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BRIEF REPORT

Mild chronic post-natal pain induces endocrine and metabolic alterations associated to enlargement in pituitary glands size in adult CD-1 male mice

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Abstract

Background Human adverse childhood experiences (ACEs) are associated with various types of mental and physical pathological outcomes in adulthood. Among them, they may present the enlargement of the pituitary gland and have been suggested to be a risk factor for the development of Cushing syndrome. Previously, we showed on outbred CD-1 male mice that chronic pain induced during the weaning time by pharmacological experimental design procedures caused endocrine and metabolic alterations in adulthood, suggestive of human mild hypercortisolism. Specifically, we observed an increase in pituitary glands weight and in adrenocorticotropic hormone (ACTH) expression, associated with the lack of the negative feedback mechanisms exerted by corticosterone that controls proopiomelanocortin- derived ACTH secretion.

Methods Here, to better understand the phenotype of mice subjected to early-life pain (ELP), their pituitary glands were examined. Mice tissues and plasma hormones measurements were conducted by ELISA assays. Analysis of brain and pituitary gland was performed using anatomic and diffusion-weighted magnetic resonance imaging. Hematoxylin and eosin-stained sections of pituitary glands were also examined.

Results Mice subjected to ELP showed an increase in total body weight, in pituitary ACTH expression and in plasmatic corticosterone levels. Imaging of the pituitary glands revealed a significant increment of their volume without apparent pathological alterations.

Conclusion The findings of this study may support the role of ELP as a risk factor for ACTH-dependent hypercortisolism in adulthood associated with an enlarged pituitary gland.

Keywords Early-life pain · Cushing syndrome · Magnetic resonance imaging · Pituitary gland

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Introduction

Chronic pain is known to affect the endocrine system, promoting sustained cortisol production [1]. Similarly, early-life pain (ELP) exposure is linked with impaired neurodevelopment and enduring metabolic and endocrine alterations both in humans [2] and rodents [3] newborns. These studies focused on the consequences of neonatal intensive care units, which are considered among adverse childhood experiences (ACEs) [4].

Following a classic post-natal pharmacological treatment protocol on male CD-1 mice, we observed that a daily subcutaneous (s.c.) injection throughout the weaning time caused, in adult age, a phenotype including an increase in body weight, hyperglycemia and hyperinsulinemia, an increase in triglycerides and leptin plasmatic levels, and endocrine alterations suggestive of human ACTH-dependent hypercortisolism [5]. These ELP effects appear gender dependent [6]. The proposed pathogenic mechanism of this ELP model appeared to be a stress-induced failure of pituitary negative feedback mechanisms that control pro-opiomelanocortin (POMC)-derived ACTH-corticosterone [7]. ELP mice presented a higher fresh pituitary weight and ACEs have been linked to an enlargement of pituitary gland size in humans [8, 9]. Since clinical studies suggested a relationship between stressful life events and Cushing syndrome (CS) [10], we evaluated the size of pituitary gland and eventual signs of pituitary adenomas using anatomic magnetic resonance imaging (MRI) and histological analysis.

Methods

Ethics guidelines

All the procedures were carried out in accordance with the guidelines of the Council of European Communities (European Communities Council Directive of November 24th, 1986, 86/609/EEC) and following the approval of the Bioethical Committee of the Italian National Institute of Health (Istituto Superiore di Sanità), and the Italian Ministry of Health (n. 729/2019-PR, 04/11/2019).

Animals and stress procedures

The detailed ELP protocol is described in [11]. Briefly, pregnant multiparous outbred laboratory-born CD-1 mice were sent by the factory (Charles River Italia, Calco, Italy) and arrived at the 14th day after conception age in the ISS vivarium. All mice were housed in single cages in a central facility and maintained under controlled conditions of $55 \pm 5\%$ humidity and temperature of 21 ± 1 °C, in a photoperiod of 12 h light and dark, with the light turned on at 07:00. Mice were fed a standard diet (6.55% kcal from fat; 4RF21, Mucedola, Italy), and food and water were available ad libitum. Following delivery, in about 12 h, only male mice were put together and randomly culled to the exact number of six pups per mother. Then, each litter was randomly assigned to one of the following groups (each group consisting of two litters, $n = 12$): (a) control (CTR) group: the pups were left undisturbed with their mother, except for cage cleaning twice a week; (b) stressed mice (SM) group: for 21 days, the pups were daily removed (10 min) from the home cage and grouped in a container with fresh bedding material. Each pup was weighed and subcutaneously (s.c.) injected with sterile saline (1 ml/kg body weight of sodium chloride 0.9%) (Fig. 1A).

Then, they returned to the home cage with their mother. On postnatal day (PND) 21, all animals were re-housed three for each cage, four cages per each experimental group ($n = 12$).

Brain morpho-functional MRI analyses

On PND 140, animals underwent MRI analyses on a 4.7 T Agilent/Varian Inova preclinical system (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a combination of volume and surface coils (RAPID Biomedical GmbH, Rimpar, Germany). The animals were anesthetized with 2.5% isoflurane in oxygen 1 L/min (Isoflo, Abbott SpA, Latina, Italy) within an induction chamber and then transferred to a stereotaxic head frame, in prone position, under the continuous supply of anesthetic gases through a facemask, and fixed by using tooth bar, earplugs and adhesive tape to reduce head movement. During the MRI analyses, anesthesia was maintained to 2.5–1.5% isoflurane in oxygen (1 L/min). An integrated heating system allowed to maintain the animal body temperature at 37.0 ± 0.1 °C. Mice were left to spontaneous breathing, with no mechanical ventilation, during the whole experiment. To detect pituitary morphological differences between the two experimental groups, T1-weighted spin echo anatomical images were acquired on the brain (TR/TE = 600/18 ms, thickness = 0.6 mm, field of view (FOV) = 20×20 mm^2; Matrix 256×128, Voxel dimensions = $78 \times 156 \times 600$ µm) in the three projections (axial, coronal and sagittal). Volumetric analyses of the whole brain and the pituitary gland have been performed (Fig. 1B) using dedicated software (Browser, Agilent Technologies). Briefly, the volume of the pituitary gland was measured in the two coronal 2D contiguous slices where the gland was visible, by manual delineation of the gland. The volume of the whole brain was calculated by manual delineation of the forebrain (from olfactory bulb to cerebellum excluded) in 2D contiguous slices. Estimation of the 3D volume from the 2D slices was performed by the Browser program by using an iterative method which minimizes the

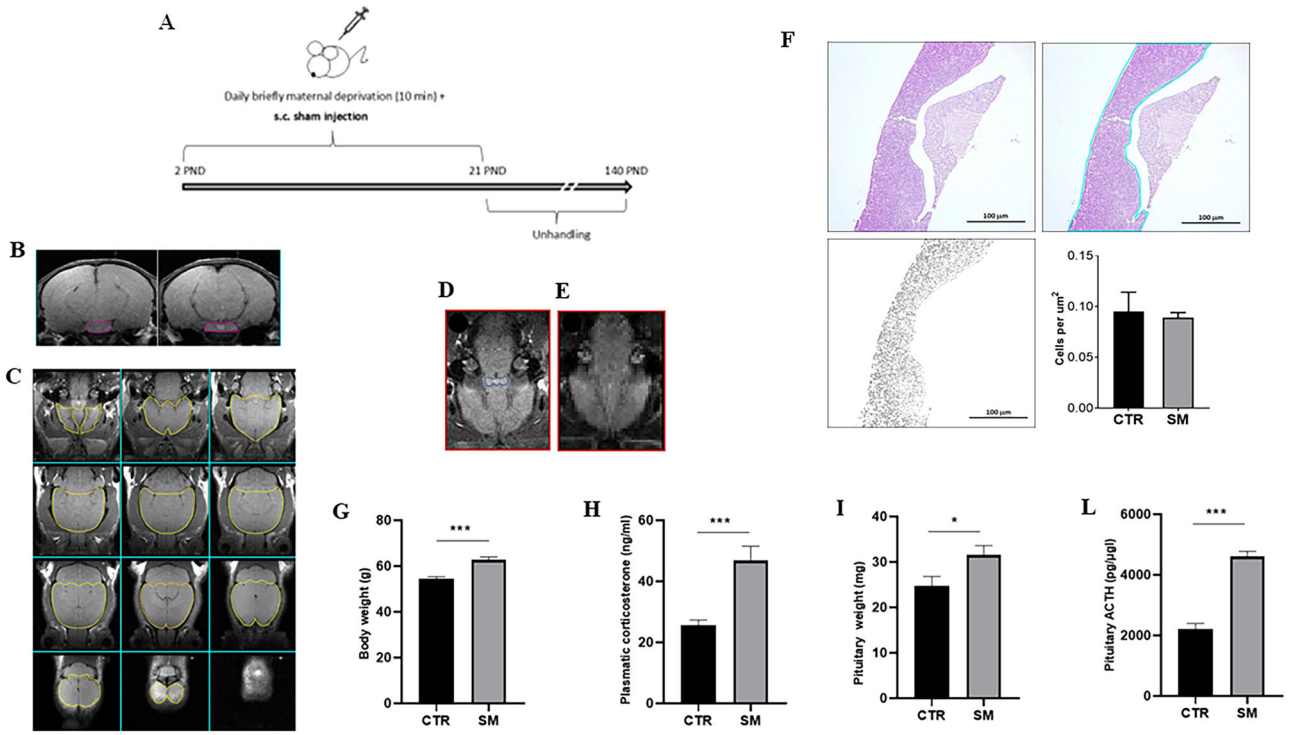

Fig. 1 **A** From the postnatal day (PND) 2 to the PND 21, pups underwent mild nociceptive stress consisting in a subcutaneous (s.c.) injection of physiological solution. To perform s.c. injection, mice were subjected to handling and a brief maternal separation. After weaning, mice were left undisturbed up to PND 140, when they started the MRI protocols. **B** T1-weighted axial anatomical MRI images for volumetric analyses of the pituitary gland. **C** Example of T1-weighted coronal anatomical MRI images for volumetric analyses of the whole brain. The pink line circumscribes the pituitary gland. The yellow line shows the brain regions considered for brain volume determination. Axial T1-weighted MRI images **D** showing pituitary gland (blue line) of a representative SM mouse and its corresponding DWI image **E** showing no hyperintensity in the pituitary area. **F** Representative H&E staining on pituitary gland 2 µm-thin sections (from CTR), and imaging elaboration on *pars distalis*. Histomorphological analysis showed on graph no evident sign of alteration and difference in cellular density in CTR (0.0950 ± 0.0190 cells/µm^2) vs SM (0.0889 ± 0.0050 cells/µm^2) ($n = 3$ per group). Cyan lines in the upper right panel indicate the *pars distalis* ROI boundaries of the pituitary. **G** On PND 150, SM show a significant ($P < 0.001$) increase in total body weight (62.7 ± 1.3 g) compared to CTRs (54.5 ± 0.9 g) ($n = 12$ per group). **H** SM presented levels of basal plasmatic corticosterone higher ($P < 0.001$) than CTRs (46.9 ± 4.6 ng/ml and 25.1 ± 1.8 ng/ml, respectively) ($n = 9$ per group). **I** SM pituitary glands showed a significant mean increase ($P < 0.039$) in fresh weight (3.15 ± 0.2 mg) as compared to CTR (2.47 ± 0.22 mg) ($n = 9$ per CTR and $n = 8$ per SM). **L** Difference ($P < 0.001$) in ACTH pituitary content between SM (4601 ± 174 pg/µg of proteins) and CTR (2204 ± 190 pg/µg of proteins) ($n = 9$ per group). CTR indicates the undisturbed mice group; SM indicates mice underwent ELP procedures. Statistical analyses were performed using unpaired t-test. Values are expressed as mean ± SE. *$P < 0.05$ and ***$P < 0.001$ for CTR vs SM

uncertainty due to partial volume effect. For the diagnosis of the presence of hypercellular masses, functional images were also acquired such as diffusion-weighted MRI or DWI (TR/TE = 2500/50 ms, thickness = 1 mm, FOV = 20×20 mm^2, Matrix 64×64, Voxel size = $312 \times 312 \times 1000$ µm) with the diffusion gradient = 0, 1, 1.5, 1.8, 2.55, 2.3, 4.8, 6 G/cm which correspond to b values ranging between 0 and 1105 s/mm^2. The ADC parameter was measured considering a mono-exponential diffusion model.

Pituitary-ACTH and plasma-corticosterone determination

On PND 150, mice were sacrificed between 10:00 and 12:00 h a.m.. Since mice have an inverted sleep-wake rhythm compared to humans, mice tissues were collected in the late morning to evaluate serum basal corticosterone [12]. Animals were rapidly sacrificed and trunk blood was collected in ice-chilled EDTA-containing tubes according to instructions for the dosage of single hormones and spun at $3500 \times g$ for 10 min at 4 °C. Plasma was collected and stored at −80 °C until the time of assay. The pituitary was dissected and immediately weighed (Gibertini Crystal 200, Novate Milanese, Italy), and stored at −80 °C until the assay. Plasmatic corticosterone and pituitary ACTH were assayed in duplicate using the appropriate ELISA kit (Elabscience, Huston, TX, USA) following instructions. All extracts were processed on the same days and peptides were measured in duplicate and intra assay coefficients of variation were <2%.

Table 1 Volume of the pituitary gland, volume of total brain (with the exclusion of olfactory bulb), pituitary gland volume-total brain volume ratio, pituitary gland volume-body weight ratio, and ADC mean and median values (×10-3 mm^2/s) derived from the monoexponential analysis of DWI images from stressed and control mice ($n = 6$)

	CTR	SM	P-value
$Vol_{pituitary}$ (mm^3)	3.8 ± 0.1	4.7 ± 0.3	**0.04***
Vol_{brain} (mm^3)	363.7 ± 11.2	360.5 ± 10.8	0.84
[$Vol_{pituitary}$ (mm^3) /Vol_{brain} (mm^3)] ×10^3	10.6 ± 0.2	12.9 ± 0.7	**0.02***
[$Vol_{pituitary}$ (mm^3)/body weight (g)] ×10^3	71.2 ± 9	76.0 ± 12.2	0.46
ADC mean (mm^2/s) ×10^4	9.1 ± 0.6	8.7 ± 0.5	0.69
ADC median (mm^2/s) ×10^4	8.0 ± 0.2	8.2 ± 0.4	0.70

Statistical analyses were performed using unpaired t-test. Values are expressed as mean ± ES

*$P < 0.05$ for CTR vs SM

Hematoxylin and eosin staining

Formalin-fixed and paraffin-embedded mouse pituitary glands were cut into 2 µm-thin sections with a rotary microtome (HM355S, ThermoFisher Scientific, Waltham, MA, USA). Hematoxylin–eosin (H&E) staining on deparaffinized sections with Eosin and Mayer's Haemalaun solution and morphological evaluation were performed. To evaluate nuclei counts in the *pars distalis* of the pituitary gland, the imaging software ImageJ and its plugin Analyze Particles have been employed. To this purpose, each microphotograph was used to create a specific manual region of interest (ROI) for the *pars distalis* of the pituitary gland. Images (RGB) were converted to 16 bit types. At this point, a manual threshold (using the Default algorithm) has been setup for each image inside the *pars distalis* ROI. Thresholded ROIs have been subjected to automated nuclei counts (representing cells) by using the Analyze Particle tool. A size between 0.18 and 3.6 mm^2 has been setup to exclude non-nuclei patterns. ROI-restricted nuclei masks and counts are automatically generated for each thresholder *pars distalis* ROI into CTR and SM images. Finally, to obtain the cell density (no. of cells per mm^2) for each *pars distalis* into CTR and SM microphotographs, nuclei counts were divided by ROI area (mm^2).

Statistical analysis

All data are expressed as means ± SEM and were analysed using t-test or Mann-Whitney comparison between the CTR and SM groups (GraphPad Prism 8), as appropriate; $P < 0.05$ was considered as a threshold for significant difference.

Results

SM present an enlarged pituitary

Significant differences emerged in the mean volume of the pituitary gland while no differences have been detected in total brain volume. The mean pituitary volume between SM and CTR showed no statistical difference after adjustment for body weight. Conversely, the mean pituitary gland volume was significantly increased in SM compared to CTR after adjustment for the total brain volume (Table 1).

Moreover, the SM pituitary glands showed a significant mean increase in fresh weight as compared to CTR (Fig. 1I). Notably, the analysis of diffusion-weighted images did not lead to the identification of abnormal hyperintensity areas within the pituitary gland, as shown in Fig. 1D (CTR) and E (SM). Furthermore, quantitative analysis did not show significant differences in the ADC parameter corresponding to the cellularity of the tissue (Table 1). H&E staining and cell densities in the *pars distalis* of the pituitaries of stressed and CTR mice confirmed the absence of gross morphological and histo-pathological alterations (Fig. 1F).

ELP models' metabolic and neuroendocrine parameters

As expected, ELP procedures produced a significant increase in total body weight compared to CTRs (Fig. 1G). Moreover, SM presented levels of basal plasmatic corticosterone and pituitary ACTH higher than CTRs (Fig. 1H, L).

Discussion

Similarly to human adolescents subjected to ACEs [8], for the first time, findings showed an enlargement of the pituitary in an early-life stress mouse model. No abnormal hyperintensity areas within the pituitary gland were detected, and quantitative analysis did not show significant differences in the cellularity of the tissue, suggesting the absence of neoplasia, which is known to cause hypercortisolism in the endogenous CS. The apparent absence of tumors and of cell morphologic alterations in the *pars distalis* of the pituitary was further supported by H&E staining. Hence, we speculate that, in our model, the chronic hypothalamic stimulation on the corticotroph during the lactation period [7] could have led in adulthood to a corticotroph hyperplasia rather than to a corticotroph adenoma, thus explaining the absence of increased cellularity in SM [13]. In fact, an enlargement of the pituitary gland may be the effect of the enhanced corticotroph output to support the

increase in CRH-induced excitability [14, 15], due to a dynamical compensation by which the gland mass adjusts over time to buffer the variation in ACTH production [16]. Besides the anatomical data, an increment in pituitary ACTH content was also found suggesting a change in pituitary function. Since SM increase in body weight is caused by prolonged exposure to higher levels of corticosterone [17], it should not affect the pituitary volume.

From a translational point of view, our results support the link between the ACEs, pituitary size and cortisol level in adults [7]. Moreover, the absence of cellular alterations in the pituitary of the ELP model raises questions about where to frame its pathological phenotype within the clinical context. The evidence that ELP stressful procedures have effect on almost all male outbred mice would suggest a higher incidence of such subtle hypercortisolism by ACEs in humans, as well. Since early-life stress is a known risk factor for psychiatric diseases and adult obesity [14], high blood pressure and type 2 diabetes [15], it is possible that an unexpected number of patients may have subclinical hypercortisolism [16–18], which could be difficult to be suspected due to the lack of a specific phenotype [19]. On the other hand, the suggested correlation between ACEs and pituitary-dependent CS cannot exclude the ELP involvement in CS aetiology in predisposed subjects to develop ACTH-secreting pituitary hyperplasia [20–24]. However, despite our results finding many matches with clinical observations, differences between rodents and humans need to be considered as a limitation of this study, as the effective translational value of such an ELP model has still to be determined. Another limitation of this study to keep in mind is that the model analyzed is older compared to the phenotype of our previously described ELP mouse [7]. Therefore, although the pituitary ACTH and plasma corticosterone levels presented here reasonably suggest a long-lasting phenotype, the lack of data such as CRH levels also at this age prevents us from being certain of this.

Data availability

Data supporting this study are openly available in Mendeley data at: https://doi.org/10.17632/gnyynhcyzt.1.

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Author contributions G.C., R.C., S.L., and I.C. made substantial contributions to the conception and design of the work, defined the methodology, and supervised the entire process. T.D., A.F., F.M., O.M., V.Z., T.S., G.P., N.S.P., and R.R. made substantial contributions to the investigation and formal analysis. R.C., S.L., and I.C. wrote the original draft. V.F., N.C., Z.M., A.L., N.S.P., and S.L. critically revised and edited the manuscript providing important intellectual content. All authors approved the version to be published and agreed to be accountable for all aspects of the work to ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval This study was carried out in accordance with the guidelines of the Council of European Communities (European Communities Council Directive of November 24th, 1986, 86/609/EEC), and reviewed and approved by the Bioethical Committee of the Italian National Institute of Health (Istituto Superiore di Sanità — ISS), and the Italian Ministry of Health (n. 729/2019-PR, 04/11/2019).

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