Prenatal Ursolic acid Programmes Durable Epigenetic, Neuroendocrine and Neuroinflammatory Resilience against Sequential Early-life and Adult Glucocorticoid Stress in Rats

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Abstract

Background: Early-life adversity programmes lifelong vulnerability to stress-related disease, and that vulnerability is compounded when adversity is followed by further challenge in adulthood. Interventions capable of conferring durable resilience remain scarce. We asked whether prenatal ursolic acid, a dietary pentacyclic triterpenoid, protects the rat prefrontal cortex against sequential early-life and adult stress. **Methodology**: Pregnant Wistar dams received ursolic acid (10 mg kg^{-1} per day, orally) or vehicle throughout gestation. Male offspring were divided into four groups (n = 12) combining prenatal treatment with maternal separation on postnatal days 2–14. Prefrontal DNMT3a and H2AX transcription was quantified by reverse-transcription PCR, and serum corticosterone and prefrontal interleukin-1β, Nrf2 and brain-derived neurotrophic factor by ELISA. Six offspring from each group then matured to postnatal day 42, when the maternally separated groups received dexamethasone (0.5 mg kg^{-1} per day for seven days) as a second challenge. Behaviour was assessed by forced swim, novel object recognition and elevated plus maze; structure by Golgi impregnation and haematoxylin and eosin staining. **Results**: Prenatal ursolic acid approximately doubled DNMT3a and H2AX transcription. Maternal separation raised corticosterone by 50%, more than doubled interleukin-1β and reduced Nrf2 by two-thirds; prenatal exposure abolished each effect. Following the adult challenge, prenatally exposed offspring maintained control-level corticosterone, interleukin-1β, Nrf2 and brain-derived neurotrophic factor, preserved dendritic arborisation, spine density and neuronal cytoarchitecture, and showed unimpaired performance across all three behavioural paradigms. **Conclusion**: Prenatal ursolic acid programmes durable, multilevel resilience that withstands sequential early-life and adult stress, identifying prenatal nutritional programming as a tractable route to lifelong stress resilience.

Keywords: DNMT3a; H2AX; Nrf2; brain-derived neurotrophic factor; maternal separation.

Introduction

The architecture of the mammalian brain is laid down over a protracted developmental window in which proliferation, migration, differentiation, synaptogenesis and myelination proceed in a tightly ordered sequence (Fenlon, 2022). Because this sequence is experience-sensitive, conditions encountered before and shortly after birth do not merely influence contemporaneous physiology; they set enduring trajectories. Early-life adversity in rodents produces a reproducible and long-lasting phenotype comprising hyperactivity of the hypothalamic–pituitary–adrenal (HPA) axis, elevated pro-inflammatory signalling, depressed neurotrophic support, and behavioural profiles resembling anxiety and depression; these

consequences persist long after the stressor is withdrawn (Speranza et al., 2024). Therefore, the central translational problem is understanding how such programming might be redirected rather than simply described.

Vulnerability programmed early is seldom expressed in isolation. The clinically relevant situation is that of a person sensitised by early adversity who then experiences a further stressor, and the two burdens summate (Bartholomeusz, 2019; Hanson and Nacewicz,, 2021). Experimental designs that impose a second challenge on animals that have already experienced early-life stress thus more faithfully model the human condition than either insult alone, and provide a more stringent test of any protective intervention (Ngala, 2025; Chen et al., 2026).

The problem is worst in the frontal cortex. It is responsible for working memory, executive function, and top-down emotion regulation. The latter is the last of cortical regions to mature and has a period of vulnerability that extends well beyond that of subcortical structures (Abayomi et al., 2022; Rowe et al., 2024). It also has copious amounts of glucocorticoid receptors, making it a direct target for the very hormones that are mobilised by stress. Chronic glucocorticoid excess induces dendritic atrophy, spine loss and depletion of brain-derived neurotrophic factor (BDNF) in this region, and the reciprocal relationship between glucocorticoid signalling and BDNF is now recognised as a principal axis through which stress remodels cortical circuitry (Tsimpolis et al., 2024; Takahashi et al., 2025).

Two molecular systems mediate much of the damage. The first is redox–inflammatory: nuclear factor erythroid 2-related factor 2 (Nrf2) is the master transcriptional regulator of cytoprotective and antioxidant genes in the brain, operating across neurons, astrocytes, microglia and oligodendrocytes, and its activity is inversely coupled to nuclear factor-κB-driven inflammatory transcription (Navarro and Esteras, 2024; Heurtaux et al., 2022). Depressive phenotypes across animal and human studies are consistently accompanied by attenuation of this pathway (Simonetti et al., 2023).

The second system is epigenetics. Developmental programming requires some mechanism to translate a transient exposure into a stable, cell-heritable change in transcriptional set-point and DNA methylation provides one (Molnár et al. 2020; You et al., 2025). The de novo methyltransferase DNMT3a establishes methylation patterns throughout cortical development and the histone variant H2AX maintains chromatin and genomic stability during the intense proliferative activity of the developing brain. Hence, any candidate intervention that purports to programme durable resilience should be demonstrated to engage this machinery, rather than merely to produce a contemporaneous pharmacological effect (Legoff et al., 2019; Merighi et al., 2021). Ursolic acid is a good candidate on mechanistic grounds. It is a pentacyclic triterpenoid found in abundance in apple peel and many culinary and medicinal plants, and has been shown in a systematic review and meta-analysis of animal and in vitro studies to reduce inflammatory cytokine concentrations and increase antioxidant enzyme activity (Zhao et al., 2023). The antioxidant activity of the extract is mainly mediated by Nrf2 pathway upregulation. Prenatal administration of the lipophilic, orally active, dietary component is a realistic rather than purely experimental proposition because of the cheap nature of the compound. What has not been shown is whether prenatal exposure can establish a protective phenotype that persists into adulthood and is available when a second stressor occurs weeks later.

The study was designed as a three-phase study. In the first phase of the study, we asked whether the prenatal ursolic acid interacts with the epigenetic machinery of the developing prefrontal cortex by quantifying the DNMT3a and H2AX transcription. The second phase of the study asked whether it protects against a proximate stressor. The study used maternal separation and measured HPA activation, IL-1β

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and Nrf2. The third and most important test involved exposing some of the same animals to dexamethasone in adulthood, to assess protection against the cumulative burden of two sequential stressors separated by a developmental interval.

Materials and Methods

Experimental Animals

Twenty-eight (28) adult female and fourteen (14) adult male Wistar rats (approximately 160 g) were obtained from Osun State University Animal Breeding Facility and housed at 22 ± 2 °C, 50–60% relative humidity, under a 12-h light/dark cycle with standard chow and water *ad libitum*. Animals were acclimatised before the experiment began. All procedures were approved by the Institutional Animal Ethics Committee and conducted under the principles of replacement, reduction and refinement, with reporting in conformity with the ARRIVE 2.0 guidelines.

All offspring studied were male to avoid confounding by the oestrous cycle on stress and neuroendocrine measures; female offspring were not analysed. Nulliparous dams were of similar age (~10–12 weeks) and weight (160 ± 10 g) at mating and were randomly assigned to prenatal treatment. The dams (control and ursolic acid) were kept under the same temperature, humidity, lighting, and dietary conditions throughout gestation, handled on the same schedule, and received the same volume of oral vehicle or compound, such that the only systematic difference between control and treated gestations was the presence of ursolic acid. On the day of birth (postnatal day 0), litters were assessed for viability and culled as needed to a standard size of eight pups with an approximately equal sex ratio, to equalise maternal provisioning among litters. Control and treated litters were raised in parallel under the same conditions and weaned at postnatal day 21.

Compounds and dosing

Ursolic acid (Sigma-Aldrich, USA) was dissolved in distilled water, pH-adjusted to 7.4 with 0.1 M phosphate-buffered saline (PBS), prepared fresh daily and stored at 4 °C. Dams received ursolic acid 10 mg kg^{-1} per day, or 0.2 mL distilled water, by oral cannula throughout gestation. Oestrous cycles were synchronised with pregnant mare serum gonadotropin and monitored by vaginal smear; proestrus females were paired overnight with males, and the morning on which spermatozoa or a copulatory plug was identified was designated gestational day 1.

Experimental design

The study proceeded in three connected phases using a single cohort of offspring (Table 1). Phase I examined the effect of prenatal exposure alone on prefrontal epigenetic markers. Phase II applied maternal separation as the early-life stressor: pups assigned to separation groups were removed from the dam for 3 h daily from postnatal day (PND) 2 to PND 14, under temperature control and with handling limited to that required by the procedure, while controls remained undisturbed. Male offspring were assigned to four groups of twelve animals each, in a factorial arrangement combining prenatal treatment (vehicle or ursolic acid) with early-life stress (absent or present).

Phase III did not employ a new cohort. Six of the twelve offspring in each Phase II group were retained and allowed to mature, unchallenged, to PND 42, at which point the two groups that had undergone maternal separation received dexamethasone (0.5 mg kg^{-1} per day, intraperitoneally, for seven consecutive days) as a second stress challenge, while the two groups that had not undergone maternal separation received an equivalent volume of normal saline. Each Phase III group is therefore the direct continuation of a defined Phase II group, and the animals receiving the adult challenge carry the cumulative burden of two sequential stressors. To minimize the impact of litter-based confounding, the experimental groups were drawn from several independent litters and, whenever feasible, littermates were distributed across groups. This ensured that any differences observed between the groups could not be explained by the unique characteristics of any one litter.

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Table 1: Experimental design.

Phase	Group	Prenatal treatment	Early-life stress (PND 2–14)	Adult challenge (PND 42–48)
I	Control	Distilled water	—	—
I	UA	Ursolic acid 10 mg kg^{-1}	—	—
II (n = 12)	Control	Distilled water	None	—
II (n = 12)	ELS	Distilled water	Maternal separation	—
II (n = 12)	UA	Ursolic acid	None	—
II (n = 12)	UA + ELS	Ursolic acid	Maternal separation	—
III (n = 6)	Adult control	Distilled water	None	Saline
III (n = 6)	Adult stress	Distilled water	Maternal separation	Dexamethasone
III (n = 6)	Prenatal UA control	Ursolic acid	None	Saline
III (n = 6)	Prenatal UA + adult stress	Ursolic acid	Maternal separation	Dexamethasone

Each Phase III group comprises six animals drawn from the correspondingly matched Phase II group of twelve: Adult control from Control; Adult stress from ELS; Prenatal UA control from UA; and Prenatal UA + adult stress from UA + ELS. UA, ursolic acid; ELS, early-life stress; PND, postnatal day.

Behavioural assessment

Testing was performed under standardised, uniformly illuminated conditions between 09:00 and 15:00, with the apparatus cleaned with 70% ethanol between animals to eliminate olfactory cues. All sessions were video-recorded and scored by an observer blinded to group allocation. Tests were conducted in ascending order of aversiveness, the forced swim test last.

Novel object recognition

Animals were habituated to an empty open-field arena (60 × 60 × 40 cm) for 10 min. During familiarisation, two identical objects were placed in diagonally opposite quadrants approximately 10 cm from the walls, and the animal explored freely for 10 min. After a defined inter-trial interval, one object was replaced by a novel object of comparable size but differing shape, colour and texture, and exploration was recorded for 5 min. Exploration was defined as directing the nose to within 2 cm of an object, or contacting it with the nose or vibrissae; climbing or sitting upon an object without directed sniffing was not scored. The position of the novel object was counterbalanced across animals. Time exploring the novel object was recorded, and the discrimination index computed as $(T_{novel} - T_{familiar}) / (T_{novel} + T_{familiar})$.

Elevated plus maze

The maze comprised two open and two enclosed arms (50 × 10 cm) extending from a central platform, elevated 50 cm above the floor. Each animal was placed on the central platform facing an open arm and allowed to explore freely for 5 min. Entries into, and time spent in, the open and closed arms were recorded, an entry being scored when all four paws had crossed into an arm. Increased time in the closed arms was interpreted as an anxiety-like profile.

Porsolt forced swim test

Animals were placed individually in a transparent cylinder (height 45 cm, diameter 20 cm) containing water at 24 ± 1 °C to a depth sufficient to prevent the tail touching the base. Following a 15-min pre-test session, the 5-min

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test session was conducted 24 h later. Struggling (active escape-directed movement of the forelimbs against the cylinder wall) and immobility (floating with only movements necessary to keep the head above water) were scored. Reduced struggling time was interpreted as a depressive-like increase in behavioural passivity. Animals were dried and warmed immediately after each session.

Tissue collection

For histology, animals were euthanised and transcardially perfused with 0.1 M PBS followed by 4% paraformaldehyde; brains were excised, post-fixed, cryoprotected in graded sucrose and stored at 4 °C. For molecular and biochemical analysis, animals were decapitated without anaesthesia to avoid pharmacological interference, and the prefrontal cortex was dissected on ice according to stereotaxic coordinates, snap-frozen and stored until assay. Trunk blood was collected for serum corticosterone.

Reverse-transcription PCR of DNMT3a and H2AX

Prefrontal tissue was lysed and total RNA was extracted using TRIzol reagent following the manufacturer's instructions. Tissue was homogenised in 1 mL TRIzol, incubated for 5 min at room temperature and phase separated with 0.2 mL of chloroform followed by centrifugation at 12,000 × g for 15 min at 4 °C. RNA was precipitated from the aqueous phase with isopropanol, washed in 75% ethanol, air dried and re-suspended in nuclease free water. Spectrophotometrically purity and concentration were measured and accepted RNA with A260/280 ratio of 1.8–2.0. Integrity was checked on a 1% agarose gel.

First strand complementary DNA was reverse transcribed from total RNA (1 µg) using a reverse transcriptase kit and oligo (dT) primers in a 20 µL reaction (25 °C for 10 min, 42 °C for 60 min and 70 °C for 10 min to inactivate the enzyme). Amplification was done in a 25 µL reaction mixture containing cDNA template, forward and reverse primers, dNTPs, MgCl2 and Taq polymerase under the following conditions: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at the primer specific temperature for 30 s and extension at 72 °C for 45 s; and final extension at 72 °C for 7 min. Gene specific primers were used to amplify DNMT3a, H2AX, GAPDH (internal reference). Products were separated on a 2% agarose gel stained with ethidium bromide with a 100-bp molecular-weight ladder, visualized by ultraviolet transillumination and photographed. Band intensity was quantified densitometrically with Image J (National Institutes of Health, USA). Target expression is reported as the ratio of target to GAPDH band intensity. The amplicon sizes were approximately 165 bp for H2AX and 150 bp for GAPDH.

Table 2: Primers

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Amplicon Size
DNMT3a	AGCTGGAAGTGGAAGGAGAA	TCCTTCTGCTCTTCTTGGTC	~120–165 bp
H2AX	GGAGGAAGAAGCGGAAGAAG	CCAGGTTGTTGAGGATGGAG	~100–170 bp

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GAPDH	AGGTCATCCCTGAGCTGAAC	TGAGTCCTTCCACGATACCA	~120–150 bp

Enzyme-linked immunosorbent assays

Prefrontal tissue was homogenised in ice-cold PBS (0.01 M, pH 7.4) containing protease inhibitor cocktail at a ratio of 1:9 (w/v), centrifuged at 5,000 × *g* for 10 min at 4 °C, and the supernatant retained. Total protein was determined by the Bradford method with bovine serum albumin as standard, permitting normalisation of all tissue analytes to protein content. Serum for corticosterone was obtained by allowing whole blood to clot, followed by centrifugation at 3,000 rpm for 15 min.

IL-1β, BDNF and Nrf2 were quantified in prefrontal supernatants, and corticosterone in serum, using commercially available rat-specific sandwich ELISA kits according to the manufacturer's protocols. In brief, 100 µL of standard or sample was added to wells pre-coated with the relevant capture antibody and incubated at 37 °C for 90 min. Wells were decanted and incubated with biotinylated detection antibody for 60 min at 37 °C, washed, and incubated with horseradish peroxidase–avidin conjugate for 30 min at 37 °C. After further washing, tetramethylbenzidine substrate was added and the plate incubated in the dark for approximately 15 min; the reaction was terminated with stop solution and absorbance read at 450 ± 2 nm on a microplate reader. Concentrations were interpolated from four-parameter logistic standard curves constructed from the kit standards. All samples were assayed in duplicate and repeated freeze–thaw cycles were avoided. Tissue analytes are expressed per milligram of total protein (IL-1β and BDNF, pg mg^{-1}; Nrf2, ng mg^{-1}) and serum corticosterone in ng mL^{-1}.

Golgi impregnation

Dendritic and spine morphology were examined using the Golgi–Cox method. The brains were quickly removed and immersed without perfusion fixation in the Golgi–Cox solution (potassium dichromate, mercuric chloride and potassium chromate in distilled water) in the dark at room temperature for 14 days, changing the solution after the first 48 h. The tissue was then cryoprotected in 30% sucrose for at least 48 h at 4 °C. Coronal sections of the prefrontal cortex were cut at 100-150 µm on a vibratome and mounted on gelatin-coated slides. Sections were developed in ammonium hydroxide for ~30 min in the dark, rinsed in distilled water, fixed in photographic fixer for 30 min, and rinsed again. Dehydrated in an ethanol series, cleared in xylene and coverslipped with a resinous mountant. Only neurons that had been fully and uninterruptedly impregnated, with the soma within the region of interest and dendrites not obscured by neighboring neurons or precipitate were analyzed. Dendritic branching and spine density were analyzed in secondary and tertiary dendritic segments at ×1000 under oil immersion. Spine density was presented as number of spines per 10 µm of dendritic length. All analyses were performed blind to group allocation.

Hematoxylin and eosin staining

Paraffin sections were dewaxed, rehydrated, stained in Harris haematoxylin, differentiated in 1% acid alcohol, blued, counterstained in 1% eosin, dehydrated, cleared and mounted. Sections were examined for cortical cytoarchitecture and for degenerative features including neuronal shrinkage, nuclear pyknosis, cytoplasmic hypereosinophilia and pericellular vacuolation.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA) with the individual animal as the experimental unit. In two-way analyses, prenatal treatment (vehicle or ursolic acid) and stress (absent or present) were entered as fixed factors and the interaction between prenatal treatment × stress was explicitly tested. A significant main effect or interaction was followed by Tukey's multiple-comparison test. Exact P values and

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symbols denoting significant pairwise differences are reported in the corresponding figure legends. Data are expressed as mean ± standard error of the mean (SEM) and a two-tailed $P < 0.05$ was considered statistically significant.

Results

Prenatal ursolic acid engages the epigenetic machinery of the developing cortex

Prenatal ursolic acid approximately doubled prefrontal DNMT3a transcription, the GAPDH-normalised expression ratio rising from 0.53 ± 0.04 in controls to 1.12 ± 0.08 ($P < 0.05$; Fig. 1). Densitometry was corroborated by the gel, in which the DNMT3a amplicon was consistently stronger in treated lanes against a uniform GAPDH band. H2AX followed the same pattern, increasing from 0.48 ± 0.05 to 0.99 ± 0.04 ($P < 0.05$; Fig. 2). Coordinated upregulation of a *de novo* methyltransferase and a chromatin-stability marker indicates that the compound acts on the transcriptional-programming machinery of the developing cortex, and not solely as a contemporaneous antioxidant.

Prenatal ursolic acid abolishes the neuroendocrine, inflammatory and redox consequences of early-life stress

Maternal separation raised serum corticosterone from 203 ± 5 to 305 ± 6 ng mL^{-1}, an increase of approximately 50% ($P < 0.05$; Fig. 3). Prenatal ursolic acid prevented this entirely (UA + ELS, 207 ± 6 ng mL^{-1}), a value indistinguishable from the UA-only group (198 ± 5 ng mL^{-1}). Ursolic acid alone did not alter basal corticosterone, indicating that the compound restrains the stress response without suppressing the axis at rest.

The same pattern held for the inflammatory and redox markers. IL-1β more than doubled with early-life stress, from 15.5 ± 1.0 to 33.0 ± 1.2 pg mg^{-1} protein, and was returned to control levels by prenatal exposure (UA + ELS, 17.0 ± 0.8 pg mg^{-1}; Fig. 4). Reciprocally, Nrf2 fell by two-thirds, from 5.77 ± 1.5 to 1.92 ± 1.2 ng mg^{-1} protein, and was fully preserved by prenatal ursolic acid (UA + ELS, 5.63 ± 1.4 ng mg^{-1}; Fig. 5). The inverse movement of Nrf2 and IL-1β, and their joint normalisation, is consistent with the compound acting on the redox–inflammatory axis rather than on either arm in isolation.

Protection persists against a second, adult challenge

The decisive test was whether protection remained available in offspring carried forward from Phase II and challenged again in adulthood. In animals that had undergone maternal separation and subsequently received dexamethasone, corticosterone rose from 200 ± 7 ng mL^{-1} in the adult control group to 275 ± 5 ng mL^{-1} ($P < 0.05$; Fig. 6). Prenatally exposed offspring subjected to the same sequence were unaffected (prenatal UA + adult stress, 197 ± 3 ng mL^{-1}), and the prenatal UA control group recorded 186 ± 4 ng mL^{-1}.

A single prenatal course therefore, restrains the corticosterone response even when an early-life stressor and an adult glucocorticoid challenge are imposed in sequence. Behaviour showed the same dissociation across all three paradigms (Fig. 7). In the forced swim test, struggling time fell from 38.5 ± 1.5 s to 21.0 ± 2.0 s in the stressed group, indicating increased behavioural passivity, whereas prenatally exposed animals maintained 37.5 ± 1.5 s. In novel object recognition, exploration of the novel object collapsed from 192 ± 5 s to 62 ± 8 s but was fully preserved at 199 ± 5 s. In the elevated plus maze, closed-arm time rose from 46 ± 3 s to 142 ± 6 s, and returned to 48 ± 3 s. Mood-related, cognitive and anxiety-related domains were thus protected concurrently.

Molecular endpoints followed. IL-1β more than doubled after the adult challenge, from 11.5 ± 0.8 to 24.0 ± 0.8 pg mg^{-1} protein, and was held near control by prenatal exposure (13.5 ± 0.8 pg mg^{-1}; Fig. 8). Nrf2 was likewise preserved (Fig. 9). BDNF, which fell from 43.5 ± 2.5 to 25.0 ± 3.0 pg mg^{-1} protein in the stressed group, was maintained at 42.0 ± 3.0 pg mg^{-1} in prenatally exposed offspring (Fig. 10), indicating that neurotrophic support survived the sequence.

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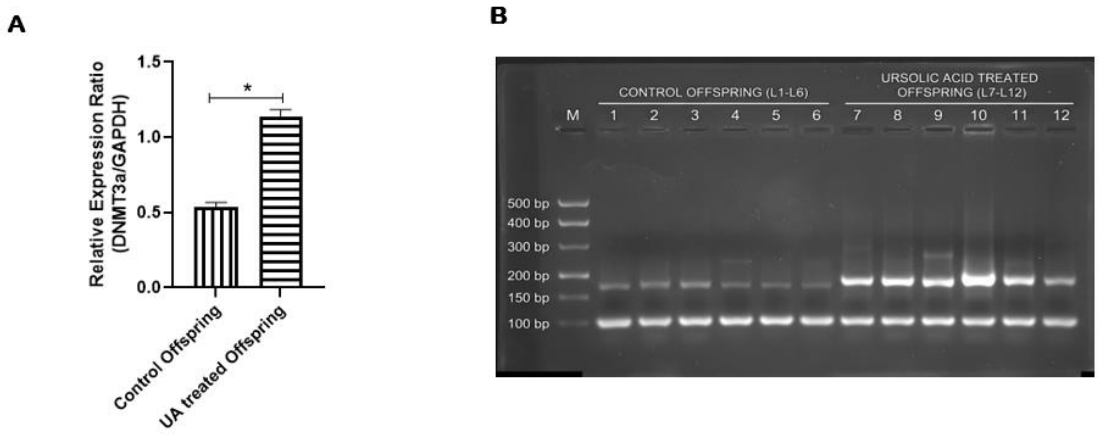

Figure 1: Effect of prenatal ursolic acid exposure on DNMT3a messenger RNA expression in the prefrontal cortex of male offspring. (A) Relative expression ratio of DNMT3a normalised to GAPDH in control and ursolic acid-treated offspring. (B) Representative agarose gel showing the DNMT3a and GAPDH amplicons for control offspring (lanes 1 to 6) and ursolic acid-treated offspring (lanes 7 to 12), with the molecular weight marker (M) in base pairs. Data are expressed as mean ± SEM. * $p < 0.05$ versus control offspring.

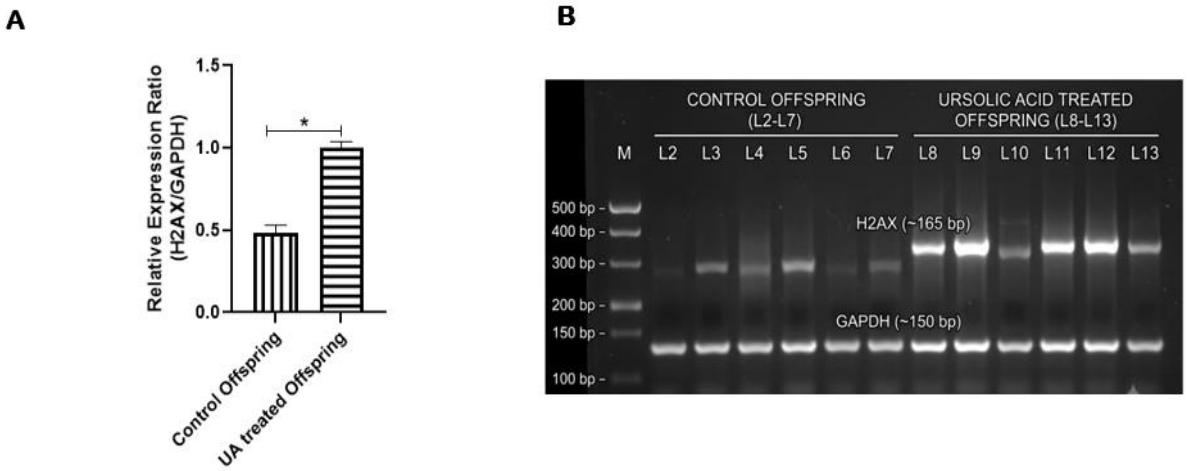

Figure 2: Effect of prenatal ursolic acid exposure on H2AX messenger RNA expression in the prefrontal cortex of male offspring. (A) Relative expression ratio of H2AX normalised to GAPDH in control and ursolic acid-treated offspring. (B) Representative agarose gel showing the H2AX amplicon (approximately 165 base pairs) and the GAPDH amplicon (approximately 150 base pairs) for control offspring (lanes 2 to 7) and ursolic acid-treated offspring (lanes 8 to 13), with the molecular weight marker (M). Data are expressed as mean ± SEM. * $p < 0.05$ versus control offspring.

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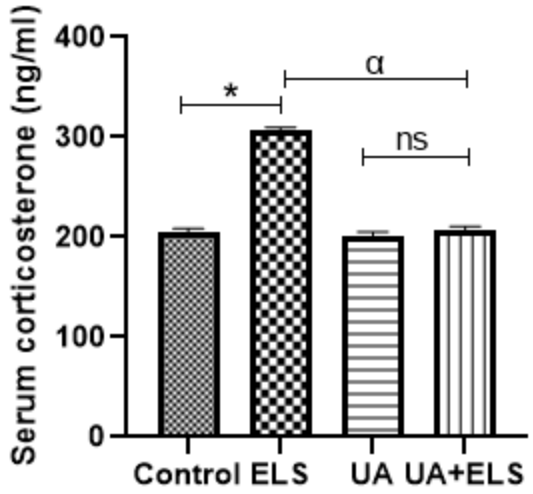

Figure 3: Effect of prenatal ursolic acid exposure and early life stress on serum corticosterone concentration in male offspring. Groups are control, early life stress (ELS), ursolic acid (UA), and ursolic acid plus early life stress (UA+ELS). Data are expressed as mean ± SEM. Symbols denote statistical significance ($p < 0.05$): * versus Control, α versus UA+ELS. ns, not significant.

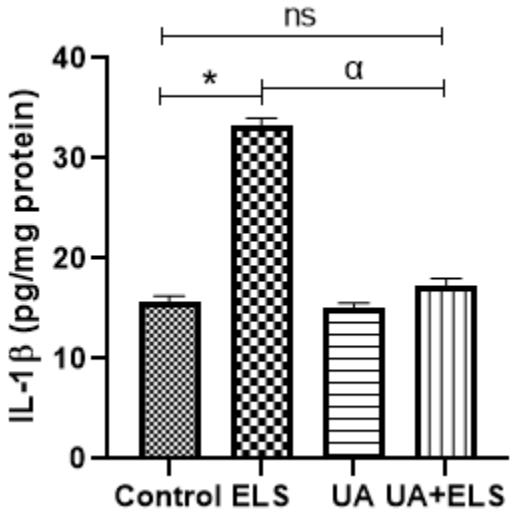

Figure 4: Effect of prenatal ursolic acid exposure and early life stress on interleukin-1 beta (IL-1β) concentration in the prefrontal cortex of male offspring. Groups are control, early life stress (ELS), ursolic acid (UA), and ursolic acid plus early life stress (UA+ELS). Data are expressed as mean ± SEM. Symbols denote statistical significance ($p < 0.05$): * versus Control, α versus UA+ELS. ns, not significant.

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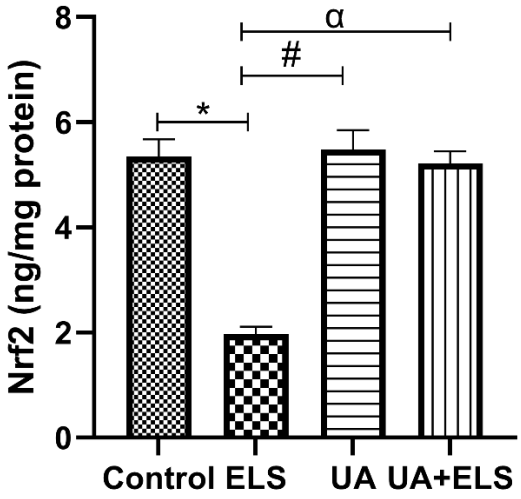

Figure 5: Effect of prenatal ursolic acid exposure and early life stress on nuclear factor erythroid 2–related factor 2 (Nrf2) concentration in the prefrontal cortex of male offspring. Groups are control, early life stress (ELS), ursolic acid (UA), and ursolic acid plus early life stress (UA+ELS). Data are expressed as mean ± SEM. Symbols denote statistical significance ($p < 0.05$): * versus Control, α versus UA+ELS

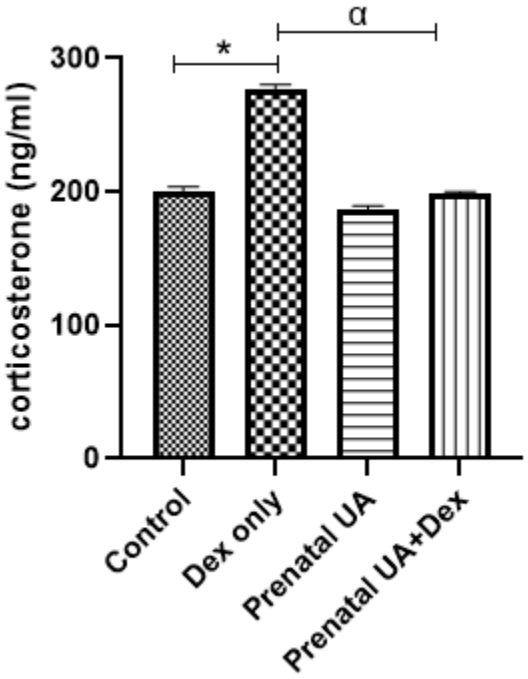

Figure 6: Effect of prenatal ursolic acid exposure on serum corticosterone concentration in male offspring subjected to an adult dexamethasone challenge. Groups are control, dexamethasone only (Dex), prenatal ursolic acid (Prenatal UA), and prenatal ursolic acid plus dexamethasone (Prenatal

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UA+Dex). Data are expressed as mean ± SEM. Symbols denote statistical significance ($p < 0.05$): * versus Control, α versus Prenatal UA+Dex.

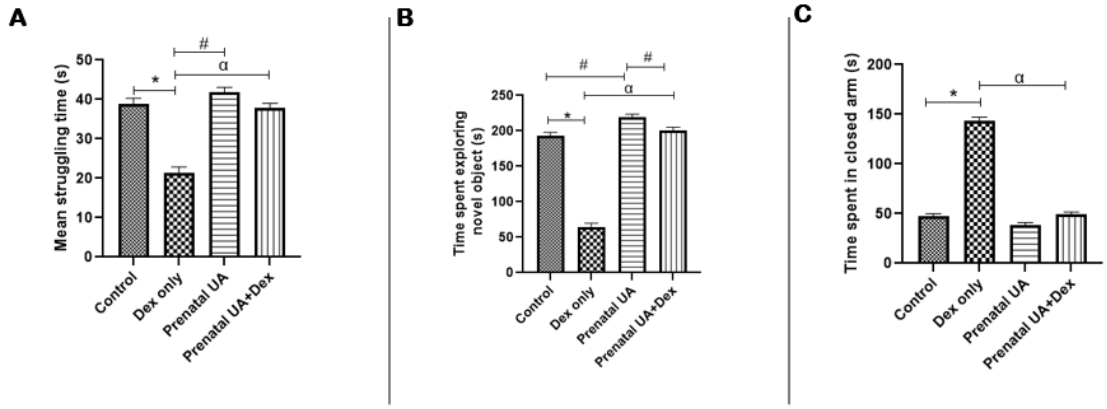

Figure 7: Effects of prenatal ursolic acid treatment on behavioural parameters. (A) Mean struggling time in the Porsolt forced swim test, (B) time spent exploring a novel object in the Novel Object Recognition test, (C) time spent in the closed arm in the Elevated Plus Maze test. Data are expressed as mean ± SEM. Symbol variations indicate statistical significance ($p < 0.05$): * versus Control, # versus Prenatal UA, α versus Prenatal UA+Dex. ns, non-significant.

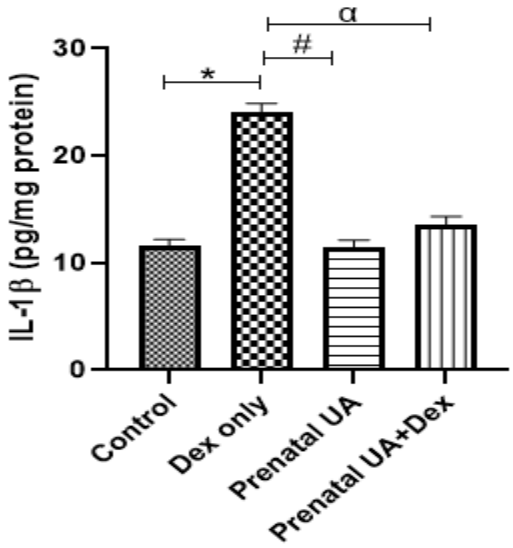

Figure 8: Effect of prenatal ursolic acid exposure on interleukin-1 beta (IL-1β) concentration in the prefrontal cortex of male offspring subjected to an adult dexamethasone challenge. Groups are control, dexamethasone only (Dex), prenatal ursolic acid (Prenatal UA), and prenatal ursolic acid plus dexamethasone (Prenatal UA+Dex). Data are expressed as mean ± SEM. Symbols denote statistical significance ($p < 0.05$): * versus Control, # versus Prenatal UA, α versus Prenatal UA+Dex.

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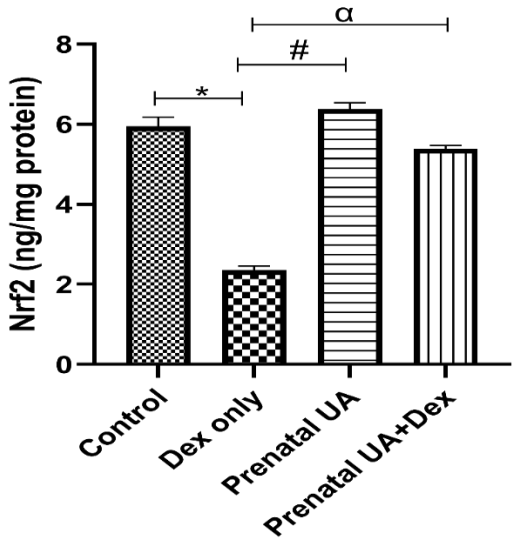

Figure 9: Effect of prenatal ursolic acid exposure and early life stress on Nuclear factor erythroid 2–related factor 2 (Nrf2) concentration in the prefrontal cortex of male offspring. Groups are control, dexamethasone only (Dex), prenatal ursolic acid (Prenatal UA), and prenatal ursolic acid plus dexamethasone (Prenatal UA+Dex). Data are expressed as mean ± SEM. Symbols denote statistical significance ($p < 0.05$): * versus Control, # versus Prenatal UA, α versus Prenatal UA+Dex.

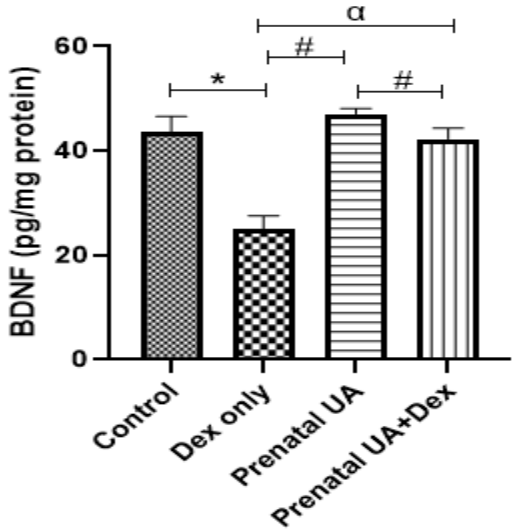

Figure 10: Effect of prenatal ursolic acid exposure on brain-derived neurotrophic factor (BDNF) concentration in the prefrontal cortex of male offspring subjected to an adult dexamethasone challenge. Groups are control, dexamethasone only (Dex), prenatal ursolic acid (Prenatal UA), and prenatal ursolic acid plus dexamethasone (Prenatal UA+Dex). Data are expressed as mean ± SEM. Symbols denote statistical significance ($p < 0.05$): * versus Control, # versus Prenatal UA, α versus Prenatal UA+Dex.

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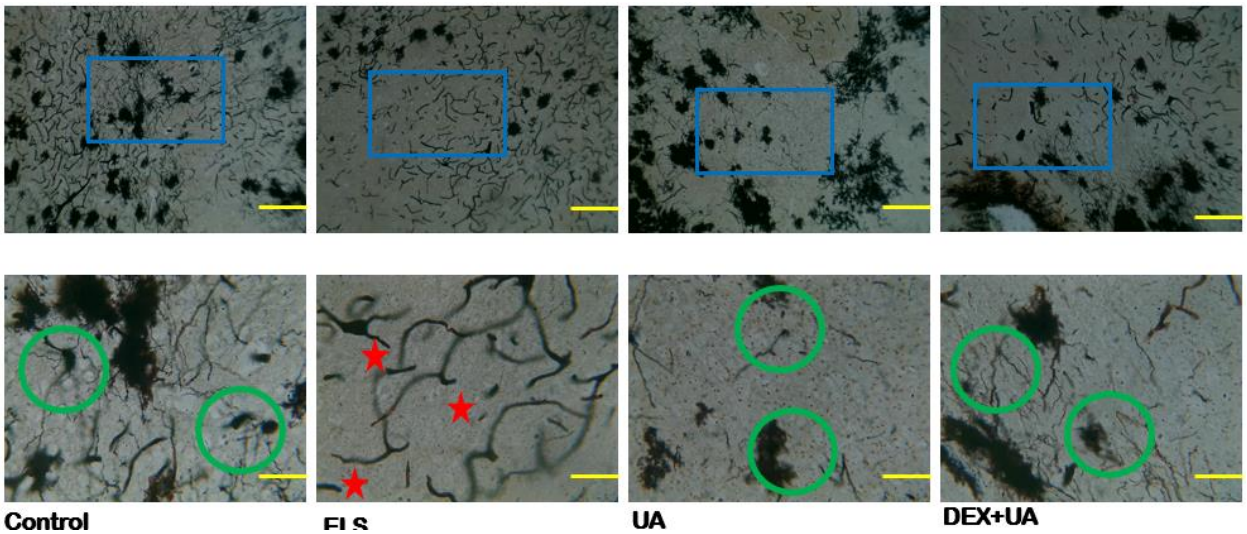

Figure 11: Photomicrographs of Golgi-impregnated neurons in the prefrontal cortex of male offspring subjected to an adult dexamethasone challenge. Groups are control, dexamethasone, ursolic acid (UA), and dexamethasone plus ursolic acid (DEX+UA). The lower row shows the boxed regions at higher magnification. Green circles highlight dendritic segments with normal spine density and red stars indicate regions of dendritic thinning and spine loss. Scale bar: 50 and 25 µm.

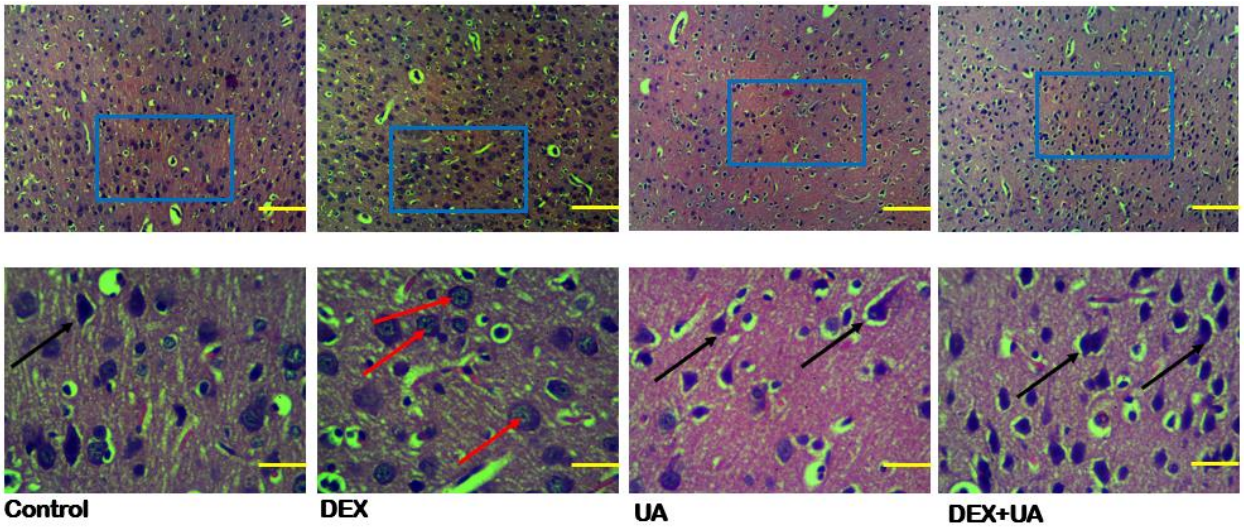

Figure 12: Photomicrographs of haematoxylin and eosin-stained sections of the prefrontal cortex of male offspring subjected to an adult dexamethasone challenge. Groups are control, dexamethasone (DEX), ursolic acid (UA), and dexamethasone plus ursolic acid (DEX+UA). The lower row shows the boxed regions at higher magnification. Black arrows indicate healthy neurons and red arrows indicate shrunken, pyknotic (degenerating) neurons. Scale bar: 50 and 25 µm.

Structural correlations of the protected phenotype

Golgi impregnation showed well-branched dendrites bearing a normal complement of spines along smooth, continuous shafts in adult control and prenatal ursolic acid sections. The stressed group showed dendritic thinning, varicose swelling and visible spine loss; in prenatally exposed offspring subjected to the same sequence, arborisation and spine density resembled the control pattern (Fig. 11). Haematoxylin and eosin staining was concordant:

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control and ursolic acid sections contained healthy neurons with round, open nuclei, prominent nucleoli and intact perikarya in orderly cortical arrangement, whereas the stressed group showed shrunken angular neurons with pyknotic nuclei and pericellular spaces. Prenatal exposure preserved neuronal morphology and cortical organisation (Fig. 12). The structural findings therefore anchor the biochemical and behavioural results in the physical substrate of connectivity.

Discussion

This study shows that a prenatal dose of ursolic acid induces a latent, wide-ranging and resilient protective phenotype in the rat prefrontal cortex able to withstand two sequential stressors. This not only confirms that the compound is anti-inflammatory, as previously shown (Zhao et al., 2023) but also demonstrates that exposure limited to gestation continues to influence the cortex's response to a challenge in adulthood, after an intervening developmental interval and over and above an earlier insult. Three aspects of the data support this interpretation: the involvement of epigenetic machinery in Phase I, the consistency of protection across independent measurements, and the durability of the effect over the sequence separating Phases II and III.

An epigenetic route from transient exposure to durable set-point

Any claim of programming requires a mechanism capable of outlasting the exposure, and DNA methylation supplies one (Alum et al., 2025). The coordinated doubling of DNMT3a and H2AX transcription indicates that ursolic acid engages both the *de novo* methylation apparatus and the chromatin-stability machinery of the developing cortex. This is mechanistically coherent: DNMT3a establishes methylation patterns that persist through subsequent cell divisions and thereby fix transcriptional set-points. At the same time, H2AX maintains genomic integrity during the intense proliferative activity of cortical development. Upregulation of the two together is more informative than either alone, since it suggests a broad chromatin-level adjustment rather than an isolated change at a single locus (Qing et al., 2023; Liu et al., 2025).

The process of methylation marks being established by DNMT3a and then replicated by maintenance methyltransferases is known as de novo transfer of methyl groups from S-adenosyl-methionine to cytosine residues. Therefore, a change in DNMT3a activity during the prenatal window can fix a transcriptional set-point for the cell's lifetime (Legoff et al., 2019; Liu et al., 2025). Ursolic acid could affect this machinery by several non-mutually exclusive routes. Given the compound's primary documented action is enhancement of the Nrf2 antioxidant pathway (Zhao et al., 2023), and the fact that the DNA-methylating enzymes and ten-eleven-translocation demethylases are themselves redox-sensitive, a lowering of the oxidative burden of the developing cortex would be expected to alter both the activity of DNMT3a and the accessibility of its target loci. Oxidative stress is a recognised modifier of genomic DNA-methylation patterns, and its relief is one plausible bridge between the compound's antioxidant chemistry and the methylation changes inferred here. Maternal nutrition and phytochemical exposure have broadly been shown to modify offspring DNA methylation, in part through the provision of one-carbon methyl donors and in part through the regulation of methyltransferase expression (Alum et al., 2025). Meanwhile, the coordinated rise of the phosphorylated histone variant γH2AX indicates activation of the chromatin-stability and DNA-damage-response machinery that protects the genome during the rapid proliferation of cortical development (Merighi et al., 2021; Qing et al., 2023); its concurrent increase with DNMT3a is consistent with a genome-wide chromatin reorganization rather than an isolated single-gene effect.

Restraint of the stress axis without basal suppression

Prenatal ursolic acid abolished the corticosterone response to maternal separation and, more strikingly, to the subsequent adult challenge,

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while leaving basal corticosterone unaltered in unchallenged animals. This dissociation matters. Due to the importance of glucocorticoid signalling in normal physiology, an agent that merely inhibited the HPA axis would pose significant risks and have limited utility. Instead, what our data show is a shift in the axis's gain, which limits the excursion but does not change the set-point. Previous studies have also demonstrated that ursolic acid can regulate the HPA axis (Ramos-Hryb et al., 2019). Early-life adversity in rodents characteristically produces the opposite: a sensitised axis that over-responds to subsequent challenge (Speranza et al., 2024). That sensitisation is precisely what the present design should have exposed, since the animals receiving dexamethasone had already been maternally separated; prenatal ursolic acid nevertheless prevented it.

The two-hit design strengthens, and constrains, the interpretation

Because the Phase III groups were carried forward from Phase II rather than drawn from a new cohort, the animals receiving dexamethasone bore the cumulative burden of early-life stress followed by an adult glucocorticoid challenge. This has two consequences. It strengthens the central claim, since protection was demonstrated against sequential rather than single adversity, which is the more stringent and the more clinically representative test. It also constrains attribution: the Phase III effects cannot be assigned to dexamethasone alone, because the comparison groups differ in both early-life and adult exposure. Hence, our study demonstrates that adult stress (dexamethasone exposure) can unmask early-life stress–induced alterations and further exacerbate neuronal damage (Brunton, 2015).

The Nrf2–inflammation–neurotrophin axis as the probable effector

The simplest interpretation of the biochemical data is that the pattern is due to a single upstream node. Nrf2 and NF-κB are inversely coupled, in that Nrf2 activity inhibits inflammatory transcription, and loss of Nrf2 disinhibits it (Navarro and Esteras, 2024; Heurtaux et al., 2022). In Phase II and Phase III, the two markers exhibited reciprocal movement: Nrf2 decreased and IL-1β increased. Prenatal ursolic acid was able to normalise both markers simultaneously. The suppression of IL-1β is most parsimoniously explained by preservation of Nrf2, rather than by an independent anti-inflammatory action, since the antioxidant action of ursolic acid is largely due to Nrf2 upregulation (Zhao et al., 2023).

This account extends naturally to BDNF. Nrf2 supports BDNF transcription, and attenuation of the Nrf2 pathway is a consistent feature of depressive phenotypes across animal and human studies (Simonetti et al., 2023); glucocorticoid signalling and BDNF are themselves reciprocally related, such that chronic glucocorticoid excess depletes neurotrophic support (Tsimpolis et al., 2024). In our Phase III data, the stressed group showed BDNF reduced by approximately 43% alongside raised corticosterone and IL-1β and lowered Nrf2, and prenatal ursolic acid preserved all four. A single mechanism, preservation of Nrf2 tone, therefore accounts economically for the redox, inflammatory and neurotrophic findings together.

Structural and behavioural convergence

Biochemical protection would be of limited interest if the tissue and the animal were nonetheless damaged. They were not. The stressed group showed dendritic thinning, varicosity and spine loss on Golgi impregnation, together with neuronal shrinkage and pyknosis on hematoxylin and eosin, and prenatal exposure preserved both. The structural findings are the expected consequence of the biochemical ones: BDNF is a principal determinant of dendritic and spine maintenance, so its preservation and the preservation of spine density are not independent observations but two views of the same protection. The preservation of behavioural performance simultaneously in three mechanistically distinct paradigms — the forced

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swim, the novel object recognition and the elevated plus maze — indicates that the protection was functional and not just histological. The convergence of transcriptional, endocrine, inflammatory, redox, neurotrophic, structural and behavioural readouts is the major strength of the dataset, because independent measures are unlikely to converge by chance.

These results are consistent with, and even contradict, a substantial body of research on the causes of stress vulnerability in early development. Harm is the dominant theme of that literature: prenatal and early-life adversity programmes a sensitised HPA axis and a lasting predisposition to anxiety- and depression-like phenotypes (Brunton, 2015; Speranza et al., 2024; Hanson and Nacewicz, 2021), and much of the transgenerational epigenetic work has been concerned with the inheritance of maladaptive rather than protective traits (Legoff et al., 2019). "So this study is important in showing programming in a positive direction – a prenatal exposure that promotes resilience, not vulnerability. This is consistent with a smaller body of work showing that maternal nutritional and phytochemical exposures can positively influence offspring neurodevelopment (Alum et al., 2025), and with evidence that ursolic acid itself protects against corticosterone-induced neuronal injury (Ramos-Hryb et al., 2019).

Limitations and potential confounding factors

These conclusions have some limitations. First, we only studied male offspring. The programming of the HPA axis and stress-related behaviour is strongly sex-dependent and the findings cannot be generalized to females without direct test. Second, only one prenatal dose of ursolic acid was tested and thus the existence of a dose-response relationship or a no effect threshold was not demonstrated. Finally, extrapolation from a rodent two-hit model to human stress-related disease must be undertaken with caution: interspecies differences in placentation, cortical maturation and glucocorticoid physiology, together with the far greater heterogeneity of human environments, all limit direct translation. Such considerations should be taken into account in interpreting the present findings.

Conclusion

A prenatal course of ursolic acid upregulates DNMT3a and H2AX transcription in the developing rat prefrontal cortex and confers protection that persists into adulthood. The offspring that are thus exposed are able to withstand both an early-life stressor and a subsequent adult glucocorticoid challenge. They maintain normal levels of corticosterone, IL-1β, Nrf2 and BDNF, as well as preserved dendritic arborisation, spine density and neuronal cytoarchitecture. Additionally, they exhibit unimpaired performance in emotional, cognitive and anxiety-related behavioural evaluations. The fact that the protection was sustained across two sequential stressors and across transcriptional, endocrine, redox, inflammatory, neurotrophic, structural and behavioural levels suggests programming of a durable resilient phenotype rather than a transient pharmacological effect. Prenatal nutritional programming thus emerges as a tractable strategy for conferring lifelong stress resilience.

Professional and translational implications

The implications of this study extend beyond its immediate results. For developmental neuroscience, this is a proof of principle that the epigenetic programming that normally transmits early adversity can be rerouted to resilience by a defined prenatal exposure, reframing the developmental origins of disease as a potentially modifiable, and not simply predictive, process. For nutritional and preventive science it suggests a candidate for prenatal supplementation, an inexpensive, orally active dietary triterpenoid present in ordinary foodstuffs, a route more readily translatable than pharmacological intervention in pregnancy. The demonstration that a single upstream node (Nrf2 tone) can be leveraged to protect the redox, inflammatory and

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neurotrophic status of the cortex simultaneously supports multi-target, mechanism-led prevention over single-pathway approaches for the study of stress-related psychiatric illness. These implications are prospective rather than clinical: they set a direction for confirmatory animal work and ultimately for carefully controlled human studies, and they should not be read as an endorsement of ursolic acid supplementation in human pregnancy prior to dedicated safety and efficacy data.

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Conflict of interest
The authors declare no competing financial or non-financial interests.

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Data availability
The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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