepted to also accurately model childhood depression (Malkesman & Weller, 2009), specifically genetically predisposed individuals, and that information regarding the short-term (i.e. pubertal) effects of ELA is limited, research into this topic is warranted. We therefore hypothesised that ELA (i.e. MSEW) would induce depressive-like behaviour in the Flinders resistant line (FRL) rat, while exacerbating the depressive-like phenotype of the FSL rat. Finally, we further expect these behavioural alterations to be supported by decreased hippocampal monoamine levels and mitochondrial function and increased markers of oxidative stress.

Materials and methods

Study design

As summarised in Fig. 1, male and female FSL ($n = 32$) and FRL ($n = 33$) offspring were randomly and equally divided into the different intervention groups. Those undergoing MSEW were separated from the dam for 3 h every day from PND02 and weaned on PND17, instead of PND21, when the pups from the non-MSEW groups were weaned (George *et al.*, 2010). Early-life depressive-like behaviour was analysed on PND21 and then reassessed on PND36 and 37, whereafter animals were euthanised by decapitation, hippocampi removed, and stored at -80°C for neuro- and biochemical analyses.

Animals

Animals were bred, supplied and housed at the Vivarium (SAVC no. FR15/13458; AAALAC accreditation international file #1717) of the Pre-Clinical Drug Development Platform, NWU, RSA. The study consisted of four experimental groups (16 rats/group; 50:50 female: male; Fig. 1). Male and female rats were grouped together as sex differences are not expected in prepubertal animals. Still, results were visually inspected to identify any obvious sex differences. Group sizes were calculated with a predicted effect size F of 0.40 ($\eta_p^2 = 0.14$), α error of 5 and 80% power. Rats were housed 3–4 rats/cage, according to sex, with corncob bedding changed weekly and the environmental temperatures maintained at 22 ± 1 °C in a relative humidity of 55 ± 10%. A 12-h light/dark cycle was followed with food and water provided *ad libitum*.

Maternal separation and early weaning

The MSEW model was used as described previously (George *et al.*, 2010). Briefly, from PND02 to 16, pups in the MSEW groups were left in their home cages, while the dams were relocated to separate cages with *ad libitum* access to food and water for 3 h per day during their wake cycle. The non-MSEW animals were left undisturbed. MSEW animals were also weaned on PND17, as opposed to the standard PND21, when the non-MSEW animals were weaned.

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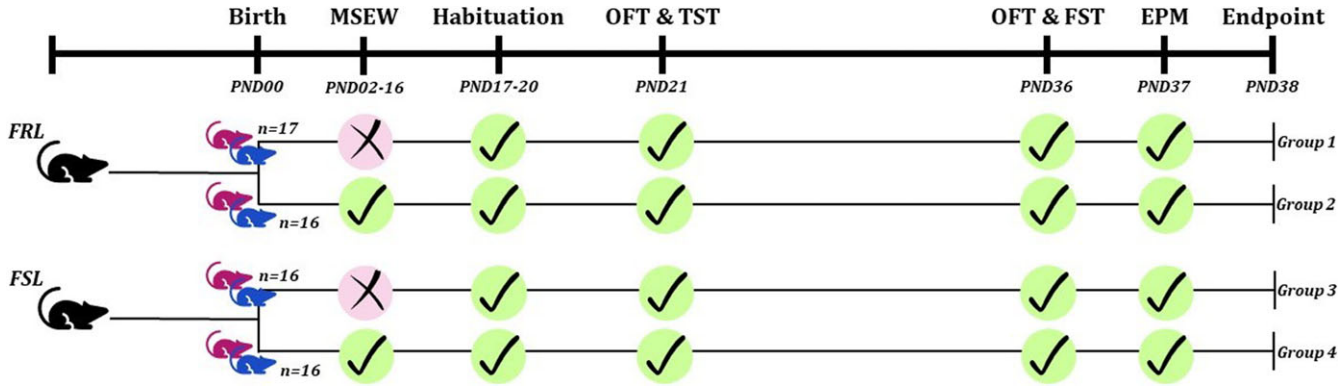

Figure 1. Graphical representation of the study layout. Pink rat icon: female rats. Blue rat icon: male rats. EPM: elevated plus maze. FRL: Flinders resistant Line. FSL: Flinders sensitive Line. FST: forced swim test. MSEW: maternal separation with early weaning. OFT: open field test. PND: postnatal day. TST: tail suspension test.

Behavioural analyses

On PND21, animals were subjected to the open field test (OFT) and tail suspension test (TST) to determine the immediate effects of MSEW on depressive-like behaviour. To investigate the long-term effects of MSEW, animals were subjected to the OFT and forced swim test (FST) on PND36, followed by the elevated plus maze (EPM) on PND37. The sequence of the behavioural analysis, specifically in terms of performing the FST before the EPM, was done to increase the chance of observing induced anxiety-like behaviour in FSL rats (Neumann *et al.*, 2011; Rea *et al.*, 2014; Bay-Richter *et al.*, 2019). To ensure normal initial foraging and activity of nocturnal animals, testing only commenced 1 h after the start of the dark cycle. Behavioural parameters of the OFT and EPM were analysed with automated software (Ethovision XT14; Noldus Information Technology BV, Wageningen, NLD), whereas that of the TST and FST were manually scored with a continuous timer (FST scoreboard 2.0, NWU, RSA) by a researcher blinded to the experimental groups.

Tail suspension test

The TST is used to identify antidepressant-like behaviour (Cryan, Mombereau, *et al.*, 2005). Although the test is best known for its use in mice, it has been successfully used in rats as well (Izumi *et al.*, 1997; Zhang *et al.*, 2013; Shinde *et al.*, 2015). The test was performed during the dark cycle under red light. As before (Castagné *et al.*, 2010; Cryan, Mombereau, *et al.*, 2005), on the day of testing, each rat was suspended by the tail with adhesive tape, positioned three-quarters of the distance from the base of the tail from a suspension hook for 6 min. To avoid injury, the suspension hook went through the adhesive tape as close as possible to the tail to ensure the animal hangs with its tail in a straight line (Castagné *et al.*, 2010). The total time spent immobile was recorded and interpreted as an indication of depressive-like behaviour.

Open field test

The OFT apparatus consisted of a 1 m^2 test arena, surrounded by opaque black, vertical walls. As previously described (Steyn *et al.*, 2020), each rat was placed in the centre of the arena and allowed to freely explore for 5 min under red light. Total distance moved during the session was interpreted as a measure of general activity.

Forced swim test

The FST is widely used to screen for depressive-like behaviour and antidepressant activity in rodents (Cryan *et al.*, 2002). The test was performed on PND36, as previously described in our laboratories. As the FSL rat is a validated animal model of depression that displays depressive-like behaviour (increased immobility), no pre-conditioning swim trial 24 h prior to the testing swim trial was conducted (Overstreet *et al.*, 2005; Overstreet & Wegener, 2013). During the dark cycle, under white light, animals were placed in an inescapable Perspex® cylinder [60 cm (*h*) × 24 cm (*ø*)], filled with 30 cm of water at a temperature of 25 ± 1°C for 6 min. During this time, behaviour was recorded by a camera mounted in front of the cylinders. The first minute of the test was discarded because animals generally engage in rapid bouts of active behaviour, during the first minute (Cryan, Mombereau, *et al.*, 2005). Behavioural analyses included immobility (floating with no active movements made, except those necessary to keep the rat's head above water), swimming (horizontal movements throughout the cylinder that included crossing into another quadrant) and struggling (upward-directed movements of the forepaws along the inside of the swim cylinder) (Cryan *et al.*, 2002; Cryan, Valentino, *et al.*, 2005). Increased immobility was considered an indication of learned helplessness or despair (depressive-like behaviour).

Elevated plus maze

The EPM is used to measure anxiety-like behaviour in rodents. The plus-shaped platform of the maze consisted of two closed [50 cm (*l*) × 20 cm (*w*) × 10 cm (*h*)] and two open arms [50 cm (*l*) × 20 cm (*w*) × 10 cm (*h*)], with a 1-cm transparent plexiglas border around the latter, to prevent animals from falling (Regenass *et al.*, 2018). The maze, constructed from black Perspex, is elevated 50 cm from the floor. On the day of testing, each rat was placed in the centre zone of the maze, facing the open arm opposite the investigator, and allowed to freely explore for 5 min under red light. Increased time spent in the closed arms was interpreted as anxiety-like behaviour. Entrance into an arm was deemed when the centre point, as defined by the automated scoring program, entered the arm zone.

Bio-analyses

Tissue collection and storage

On PND38, animals were euthanised by decapitation, whereafter brain and heart samples were harvested and weighed. Following the decapitation, right and left hippocampi were dissected on an ice-cooled dissection slab and stored separately. The right hippocampi were used for neurochemical analysis via LC–MS and snap-frozen in liquid nitrogen and stored at −80°C until the

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day of analysis and were analysed according to the methods described below. The left hippocampi were removed and immediately placed into an isolation buffer (mannitol 200 mM, sucrose 50 mM, potassium phosphate 5 mM, EGTA 1 mM, 3-(N-morpholino)propanesulfonic acid 5 mM and bovine serum albumin 0.10% pH 7.2) (Kim *et al.*, 2016), whereafter it was stored at −80°C until metabolic profiling was performed.

Quantitative analyses of hippocampal monoamines, GSH and GSSG

Each brain tissue sample was individually weighed prior to analyses. Hereafter, 250 µL of the internal standard solution was added to brain sample. The mixtures were homogenised by sonication (twice for 12 s, at an amplitude of 14 µ; MSE ultrasonic disintegrator, Nuaillé, FRA) and 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 High-Performance Liquid Chromatography (HPLC) sample vail. The analytical HPLC column used was a Venusil ASB C18 (purchased from Agela Technologies, Torrance, CA, USA), 2.1 × 150 mm, a particle size 3 µm. Ultivo Triple Quadrupole LC/MS System is controlled by the MassHunter software from Agilent Technologies©, Inc. (Santa Clara, CA 95051 US). A gradient mobile phase consisting of 0.1% formic acid/HPLC grade water and 0.1% formic acid/methanol were prepared. Serotonin turnover was expressed as the ratio between the metabolite, 5-hydroxindoleacetic acid (5-HIAA) and serotonin (5-HT) levels, while the redox state was calculated as glutathione (GSH) over glutathione disulphide (GSSG).

Metabolic profiling

Untargeted gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) was performed as previously described (Lindeque *et al.*, 2013; Terburgh *et al.*, 2019) on the left hippocampi of FSL rats. Of note, the storage buffer was used for the intended mitochondrial respiration assay, yet due to logistical reasons could not be performed and therefore influenced the metabolic results (i.e. reduced *n*-values).

A stepwise Bligh-Dyer extraction method (Wu *et al.*, 2008) was performed resulting in biphasic separation. Briefly, 400 µl of methanol, 75 µl of water and 50 µl of internal standard (50 ppm 3-phenylbutyric acid) were added to each hippocampus in safe-lock microcentrifuge tubes, together with a single steel bead (3 mm Ø). Thereafter, the brain 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 together with an additional 200 µl water was added to each sample and vortexed for 30 s. The samples were then centrifuged at 2000 × g for 5 min at 4°C to induce phase separation. Four hundred microlitre of the top polar phase and 300 µl of the bottom apolar phase of were transferred to glass vials and dried under nitrogen. The samples were stored at −80°C until derivatisation. 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 derivatised via oximation and silylation as previously performed (Lindeque *et al.*, 2013; Terburgh *et al.*, 2019). For oximation, 50 µl of 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 µl of 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 derivatisation. Each vial was then loaded onto an Agilent 7693 auto sampler for GC-TOF-MS analysis.

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 ionisation source. One microlitre of each 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 × 0.18 mm × 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, where after 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 for the next run. The transfer line and ion source temperature were kept constant at 225 and 200°C, respectively. The data were obtained at an acquisition rate of 20 spectra/s (50–800 m/z).

For data acquisition and extraction, the LECO Corporation ChromaTOF® software (version 4.5x) was utilised. Microsoft Office Excel was used for the pre-processing of the data. The NIST MS search program (version 0.2) using AMDIS (National Institute of Standards and Technology) was used to compare measured spectra to the NIST 11 mass spectral library to identify all the detected components and validate relevant metabolites. The sample sizes (indicated in Table 3) differ from that of the other analyses and may be due to the storage buffer used.

Statistical analyses

The Grubb's test ($\alpha = 0.05$) was performed to identify outliers with group sizes described in figure legends. Normality of distribution and homogeneity of variances were determined via the Shapiro–Whilk and Levene's tests, respectively, and only reported where these assumptions were violated. Two-way ANOVAs (analysis of variances) and ANCOVAs (analysis of covariances) were used for analyses, with the latter correcting for locomotor activity. Groupwise comparisons are reported as Bonferroni-adjusted values ($p < 0.05$ accepted as significant). Partial eta squared (η_p^2) and the unbiased Cohen's d (d_{unb}) values (Cumming, 2014) were used to calculate effect magnitude, with large effect sizes defined as $\eta_p^2 \geq 0.14$ (Ellis, 2010) and $d \geq 0.8$ (Sullivan & Feinn, 2012).

GC-MS data were pre-processed as before (Lindeque *et al.*, 2013) and normalised using the MSTUS normalisation method (Warrack *et al.*, 2009). The data were log-transformed prior to ANOVA analyses, to ensure normal distribution of all variables. Statistical analysis was performed in MetaboAnalyst (version 5; www.metaboanalyst.ca).

All other statistical analyses were performed in IBM® SPSS® Statistics (version 28), assisted by Laerd Statistics® (https://statisticslaerd.com) and the NWU statistical consultation services. Effect magnitude indicators were calculated in Exploratory Software for Confidence Intervals (Cumming, 2014). All graphical representations were created in GraphPad Prism® (version 9), with

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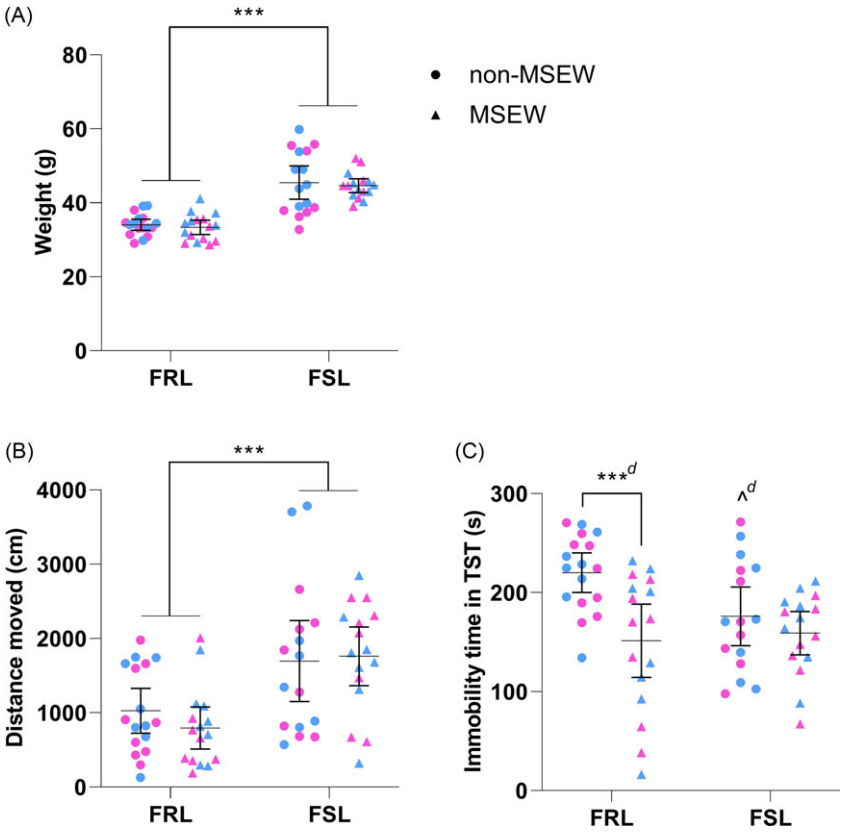

Figure 2. The immediate effects of maternal separation and early weaning on FRL and FSL rats on PND21. (A) Weight[a], (B) distance moved (over 5 min) in the OFT[a,b] and (C) time spent immobile in the TST[a] on PND21. Data points represent the mean ±95% CI, with male and female indicated in blue and pink, respectively. Statistical analyses are reported in the text with *** $p \leq 0.001$ vs. indicated group; ^ $p < 0.05$ vs. non-MSEW FRL rats, and $d \geq 0.8$ (significant large effect) vs. indicated group (or non-MSEW FRL when presented with no line). [a)] Heterogeneity of variances. [b)] Not all data sets were normally distributed. FRL: Flinders resistant line. FSL: Flinders sensitive line. MSEW: maternal separation and early weaning. TST: Tail suspension test.

the initial power analysis performed in G*Power (version 3; Universität Kiel, GER).

Results

Immediate effects of ELA

Body weight, distance moved in the OFT and immobility in the TST

Only strain affected body weight (Fig. 2A; $F_{1,61} = 80.06$, $p < 0.001$, $\eta_p^2 = 0.57$) and distance moved (Fig. 2B; $F_{1,61} = 19.63$, $p < 0.001$, $\eta_p^2 = 0.24$) on PND21 so that FSL rats (irrespective of ELA) weighed 11 g [9; 14 g] more and covered 817 cm [448; 1186 g] more than FRL controls. Although distance moved in the OFT did not affect time spent immobile in the TST (Table 1; $F_{1,60} = 0.16$, $p = 0.69$, $\eta_p^2 = 0.003$), the strain*ELA interaction ($F_{1,60} = 4.03$, $p = 0.049$, $\eta_p^2 = 0.06$) and ELA alone ($F_{1,60} = 10.83$, $p = 0.002$, $\eta_p^2 = 0.15$) influenced TST behaviour (Fig. 2C). After correcting for distance moved, non-MSEW FSL rats spent less time immobile that their non-MSEW FRL controls ($p = 0.036$, $d_{unb} = 0.9$ [0.2; 1.6]). Although ELA (regardless of strain) decreased TST immobility, this was only significant for FRL ($p < 0.001$, $d_{unb} = 1.2$ [0.5; 2.0]), and not FSL ($p = 0.37$, $d_{unb} = 0.3$ [−0.4; 1.0]) pups.

Delayed effects of ELA

Distance moved in the OFT and swimming behaviour in the FST

In Fig. 3A, the strain*ELA interaction ($F_{1,61} = 7.88$, $p = 0.007$, $\eta_p^2 = 0.11$) was significant for distanced moved on PND36. FSL rats (regardless of ELA) covered 870 cm [582; 1158 cm] more in the OFT than FRL controls ($p < 0.001$, $\eta_p^2 = 0.07$). However, MSEW only increased distance moved in the FSL ($p = 0.001$, $d_{unb} = 1.1$ [0.4; 1.9]) and not FRL ($p = 0.61$, $d_{unb} = 0.2$ [−0.9; 0.5]) rats.

FST behaviour was unaffected by distance moved in the OFT ($p > 0.05$ in all instances; Table 1). After correcting for distance moved, only immobility (Fig. 3B) was influenced by the strain*ELA interaction ($F_{1,60} = 4.73$, $p = 0.03$, $\eta_p^2 = 0.07$). Of note, both strain ($F_{1,60} = 40.76$, $p < 0.001$, $\eta_p^2 = 0.41$) and ELA ($F_{1,60} = 25.36$, $p < 0.001$, $\eta_p^2 = 0.30$) independently also influenced immobility time so that FSL rats (regardless of ELA) were 88 s [60; 115 s] more immobile than FRL controls and that MSEW (regardless of strain) increased time spent immobile by 57 s [34; 79 s]. Interestingly, statistical significance between non-MSEW and MSEW was only reached in the FRL rats ($p < 0.001$, $d_{unb} = 1.5$ [0.7; 2.3]), while a similar trend existed in the FSL rats ($p = 0.07$, $d_{unb} = 1.0$ [0.2; 1.7]).

Time spent swimming and struggling was independently influenced by both strain (Fig. 3C; $F_{1,60} = 12.90$, $p < 0.001$, $\eta_p^2 = 0.18$ and Fig. 3D; $F_{1,60} = 19.39$, $p < 0.001$, $\eta_p^2 = 0.24$) and ELA (Fig. 3C; $F_{1,60} = 13.07$, $p < 0.001$, $\eta_p^2 = 0.18$ and Fig. 3D; $F_{1,60} = 9.31$, $p = 0.003$, $\eta_p^2 = 0.13$). After correcting for distance moved, FSL rats (regardless of ELA) swam 26 s [11; 40 s] less than FRL controls as did MSEW animals in relation to non-MSEW controls (Fig. 3C). For time spent struggling (Fig. 3D), FSL rats (regardless of ELA) struggled 62 s [34; 90 s] less than FRL controls, while MSEW animals struggled 35 s [12; 58 s] less than non-MSEW controls. Interestingly, this effect was only significant in FRL ($p < 0.001$, $d_{unb} = 1.0$ [0.3; 1.8]) and not FSL ($p = 0.49$, $d_{unb} = 0.3$ [−0.4; 1.0]) rats.

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Table 1. Original and ANCOVA-adjusted immobile times in the TST and FST

Behavioural parameters in tail suspension and forced swim tests					
		Immobility in the TST (s)		Immobility in the FST (s)	
Strain	MSEW	Original ± *SD (n)*	Adjusted ±*SEM (n)*	Original ± *SD (n)*	Adjusted ±*SEM (n)*
FRL	***no***	220.2 ± 38.9 *(17)*	219.2 ± 13.1 *(17)*	103.8 ± 65.1 *(17)*	102.0 ± 11.2 *(17)*
	yes	151.4 ± 69.4 *(16)*	149.5 ± 14.0 *(16)*	185.8 ± 38.7 *(16)*	183.6 ± 11.9 *(16)*
FSL	***no***	176.1 ± 55.6 *(16)*	177.5 ± 13.7 *(16)*	214.2 ± 33.4 *(16)*	214.6 ± 11.0 *(16)*
	yes	159.1 ± 41.1 *(16)*	160.7 ± 13.8 *(16)*	242.4 ± 23.1 *(16)*	246.1 ± 13.3 *(16)*
		Swimming in the FST (s)		**Struggling in the FST (s)**	
FRL	***no***	87.3 ± 35.1 *(17)*	87.1 ± 5. 9 *(17)*	108. 5 ± 68.7 *(17)*	110.5 ± 11.4 *(17)*
	yes	64.3 ± 11.5 *(16)*	64.0 ± 6.2 *(16)*	50.0 ± 40.2 *(16)*	52.6 ± 12.1 *(16)*
FSL	***no***	59.4 ± 20.4 *(16)*	59.5 ± 5.8 *(16)*	26.4 ± 33.2 *(16)*	25.9 ± 11.2 *(16)*
	yes	39.5 ± 15.7 *(16)*	39.9 ± 7.0 *(16)*	18.3 ± 15.8 *(16)*	13.9 ± 13.6 *(16)*

Values adjusted with the mean distance moved in the OFT on PND21 (for TST) and OFT on PND36 (for FST). All statistical findings are reported in text, with these values presented here for transparency. FRL: Flinders resistant line; FSL: Flinders sensitive line; FST: forced swim test; MSEW: maternal separation and early weaning; TST: tail suspension test.

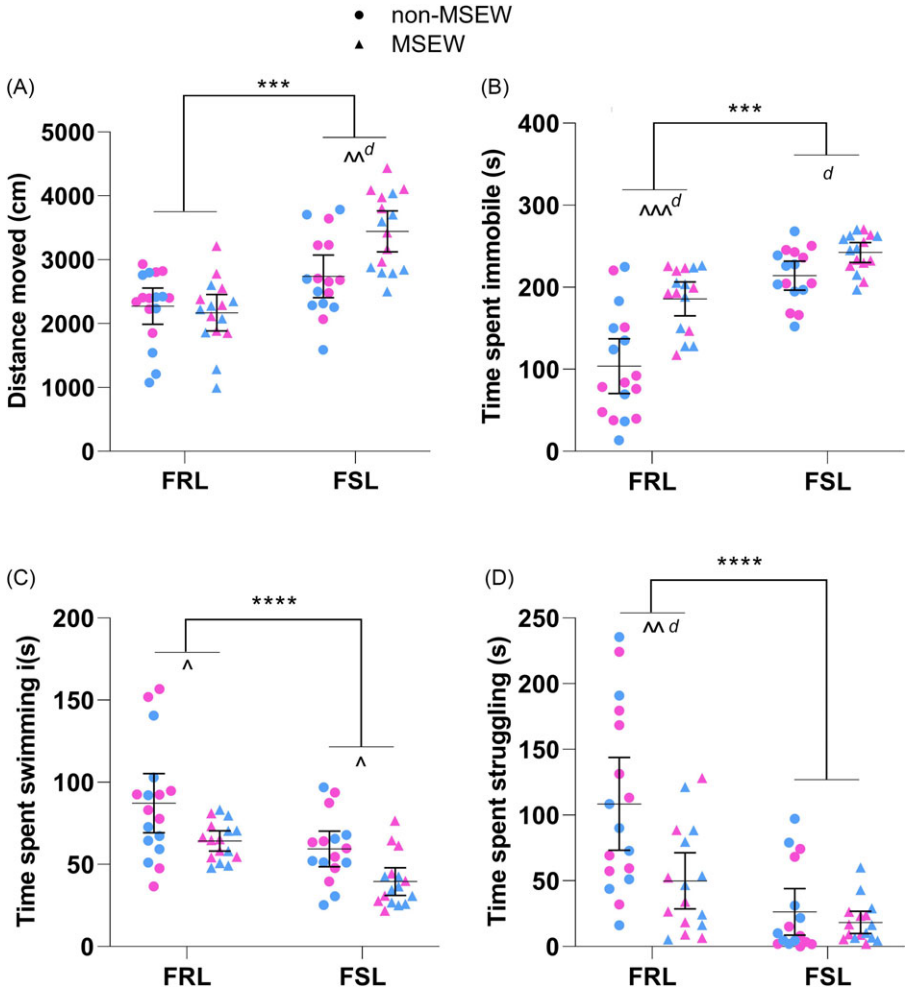

Figure 3. The delayed effects of maternal separation and early weaning. (A) Distance moved in the OFT[a]. (B) Time spent immobile[a,b], (C) swimming[a,b] and (D) struggling [a,b,c] in the FST. Data points represent the mean±95% CI, with male and female indicated in blue and pink, respectively. Statistical analyses are reported in the text with *** $p \leq 0.001$ vs. indicated group; ^ $p < 0.05$, ^^ $p \leq 0.05$ and ^^^ $p \leq 0.001$ vs. indicated group, and d ≥ 0.8 (significant large effect). [a)] Not all data sets were normally distributed. [b)] Heterogeneity of variances. [c)] Outlier identified but not excluded. FRL: Flinders resistant line. FSL: Flinders sensitive line. FST: forced swim test. MSEW: maternal separation and early weaning.

Anxiety-like behaviour in the EPM

There was no significant interaction ($F_{1,61} = 0.01$, $p = 0.91$, $\eta_p^2 \leq 0.0005$) or any main effects identified for the percentage time spent in open arms of the EPM (*data not shown*).

Brain and heart weight

Brain weight (Fig. 4A) was influenced by strain ($F_{1,60} = 78.07$, $p < 0.001$, $\eta_p^2 = 0.57$) and ELA ($F_{1,60} = 13.41$, $p < 0.001$, $\eta_p^2 = 0.18$) independently so that the brain weight of FSL rats (regardless of

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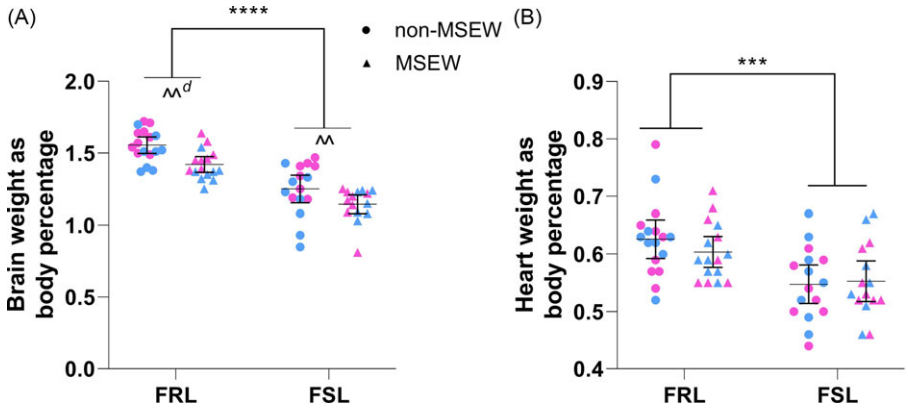

Figure 4. The effects of maternal separation and early weaning on anatomical markers. (A) Brain[a] and (B) heart weight of FSL and FRL rats, expressed as a percentage of body weight. Data points represent the mean±95% CI, with male and female indicated in blue and pink, respectively. Statistical analyses are reported in the text with *** $p \leq 0.001$ vs. indicated group; ^^ $p \leq 0.01$ vs. indicated group and $d \geq 0.8$ (significant large effect). [a)] Outlier identified but not excluded. FRL: Flinders resistant line. FSL: Flinders sensitive line.

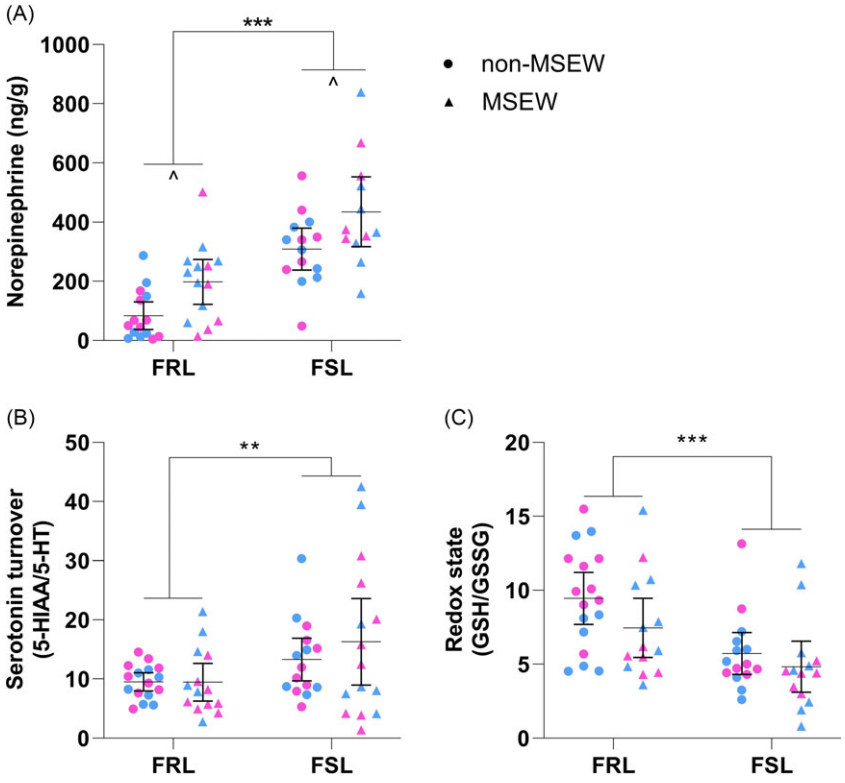

Figure 5. Hippocampal monoamine levels and redox state markers. (A) Norepinephrine levels[a,c], (B) serotonin turnover[b,c] and (C) redox state[a,c] (GSH/GSSG) of FRL and FSL rats on PND38. Data points represent the mean±95% CI, with male and female indicated in blue and pink, respectively. Statistical analyses are reported in the text with ** $p \leq 0.01$, *** $p \leq 0.001$ vs. indicated group; ^ $p < 0.05$ vs. indicated group, and $d \geq 0.8$ (significant large effect). [a)] Not all data sets were normally distributed. [b)] Heterogeneity of variances. [c)] Outliers identified and excluded. FRL: Flinders resistant line. FSL: Flinders sensitive line. MSEW: maternal separation and early weaning.

ELA) was 0.29% [0.2; 0.4%] lower than FRL controls. Similarly, the brain weight of MSEW animals (regardless of strain) weighed 0.12% [0.5; 0.2%] less than non-MSEW counterparts. Heart weight (Fig. 4B), however, was influenced only by strain ($F_{1,60} = 18.09$, $p < 0.001$, $\eta_p^2 = 0.23$) so that those of FSL rats (regardless of ELA) weighed 0.07% [0.03; 0.1%] less than that of FRL controls.

Hippocampal monoamine levels, metabolic markers and redox state

In Fig. 5A, significant main effects of strain ($F_{1,51} = 41.37$, $p < 0.001$, $\eta_p^2 = 0.45$) and ELA ($F_{1,51} = 11.12$, $p = 0.002$, $\eta_p^2 = 0.18$) were identified for hippocampal norepinephrine levels. Regardless of ELA, FSL rats had 231 ng/g [159; 303 ng/g] more hippocampal norepinephrine than FRL controls. Similarly, hippocampal norepinephrine levels of MSEW animals (regardless of strain) were 120 ng/g [48; 192 ng/g] higher than that of their non-MSEW counterparts.

Hippocampal serotonin turnover (5-HIAA/5-HT) and redox state (GSH/GSSG) (Table 2) were only influenced by strain (Fig. 5B; $F_{1,56} = 6.63$, $p = 0.01$, $\eta_p^2 = 0.11$ and Fig. 5C; $F_{1,56} = 15.25$, $p < 0.001$, $\eta_p^2 = 0.21$) so that FSL rats (regardless of ELA) had higher serotonin turnover and lower GSH/GSSG values than FRL controls.

Of the metabolic markers detected (palmitic acid, stearic acid, oleic acid, 1-monopalmitin, 1-monostearin and nicotinic acid), only 1-monopalmitin acid and nicotinic acid were significantly influenced by the investigated factors (Table 3). For 1-monopalmitin, there was a significant strain*ELA interaction ($F_{1,32} = 4.33$, $p = 0.046$, $\eta_p^2 = 0.12$) so that MSEW decreased hippocampal levels only in FSL rats relative to non-MSEW strain controls ($p = 0.002$; $d_{unb} = 1.6$ [0.5; 2.9]). Secondly, hippocampal

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Table 2. Hippocampal serotonin levels and redox state markers

		Serotonin turnover parameters		Redox state parameters	
Strain	MSEW	5-HT [*ng/g*] *mean ± SD (n)*	5-HIAA [*ng/g*] *mean ± SD (n)*	GSH [*μg/g*] *mean ± SD (n)*	GSSG [*μg/g*] *mean ± SD (n)*
FRL	***no***	25.2 ± 9.4 (*16*)	226.2 ± 73.5 (*16*)	51.7 ± 7.3 (*17*)	6.2 ± 2.2 (*17*)
	yes	27.0 ± 10.7 (*14*)	226.7 ± 107.2 (*14*)	47.8 ± 11.4 (*14*)	7.5 ± 3.7 (*14*)
FSL	***no***	19.8 ± 7.9 (*15*)	242.5 ± 101.3 (*15*)	42.4 ± 7.3 (*15*)	8.4 ± 2.8 (*15*)
	yes	19.0 ± 7.5 (*15*)	288.1 ± 262.3 (*15*)	41.8 ± 21.8 (*14*)	9.7 ± 4.4 (*14*)

Because of the significant influence of main effects, all statistical findings are reported in text. 5-HT: 5-hydroxytryptamine (serotonin); 5-HIAA: 5-hydroxyindoleacetic acid; FRL: Flinders resistant line; FSL: Flinders sensitive line; GSH: glutathione; GSSG: glutathione disulphide; MSEW: maternal separation and early weaning.

Table 3. Significant hippocampal metabolomic markers

Strain	MSEW	1-monopalmitin *mean ± SD (n)*	Nicotinic acid *mean ± SD (n)*
FRL	***no***	0.35 ± 0.16 (*10*)	5.92 ± 4.02 (*6*)
	yes	0.32 ± 0.14 (*7*)	29.86 ± 27.04 (*5*)
FSL	***no***	0.49 ± 0.15 (*5*)	58.09 ± 54.06 (*3*)
	yes	0.22 ± 0.16 (*14*)	46.99 ± 41.44 (*13*)

The values presented here are all log-transformed and therefore contain no SI unit. Group sizes differ from behavioural analyses and could be explained by the storage buffer used. Because of the significant influence of main effects, all statistical findings are reported in text. **FRL:** Flinders resistant line. **FSL:** Flinders sensitive line. **MSEW:** maternal separation and early weaning.

nicotinic levels were only affected by strain ($F_{1,23} = 4.83$, $p = 0.038$, $\eta_p^2 = 0.17$) so that FSL rats (regardless of ELA) had 98% higher levels than FRL controls.

Discussion

Juvenile strain differences

The FSL rat is a validated rodent model of depression (Overstreet & Wegener, 2013), yet bio-behavioural data during the juvenile developmental period is limited. Based on the findings of Malkesman and Weller (2009), the FSL rat can be used to model childhood depression, as it already displays its characteristic depressive-like phenotype between PND31 and 40 (Malkesman, Braw, *et al.*, 2006), despite having comparable monoaminergic levels across different brain regions at PND34 (Malkesman *et al.*, 2007; Malkesman *et al.*, 2008). Importantly, these findings were in reference to Sprague-Dawley rats and not FRL controls and were also measured during the pubertal onset period (i.e. PND35-45) (Murrin *et al.*, 2007; Drzewiecki *et al.*, 2020). Here, we screened behaviour in these animals at weaning age (i.e. prepubertal), which is useful to further validate this model as an appropriate rodent model for childhood depression. At PND21, FSL rats (regardless of sex) were heavier, displayed hyperlocomotor activity in the OFT and spent less time immobile in the TST (Fig. 2). These findings would therefore suggest that prepubertal male and female FSL rats do not display increased 'behavioural despair' or depressive-like behaviour, as measured in the TST on PND21. To the best of our knowledge, the TST has not yet been used to investigate the depressive-like phenotype of the FSL rat, and our findings therefore require confirmation, especially because body weight has been suggested to influence mobility (Bogdanova *et al.*, 2013). This may be especially relevant here, as the larger body weight of the FSL rats on PND21 could have allowed for greater physical mobility in the TST (Fig. 2C), even after correcting for locomotor differences. Importantly, others have noted that the neuronal mechanisms involved in the TST differ from those in the FST (Renard *et al.*, 2003). Adrenergic neurotransmission has been found to influence TST behaviour more than the escape-directed behaviour in the FST (Hascoët *et al.*, 1991). In this regard, we observed increased hippocampal norepinephrine levels in the FSL rats on PND38 (Fig. 5A) that may explain the decreased immobility in the TST relative to age-matched FRL controls (Fig. 2C). Importantly, that we did not measure monoamine levels at PND21 is a limitation that must be confirmed in prospective studies. Interestingly, 'irritable mood' and 'psychomotor agitation' are key diagnostic symptoms of juvenile depression (American Psychiatric Association, 2013) that may have presented here as increased locomotor activity in the OFT and TST on PND21. In fact, Mitchell *et al.* (2013) reported PND21 mice to be more mobile in the TST than PND28 or adult (PND62-90) controls. Still, this early-life hyperlocomotor activity was also present on PND36 (Fig. 3A) and therefore aligns our findings with that of others (George *et al.*, 2010) and motivates for further investigation into the proposed psychomotor agitation-like behaviour. Importantly, this increased locomotor activity is not likely to be indicative of anxiety-like behaviour as no strain differences were observed for time spent in the open arms of the EPM, even following the FST. This is in line with the literature, describing the FSL rat as 'a model of depression without comorbid anxiety' (Overstreet & Wegener, 2013), even at a juvenile age (Malkesman & Weller, 2009). Still, on PND36, FSL rats displayed increased depressive-like (i.e. time spent immobile; Fig. 3B) and decreased coping (i.e. swimming and struggling) behaviours (Figs. 3C and D) in the FST. Considered together, although the TST data may, on face value, be interpreted as representing antidepressive-like behaviour, it could in fact represent a psychomotor agitation phenotype that warrants further investigation. In contrast to this, the depressive-like behaviour observed in the FST (Fig. 3) may accurately represent behavioural despair at a young (pubertal onset) age. Although no significant sex differences were observed in the current study, it is important to note that pubertal onset in rats takes place between PND35 (females) and PND43 (males) (Murrin *et al.*, 2007; Drzewiecki *et al.*, 2020) and may therefore have influenced the outcome of the behavioural tests (Goodwill *et al.*, 2019) and therefore warrants further investigation.

Still, to corroborate these behavioural findings, we measured hippocampal neurochemical markers because of the region's recognised role in depression (Campbell & MacQueen, 2004). In line with clinical findings (Barton *et al.*, 2008; Zhuo *et al.*, 2017),

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juvenile FSL rats, regardless of sex or ELA, had lower brain weights (Fig. 4A) and increased hippocampal serotonin turnover (Fig. 5B) and an altered redox state (Fig. 5C). The GSH/GSSG ratio is a valuable biomarker of cellular redox state (Enns & Cowan, 2017), with lower levels indicating increased oxidative stress (Chai *et al.*, 1994). GSH is a highly abundant and potent intra- and extracellular antioxidant (Zitka *et al.*, 2012) that when oxidised to GSSG reduces antioxidant protection. Therefore, that the redox state in juvenile FSL rats appears dysfunctional suggests increased oxidative stress damage, specifically in the hippocampus that could have contributed to the observed brain atrophy (Fig. 4A). This finding supports increasing evidence suggesting depression to be a bio-energetic disorder, resulting from increased free radical production and an altered redox state, caused by dysfunctional mitochondria. To this end, clinical and preclinical evidence suggests mitochondrial function and numbers to be adversely affected by early-life stress (Hoffmann & Spengler, 2018; Ridout *et al.*, 2018; Zitkovsky *et al.*, 2021). Here, we unfortunately did not analyse any direct markers of mitochondrial function, such as respiration, yet still identified strain differences in terms of hippocampal nicotinic acid concentrations (Table 3). Nicotinic acid is a precursor for the mitochondrial electron carrier NAD^+ that contributes to energy production and the redox state of the mitochondria (Crowley *et al.*, 2000). That FSL rats (irrespective of sex and ELA) had significantly higher nicotinic levels may point towards a dysfunctional bio-energetic system in that FSL rats may have impaired nicotinic acid to NAD^+ conversion pathways. This is at least somewhat supported by available literature reporting hippocampal synapse and mitochondria numbers of the FSL rat to be reduced (Chen *et al.*, 2018).

Next, that we observed increased serotonin turnover in FSL rats mirrors clinical findings (Barton *et al.*, 2008) and further strengthens the validity of the FSL rat as a model for paediatric depression. Despite the limited neurochemical data on depressed children, Dahlström *et al.* (2000) reported elevated serotonin transport activity in depressed juveniles (aged 7–17 years), proposing a compensatory mechanism for the hyposerotonergic state to be at play. That similar findings were observed in adult FSL rat (Hvilsom *et al.*, 2019; Harvey *et al.*, 2021) supports this idea and our current findings. As for norepinephrine levels, although others have reported comparable (Harvey *et al.*, 2021) and reduced (Brand & Harvey, 2017) hippocampal levels in adult FSL rats, our findings (Fig. 5A) are supported by others (Zangen *et al.*, 1999; Roets *et al.*, 2023) and could be explained by lower α_2 receptor expression in the FSL rat (Landau *et al.*, 2015). Finally, a dysfunctional adrenergic system is implicated in depression and associated with comorbid impaired cardiovascular function (Alvares *et al.*, 2016). In this regard, it is interesting that juvenile FSL rats (regardless of sex or ELA) also had lower heart weights than their FRL controls (Fig. 4B), as the cardiovascular system is strongly regulated by the sympathetic nervous system, which has been shown to be impaired or dysfunctional in depressed patients (Määttänen *et al.*, 2019). These results, however, invite confirmation.

The effect of an early-life stressor on bio-behavioural parameters

Because ELA is a significant contributor to the development of mood disorders later in life, we also investigated the effects of MSEW in the Flinders line rats to determine whether genetic susceptibility would exacerbate the effects of ELA on depressive-like behaviour. Here, MSEW did not affect the weight of either strain on PND21 (Fig. 2A), which is similar to the findings of George et al. (2010). It should also be noted here that to the best of our knowledge, MSEW has not been investigated in the offspring FSL rat strain. Interestingly, we recently reported that MSEW unexpectedly induced antidepressant-like effects in the FSL postpartum dam (Roets *et al.*, 2023). As for the offspring, MSEW in the current study did not affect the locomotor activity (Fig. 2B) of PND21 pups yet had an overall decreasing effect on time spent immobile in the TST (Fig. 2C). Surprisingly, this effect was more significant in the FRL rats. Although the exact reason for this is unknown, it may involve the animal's response to a stressor. In this regard, it is worth noting that prolonged interference with maternal–offspring relationship is reported to induce a hypothalamic–pituitary–adrenal (HPA) axis hyper-responsiveness, evident by increased stress hormones (Pfeffer *et al.*, 2007). The corticosterone response of early weaned C57BL/6 mice was prolonged in animals maternally separated for 3 h/day, whilst a blunted response was observed in animals separated for only 10 min/day (Parfitt *et al.*, 2004; Kikusui *et al.*, 2006). This hypothesis is further supported by the findings of Malkesman, Maayan, *et al.* (2006) who concluded that the FSL rat may present with chronic HPA axis up-regulation following chronic stress, which may not be the case for the FRL strain. These reports, together with the increased mobility observed here, underlines the importance of investigating the corticosterone response of both strains at PND21.

Although the immediate effects of MSEW did not affect locomotor activity on PND21, our findings show that MSEW had a delayed effect on locomotor activity on PND36 (Fig. 3A), with a more prominent effect in FSL rats. Similar increased locomotor activity following maternal separation has been observed by others (George *et al.*, 2010; Wang *et al.*, 2015; Jin *et al.*, 2018) and suggest that MSEW may result in or even worsen psychomotor agitation-like behaviour (Grønli *et al.*, 2005; Linge *et al.*, 2013). Specifically, Sanders and Anticevic (2007) reported similar findings in Wistar-Kyoto rats, following maternal separation from PND01-14, and noted that these pups also exhibited tachycardia during the stress period. This, together with the different norepinephrine levels reported here (Fig. 5A), again highlights the need for further investigation into the stress response and psychomotor agitation-like behaviour of these animals following an early-life stressor. That anxiety-like behaviour was unaffected by MSEW strengthens our hypothesis that the mentioned behaviour might be indicative of a psychomotor agitation-like behaviour rather than anxiety-like behaviour. After correcting for distance moved in the OFT, MSEW (regardless of strain) increased time spent immobile in the FST (Fig. 3B), thereby enforcing the hypothesis that ELA can result in depressive-like behaviour – even in non-susceptible individuals. It must however be highlighted that time spent immobile and struggling were more prominently affected in the FRL rats (Fig. 3B and D), suggesting the need to further explore the adverse effects of ELA in a population without a genetic susceptibility for depression. That only time spent swimming was adversely affected by MSEW (Fig. 3C) suggests that the depressive-like phenotype of the FSL rat may not be exaggerated by MSEW. Still, whether it affected their response to antidepressant treatment (i.e. treatment resistant depression) remains unknown and is worth investigating. Still, our findings are in line with clinical reports, showing ELA to have negative neurodevelopmental implications (Bick & Nelson, 2016). MSEW did however not influence cardiac weight in either strain, but further studies should investigate whether this (and other cardiovascular functions) is also the case at a later age, as maternal

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separation has the potential to influence autonomic outflow (Card *et al*., 2005), which influences the cardiovascular system. To this point, childhood adversity is also strongly linked to increased risk for cardiovascular diseases, probably via an inflammatory-mediated mechanism (Obi *et al*., 2019). Regardless, that MSEW increased hippocampal norepinephrine levels in both FSL and FRL rats points to an activated stress response and supports the notion of MSEW indeed being a stressor. That MSEW appeared to induce a more robust behavioural effect in FRL rats may again highlight different stress responses at play, which is in line with the clinical setting (Zorn *et al*., 2017). That hippocampal serotonin turnover and redox state (Fig. 5B and C) was unaffected by MSEW could point towards a different mechanism through which ELA induces depressive-like behaviour and should be explored in future studies. To this end, MSEW did cause a significant reduction in 1-monopalmitin hippocampal levels in FSL rats (Table 3). 1-Monopalmitin is a lipid responsible for amongst others, storing energy and acting as a structural component of cell membranes (Qin *et al*., 2021). Consequently, that MSEW induced a reduction in hippocampal 1-monopalmitin of FSL rats (Table 3) could suggest a negative effect on mitochondrial function and is strongly supported by the robust preoteomics analysis of Mallei *et al.* (2015) on the Flinders strain and recent findings linking stress-related pathways, stress reactivity and the effects on mitochondrial function (Rappeneau *et al*., 2023). Nevertheless, it is worth noting that despite their not being any statistical strain differences in hippocampal 1-monopalmitin levels, O'Gorman *et al.* (2017) reported plasma 1-monopalmitin levels to be reduced in children and adolescents suffering from psychotic disorders. It must however be noted here that prospective studies should measure mitochondrial function to confirm our findings, specifically because the samples collected here were kept in a storage buffer used for respiration analyses (i.e. Seahorse assay). Taken together, the bio-behavioural effects caused by MSEW hints at a central role that the adrenergic nervous system, and perhaps mitochondrial function, may play in the long-term effects of early-life insults.

Conclusion

Our findings firstly support available literature, motivating the FSL rat as a suitable rodent model for juvenile depression, more specifically adolescent depression as it presents with altered early-life locomotor activity and increased depressive-like behaviour on PND35. These behavioural differences may be due to increased hippocampal norepinephrine levels and serotonin turnover, and a dysfunctional redox state, possibly caused by mitochondrial dysfunction. Next, our findings show that an early-life stressor, in the form of MSEW, is depressogenic and appears to be more prominent in the FRL rat. That MSEW also increased hippocampal norepinephrine levels and reduced rather than increased early-life immobility in the TST may point towards a central role of the stress response pathway in the behavioural alterations caused by early-life stress. Overall, our findings confirm that ELA can induce neuropsychiatric alterations but may follow a different course or mechanism in the presence or absence of genetic susceptibility.

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Author contribution. SFS conceptualised, funded and designed the layout of the study. AJW performed all the experimental work and analysed and interpreted the data with SFS. AJW and SFS also wrote the original and amended versions of the manuscript, with ZL and RK collating and finalising the paper for submission and funded the metabolic analyses (ZL). All authors contributed to the various sections of this paper.

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Competing interests. None.

Animal welfare. All animal procedures were approved by the Animal Care, Health and Safety Research Ethics Committee of the North-West University (NWU-AnimCareREC; ethics approval number: NWU-00419-21-A5) and in accordance with the relevant code of ethics. All procedures complied with national legislation that pertains to experimental animal welfare (including the Department of Health's Ethics in Health Research: Principles, Processes and Structures and the South African National Standard: The Care and Use of Animals for Scientific Purposes (SANS 10,386:2008)). This work also complied with the ARRIVE guidelines ensuring that all experimental data are reproducible, transparent, accurate comprehensive and logically ordered to promote well-written manuscripts.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the South African National Standards and institutional guides on the care and use of laboratory animals.

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