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Comparative analysis of neurosteroid levels in normal and adenomatous human pituitary tissue

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Neurosteroids (NS) are neuroactive steroid hormones, synthesized in the nervous system. Prior research on NSs in pituitary tissue relied on animal investigations. The complete knowledge regarding the presence of these distinct hormones in the human pituitary gland is lacking. The objective of this study was to examine the presence of NS in tissue samples from human pituitary adenomas (PA) and how it differs from normal pituitary tissue (NPT). In this cross-sectional comparative study, 74 samples from human PAs, collected during transsphenoidal surgery and, 19 NPT, collected from age and sex matched cadavers, were included. The presence and concentrations of 17 different NS were examined by using the liquid-chromatography-mass-spectrometry in both PA tissue and preoperative blood samples of the individuals with PA. Tissue specimens were compared with those from NPT. Levels of Pregnenolone, 7α-Hydroxypregnenolone, androsterone, progesterone, and 17-Hydroxyprogesterone were higher in PA samples in comparison to NPT($p < 0.001$,$p = 0.004$,$p = 0.007$,$p = 0.04$ and $p = 0.007$,respectively). Levels of dehydroepiandrosterone-sulfate (DHEAS), corticosterone and cortisol were higher in NPT ($p = 0.01$, $p < 0.001$ and $p < 0.001$, respectively). The detection of corticosterone, 17OHPRG, and PRG, increased concentrations of DHEAS and cortisol, favors the identification of the tissue as an NPT rather than PA. The levels of NS in the PA group also varied based on hormonal status and differences in sex. Of the NS in PA, only tissue levels of DHEAS, 17-Hydroxyprogesterone and cortisol were positively correlated with their blood levels ($r = +0.6$, $p < 0.001$; $r = +0.7$, $p = 0.005$ and $r = +0.4$, $p < 0.001$, respectively). NS is present both in PAs and in NPT without adenomas. The quantity and varieties of NS in PAs are impacted by the hormonal status of the PA and sex of the subjects. NS could be involved in the regulation of pituitary hormones and the development of adenomas.

Keywords Pituitary, Adenoma, Neurosteroid, Steroid

The pituitary gland is a major endocrine organ which controls multiple essential hormone-secreting processes. It controls adrenal and gonadal steroidogenesis through adrenocorticotropic hormone (ACTH), follicle-stimulating hormone (FSH) and, luteinizing hormone (LH) secretion.

Classically known steroid hormones, synthesized by adrenal glands and gonads, have impact on distant tissues and act through intracellular receptors. Neurosteroids (NS) are also steroid structured hormones and the pathways for synthesizing these steroids are the same as the classically known ones[1]. However, NS have certain differences: (i) *de novo* synthesis in central (CNS) and peripheral nervous systems (ii) activity through cellular surface receptors (iii) paracrine effects in addition to autocrine ones (iv) neuroactivity[2]. It has been long known that glial cells and neurons in certain brain regions have capability of NS synthesis[2]. In this respect, the nervous system is also a contributor to steroidogenesis.

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A few preceding studies have indicated the synthesis and functions of NS in the pituitary gland[3,4]. These studies were predominantly conducted using pituitary tissue from non-human specimens. It is yet to be determined whether human pituitary gland is a source or target of these special group of hormones. Moreover, to our knowledge, none of the previous studies have evaluated the changes in pituitary adenomas in this regard.

In this study we aimed to evaluate presence of NS in tissue specimens obtained from human pituitary adenomas (PA) in comparison to normal pituitary tissue (NPT).

Materials and methods

Tissues and blood samples

In this cross-sectional comparative study 74 tissue specimens were obtained from human PA. Participants with a background of head trauma, cerebrovascular event, prior brain surgery, psychosis, or substance abuse were excluded from the research.

Specimens from PAs were obtained during endoscopic transsphenoidal surgery between 2021 and 2023 and stored at − 80 °C. Of the 74 PAs 40 were non-functioning, adenoma (NFA), 20 were somatotropinomas, 9 were corticotropinomas and 5 were prolactinomas.

For evaluation of blood NS levels, preoperative venous blood samples taken at 08:00–10:00 am, while fasting. The samples were stored at − 80 °C. They were first moved to -20 °C and then stored at + 4 °C prior to the day of study. Samples were held at room temperature for an hour prior to being studied.

Pituitary tissues from 19 cadavers were acquired during forensic autopsies. The samples were preserved at − 80 °C. Subjects with past of head trauma, cerebrovascular events, or prior brain surgery were excluded. Additional information on cadavers is available in Table 1.

Analysis of NS

Analyzes related to NS were carried out at the biochemistry laboratory of Altium International Laboratory Devices Inc.The gold standard method of analysis, Liquid Chromatography Mass Spectrometry (LC-MS/MS), was employed to evaluate NS levels. Analyzes of samples were performed on Agilent Infinity 1290 HPLC system (Agilent Technologies, Santa Clara, CA, USA) consisting of binary pump(G4220A), column compartment(G1316C) and autosampler(G7167B) coupled to 6470 triple quadrupole mass spectrometry(6470 A, Agilent Technologies, Santa Clara, CA, USA) with an Agilent Jet Stream technology(AJS) electrospray ionization(ESI) source (Agilent Technologies, Santa Clara, CA, USA). For the measurement of the concentrations of steroid hormones, Jasem Steroid Hormones LC-MS/MS analysis kit (JSM-CL-6500) was used (Altium International Laboratuvar Cihazları A.Ş., Istanbul, Turkey). Kit components applied throughout the analyzes were as follows: analytical column specified for the analysis of steroid hormones (JSM-CL-6575), mobile phases (mobile phase A; JSM-CL-6501 and mobile phase B; JSM-CL-6502), stable isotope labelled steroid hormones solution as internal standards (JSM-CL-6507), sample treatment reagents (JSM-CL-6504 and JSM-CL-6505), chromatographic and mass detection parameters. Validation parameters of Jasem Steroid Hormones

Sample no	Gender	Age	Pmi	Incident	Cause of death	Medical history	Toxicology
1	Male	66	21	Collapse	Cardiovascular	NA	Negative
2	Male	69	24	Asphyxia	Asphyxia	NA	Negative
3	Male	66	15	Collapse	Unknown	NA	Negative
4	Male	43	12	Asphyxia	Asphyxia	NA	Negative
5	Male	59	18	Collapse	Cardiovascular	Cardiovascular disease	Atorvastatin, Pantaprazole, Ramipril, Clopidogrel, Quetiapine
6	Male	48	5	Collapse	Cardiovascular	Cardiovascular disease	Negative
7	Female	23	8	Drowning	Drowning	NA	Blood; Ethanol, Venlafaxine, Nordazepam, Diazepam, Medazepam, Atropine. Urine: Cocaine, Benzoylecgonine, Venlafaksine, Nordazepam, Atropine.
8	Female	69	5	Collapse	Cardiac tamponade	NA	Negative
9	Male	23	7	Drowning	Drowning	NA	Blood: Ethanol, Pantoprazole, Atropine. Urine: Benzoylecgonine
10	Female	65	14	Found dead	Peritonitis	NA	Negative
11	Female	54	12	Collapse	Cardiovascular	Diabates	Negative
12	Male	54	6	Collapse	Cardiovascular	Diabates	Negative
13	Male	60	12	Collapse	Cardiovascular	NA	Negative
14	Male	60	8	Collapse	Cardiovascular	Diabates	Negative
15	Female	35	12	Asphyxia	Asphyxia	None	Negative
16	Male	69	12	Collapse	Cardiac tamponade	Diabates	Negative
17	Female	52	13	Collapse	Cardiovascular	Hypertension	Negative
18	Female	64	16	Collapse	Unknown	Hypertension	Negative
19	Female	64	8	Collapse	Disseminated tumor	Malignancy	Pantaprazole, Clozapine, Lidocaine, Chlorphenamine, Nordazepam, Pseudoephedrine, Diazepam.

Table 1. Characteristics of Cadevers. *Pmi* post mortem interval (hours), *NA* not available.

LC-MS/MS Analysis Kit are presented in Table 2. The proposed method was validated according to the Q2(R1) ICH guidelines[5]. Data acquisition and quantification were carried out using Agilent Mass Hunter Acquisition and Quantitative Analysis software programs, respectively.

The ethical committees of Basaksehir Cam and Sakura City Hospital (2021.12.280) and The Council of Forensic Medicine of Turkey (21589509/2021/1427) approved the study. Informed consents were provided from the patients with pituitary adenomas and the study was conducted in accordance with the Declaration of Helsinki. Adhering to the Medicolegal Autopsy guidelines of our country, normal tissue samples were obtained during forensic analysis.

Data was analyzed using SPSS 25.0. Normal distribution was examined using the Kolmogorov-Smirnov test. Student's t-test and Mann-Whitney U test were applied to compare continuous variables with normal and non-normal distributions. Multiple group medians were compared through the Kruskal-Wallis test. Associations between continuous variables were assessed with Spearman's correlation, and the χ^2 test was used to compare categorical variables. $p < 0.05$ was considered statistically significant.

The data were additionally analyzed using Partial Least Squares Discriminant Analysis (PLS-DA), a robust method for classifying high-dimensional data. This technique was chosen due to its ability to handle large numbers of predictors and its capacity to reveal latent structures that distinguish between predefined groups in the dataset. The analysis was performed using the **mixOmics** package in R (version 4.4.3), with the target variable (response) being the class labels, which were categorized as factor variables. PLS-DA loading values indicate the contribution of each variable to the components, while variable importance point (VIP) scores were used to assess the discriminative power of variables within the PLS-DA model[6].

Results

The mean ages of PA and NPT groups were 51 ± 14.2 and 54.9 ± 14.5 years, respectively ($p = 0.3$). Female/Male ratio was 40/34 in PA and was 8/11 in NPT group ($p = 0.4$).

Detailed data on NS are presented in Table 3. Pregnenolone (PREG) was more abundant in NPT (100% vs. 55%, $p < 0.001$), yet its levels were elevated in PA samples ($p < 0.001$). Also, 7α-Hydroxypregnenolone (7αOHPREG), androsterone, progesterone (PRG), and 17-Hydroxyprogesterone (17OHP) were more prevalent in NPT ($p = 0.008$, $p = 0.002$, $p < 0.001$ and $p < 0.001$, respectively), albeit in elevated concentrations in PA ($p = 0.004$, $p = 0.007$, $p = 0.04$ and $p = 0.007$, respectively). The presence of 4-Androsten-3,17-dione (AE), testosterone (T), dihydrotestosterone (DHT), and aldosterone was more common in NPT (100%, 100%, 53% and 26%, respectively) than in PA (53%, 54%, 5% and 3%, respectively) ($p < 0.001$, $p < 0.001$, $p < 0.001$ and $p = 0.001$, respectively), with comparable levels in both tissue types ($p = 0.7$, $p = 0.5$, $p = 0.1$ and $p = 0.9$, respectively). Both availability and levels of corticosterone were higher in NPT ($p < 0.001$ and $p = 0.01$, respectively). In both PA and NPT, Dehydroepiandrosterone (DHEA) (80% of PA and 74% of NPT, $p = 0.6$) and DHEAS (99% of PA and 100% of NPT, $p = 0.6$) were commonly found, with similar levels of DHEA ($p = 0.6$) and higher levels of DHEAS in NPT ($p < 0.001$). 11-Deoxycortisol was found in all samples (100%) collected from PA and NPT, with similar levels ($p = 0.5$). Cortisol was present in a large percentage of samples from both PA (96%) and NPT (100%), with higher concentrations in NPT specimens ($p < 0.001$). 11-deoxycorticosterone (DOC) were measured in 30% of PA and 42% of NPT ($p = 0.3$), with comparable levels ($p = 0.2$) (Fig. 1). Of note, 17-Hydroxypregnenolone

Analytes	LOD (µg/L)	LOQ (µg/L)	Linearity (R^2)	Accuracy (%)	Repeatability (RSD%)
Pregnenolone	0.033	0.108	0.994	77.49	10.46
7α-Hydroxypregnenolone	0.012	0.038	0.998	98.54	3.8
17-Hydroxypregnenolone	0.145	0.480	0.997	100.44	9.24
Dehydroepiandrosterone	0.102	0.336	0.998	109.62	6.66
Dehydroepiandrosterone Sulfate	4.945	16.32	0.997	103.47	2.63
4-Androstene 3,17-dione	0.023	0.076	0.998	109.93	1.99
Testosterone	0.008	0.026	0.997	96.75	1.8
Dihydrotestosterone	0.012	0.041	0.997	101.98	14.36
Estradiol (Oestradiol)	0.015	0.051	0.998	109.37	6.32
Androsterone	0.035	0.115	0.994	93.7	13
Progesterone	0.033	0.108	0.994	108.37	4.45
Allopregnanolone	0.011	0.035	0.998	97.45	4.6
11-Deoxycorticosterone	0.016	0.052	0.994	95.34	6.84
Corticosterone	0.054	0.179	0.998	98.42	4.09
Aldosterone	0.011	0.035	0.998	99.81	6.34
17-Hydroxyprogesterone	0.028	0.091	0.997	97.01	4.8
11-Deoxycortisol	0.015	0.048	0.998	102.23	4.45
Cortisol	0.102	0.336	0.999	89.74	1.69

Table 2. Validation parameters of Jasem steroid hormones LC-MS/MS analysis kit. *LOD* limit of detection, *LOQ* limit of quantitation, *RSD* relative standard deviation.

	PA ($n=74$)	NPT ($n=19$)	$p^{\S}$
PREG			
Availability*	41(55%)	19 (100%)	**<0.001**
Level**	89.5 [12.6-184.7]	7.2 [3.1–15.6]	**<0.001**
7αOHPREG			
Availability*	53 (72%)	19 (100%)	**0.008**
Level**	46.4 [15.2-125.3]	16.2 [7.5–24.1]	**0.004**
17OHPREG	–	–	–
DHEA			
Availability*	59 (80%)	14 (74%)	0.6
Level**	11.1 [5.7–25.7]	9.9 [6.2–14.7]	0.6
DHEAS			
Availability*	73 (99%)	19 (100%)	0.6
Level**	26.9 [10.1–53.4]	152.3 [83.6-210.8]	**<0.001**
AE			
Availability*	39(53%)	19(100%)	**<0.001**
Level**	0.9 [0.4–1.7]	1 [0.5–1.7]	0.7
T			
Availability*	40 (54%)	19 (100%)	**<0.001**
Level**	0.8 [0.5–1.4]	1.2 [0.5–1.7]	0.5
DHT			
Availability*	4 (5%)	10 (53%)	**<0.001**
Level**	39.5 [1.2–93.8]	0.7 [0.6–0.8]	0.1
E2	-	-	-
Androsterone			
Availability	42(58%)	18 (95%)	**0.002**
Level**	14.8 [3.2–34.3]	4.5 [2.4–5.9]	**0.007**
PRG			
Availability*	7 (10%)	15 (79%)	**<0.001**
Level**	0.9 [0.5–5.3]	0.4 [0.2–0.6]	**0.04**
ALLO			
Availability*	1 (1%)	-	-
Level**	148.5		
DOC			
Availability*	22 (30%)	8 (42%)	0.3
Level**	0.5 [0.4–0.9]	0.4 [0.2–0.6]	0.2
Corticosterone			
Availability*	15 (20%)	19 (100%)	**<0.001**
Level**	0.9 [0.6–4.1]	13.2 [1.4–17.6]	**0.01**
Aldosterone			
Availability*	2 (3%)	5 (26%)	**0.001**
Level**	0.7 [0.4-]	0.4 [0.3–1.1]	0.9
17OHPRG			
Availability*	14 (19%)	16 (84%)	**<0.001**
Level**	0.8 [0.5–1.7]	0.3 [0.1–1.1]	**0.007**
11 Deoxycortisol			
Availability*	74 (100%)	19 (100%)	
Level**	1.2 [0.8–2.8]	1.3 [0.5–2.5]	0.5
Cortisol			
Availability*	70 (96%)	19 (100%)	0.4
Level**	6 [2.6–10.4]	56.5 [9.7–88.3]	**<0.001**

Table 3. Comparison of neurosteroid availability and levels between pituitary adenoma and normal tissue. ❡Statistically significant p values are shown in bold * n (%) ** ng/g, Levels presented as median and interquartile range [IQR] PA-Pituitary adenoma, NPT-Normal pituitary tissue, PREG-Pregnenolone, 7αOHPREG- 7α-Hydroxypregnenolone, 17OHPREG-17-Hydroxypregnenolone, DHEA-Dehydroepiandrosterone, DHEAS Dehydroepiandrosterone sulfate, AE-4-Androstene 3,17-dione, T-Testosterone, DHT-Dihydrotestosterone, E2- Estradiol, PRG-Progesterone, ALLO-Allopregnanolone, DOC-11deoxycorticosterone, 17OHPRG-17Hydroxyprogesterone.

(17OHPREG) and estradiol (E2) were not available in the tissues from PA or NPT. Allopregnanolone (ALLO) was available in one tissue sample obtained from an NFA.

Figure 2 and Table 4 demonstrate results of PLS-DA which suggest that availability of corticosterone (VIP score: 1.74), 17OHPRG (VIP score: 1.61) and PRG (VIP score: 1.67) contributed significantly to the model's ability to distinguish between the tissue groups (for NPT-AUC: 1, [CI:1, 1] and, for PA AUC: 0.63, [CI: 0.58, 0.68]). When the results shown in Table 3 are taken into account, this investigation demonstrated that the presence of corticosterone, 17OHPRG, and PRG signifies that the tissue is to be categorized as NPT. Figure 3 and Table 5 demonstrate results of PLS-DA based on the tissue levels of NS. Higher tissue levels of DHEAS (VIP score: 2.51) and cortisol (VIP score: 1.78) contributed significantly to the model's ability to distinguish between the tissue groups. Together with this analysis and the data represented in Table 3, enhanced levels of DHEAS and cortisol were noted to be in alignment with NPT.

Details regarding NS in PA subgroups are outlined in Table 6. Tissue PREG abundance (85%) and level (105.2 [IQR:54.9-214.4] ng/g) was highest in the samples obtained from NFA ($p < 0.001$ and $p = 0.001$, respectively). Samples from corticotropinomas had the lowest availability of 7αOHPREG (33%, $p = 0.002$). The levels of 7αOHPREG were similar across subtypes of PAs, although tissues from corticotropinomas and prolactinomas tended to have lower levels ($p = 0.05$). Tissue levels of DHEA was highest in prolactinoma samples and lowest in NFA ($p = 0.006$). T was found to be least present in corticotropinoma samples (22%) and prolactinomas (20%), while being present in 68% of NFA and 50% of somatotropinomas ($p = 0.03$). Its levels were comparable among the subgroups ($p = 0.2$). Despite 100% availability of 11-Deoxycortisol in each subgroup, the levels varied among the subgroups, lowest in the tissue samples of NFA and highest in the tissue samples from prolactinomas ($p < 0.001$).

Table 7 shows blood levels of NS in cases with PAs. In cases with PA tissue levels of 7αOHPREG tended to be negatively correlated with its blood levels (r=-0.3, $p = 0.05$). Tissue levels of DHEAS, 17OHPRG and cortisol were positively correlated with their blood levels ($r = +0.6$, $p < 0.001$; $r = +0.7$, $p = 0.005$ and $r = +0.4$, $p < 0.001$, respectively). There was not any other correlation between the tissue and/or blood levels of NS (data not shown here).

The comparison of NS levels and availability in NPT and PA tissues between males and females is depicted in Table 8. Female cases showed a greater presence of DOC and aldosterone in specimens from NPT compared to male cases ($p = 0.01$ and $p = 0.05$, respectively). Nevertheless, their levels were similar between female and male subjects ($p = 0.07$ and $p = 0.4$, respectively). Levels of PREG, AE, PRG, 17OHP, and 11-Deoxycortisol were elevated in samples from NPT of female cases ($p = 0.001$, $p = 0.02$, $p = 0.001$, $p = 0.03$ and $p = 0.009$, respectively), even though their availability were alike. Among cases with PA availability of PREG and T were higher in specimens obtained from males ($p = 0.02$ and $p < 0.001$, respectively). In PA group 11-Deoxycortisol levels tended to be higher in specimens obtained from females ($p = 0.05$). There was no additional differentiation between males and females in terms of the availability or levels of other NS.

Discussion

The current study reveals the existence of neurosteroids in both normal pituitary and pituitary adenoma tissues in humans, with certain variations in the levels and proportions of specific neurosteroids. Also, the types and levels of specific neurosteroids may change according to the hormonal activity of pituitary adenomas. Additionally, sex disparities could impact the diversity in neurosteroid levels within tissues.

Hormones with a steroidal structure, which are synthesized in the nervous system and act through cell surface receptors are known as NS[2,7]. NS execute their activities mainly through γ-aminobutyric acid (GABA-A) receptors[2]. Within the nervous system, they regulate behavioral, neuroendocrine and metabolic activities[7]. In addition, due to their lipophilic structure, the blood-brain barrier (BBB) is permeable to NS[2]. Similar to other parts of the CNS, pituitary gland also expresses steroidogenic enzymes[8,9]. Moreover, GABA receptors, primary targets of NS, have been revealed in the pituitary gland and they have inhibitory effects on the hypothalamic-pituitary-adrenal axis (HPA)[3,10]. It has been established that the HPA axis and an array of NS exhibit mutual interactions[11–15]. NS-pituitary hormone interaction is not restricted to the HPA axis only. Moreover, interactions can be identified between prolactin (PRL) and NS[3,4,16,17]. NS might also affect hormone release from the posterior pituitary[18]. These associations between NS and pituitary hormones imply that the production of pituitary hormones could be more intricate and multifaceted than the familiar feedback mechanisms which we are aware of. Previous evidence for the existence and functions of NS in the pituitary gland was founded on animal experiments. To date, there have been no studies that demonstrated the existence or concentrations of NS in human tissue samples from the anterior pituitary gland or PA.

In this study DHEA, DHEAS, 11-Deoxycortisol, and cortisol were found to be significantly present in tissues from NPT as well as in tissues from PAs. While DHEA and 11-Deoxycortisol levels were comparable

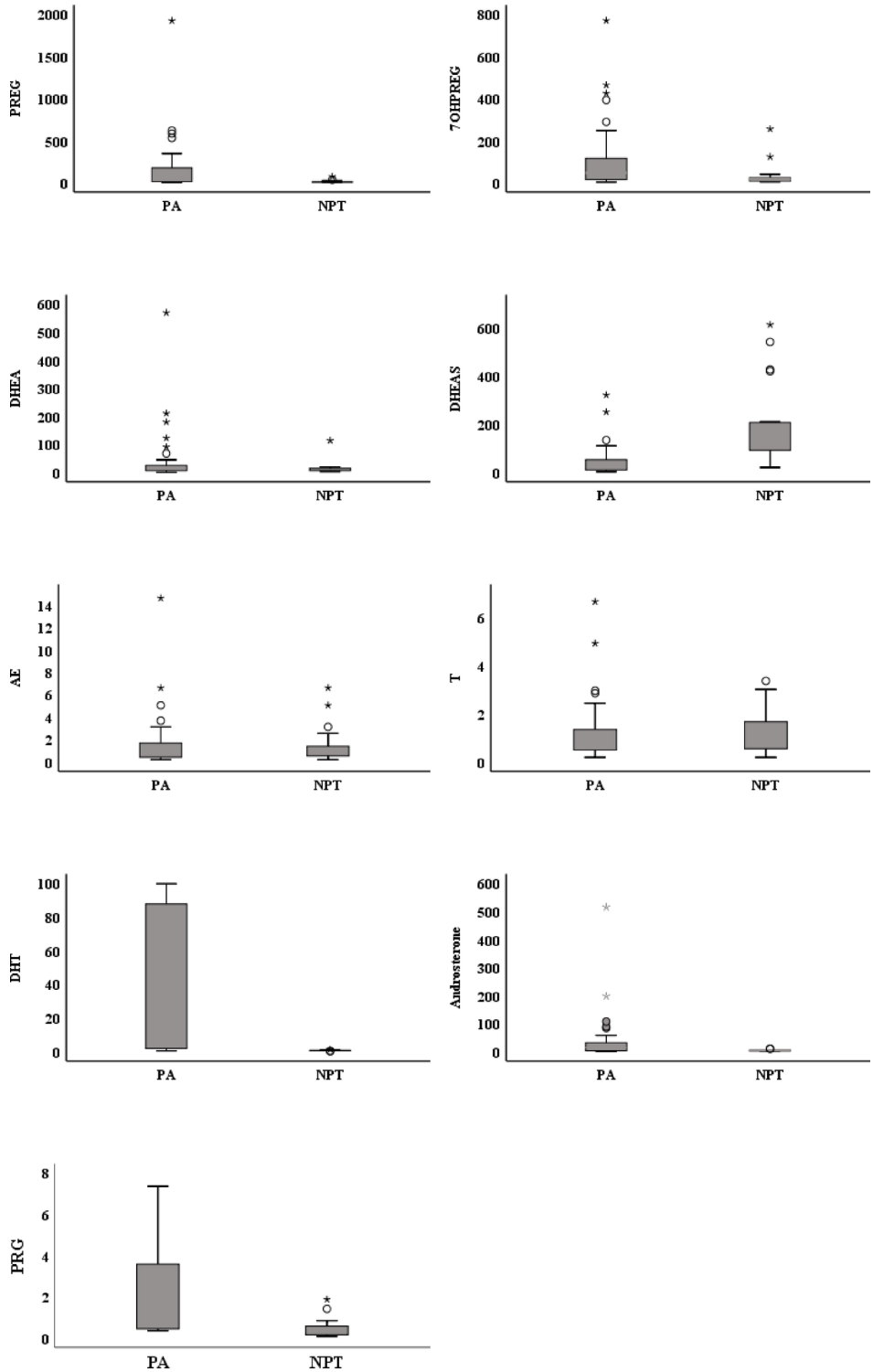


Fig. 1. Comparison of NS levels between tissue samples obtained from human PA and NPT. PA-Pituitary adenoma, NPT-Normal pituitary tissue PREG-Pregnenolone, 7αOHPREG--7α-Hydroxypregnenolone, DHEA - Dehydroepiandrosterone, DHEAS-Dehydroepiandrosterone sulfate, AE-4-Androstene3,17-dione, T-Testosterone, DHT-Dihydrotestosterone, PRG-Progesterone, DOC-11deoxycorticosterone, 17OHPRG-17Hydroxyprogesterone.

in both tissue types, DHEAS and cortisol concentrations were notably higher in NPT. In the analysis of PA subgroup by hormonal status, the highest levels of DHEA and 11-Deoxycortisol were observed in prolactinoma specimens, with elevated cortisol levels seen in both prolactinomas and corticotropinomas. Previous studies have demonstrated that DHEA boosts the release of PRL from the pituitary gland[19]. The presence of higher DHEA

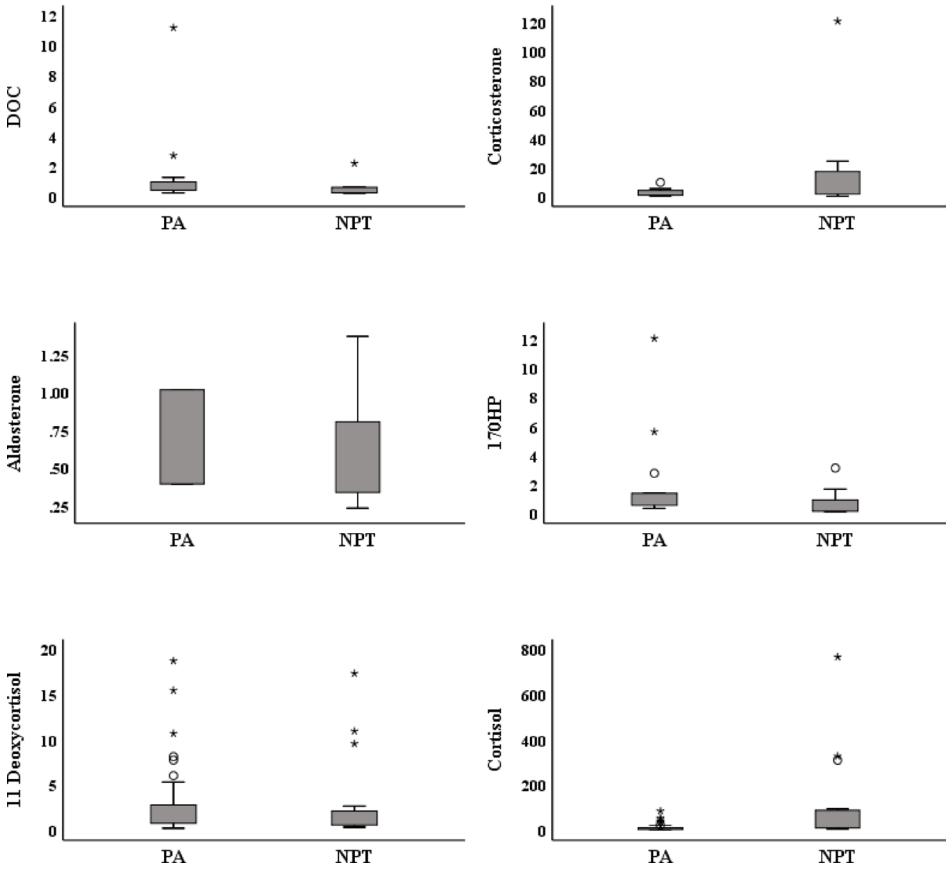


Fig. 1. (continued)

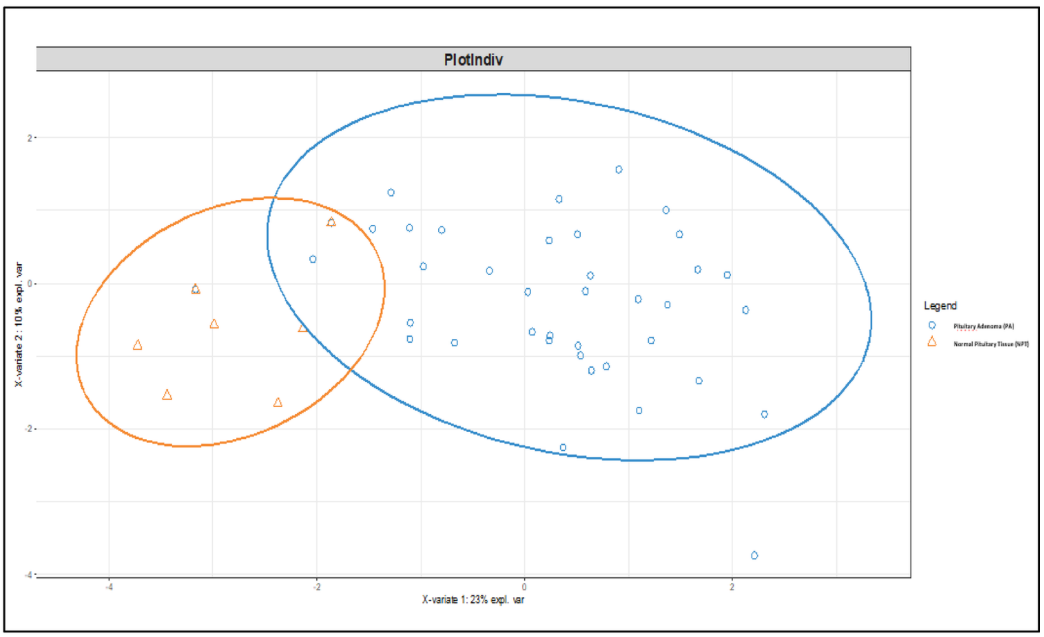
levels in prolactinomas may potentially impact hormone production in this subset of PAs. Earlier research has also outlined the link between high levels of PRL in the blood and an increase in the production of adrenal steroid hormones, specifically DHEAS[20]. Elevated PRL levels in prolactinomas might play a role in the increased levels of DHEA, 11-Deoxycortisol, and cortisol found in prolactinoma tissues in this research. The question of whether the secreted hormone modifies NS levels in PA or if NS contribute to adenoma secretion through an influence on the hormonogenesis remains unanswered and falls outside the scope of this study.

PREG, the first precursor of NS, with 100% availability was more frequently available in NPT in comparison to PA. During the subgroup analysis, it was found that NFA exhibited greater availability and the highest concentrations of PREG, which accounted for the higher concentrations of PREG in PAs compared to NPT. PREG was less prevalent in other PA categories. PREG and its sulfate derivative have a neuroprotective effect in both the CNS and peripheral nervous system[21]. The sulfated form of PREG has been demonstrated to stimulate GH3 cell proliferation in the rat pituitary gland[4]. After observing this result in rats and the high levels of PREG in NPT and NFA in our study, it sparks speculation about the role of PREG in the pituitary gland.

Furthermore, 7αOHPREG, PRG, 17OHP, and androsterone, were more commonly found in NPT. Despite this, PAs had greater levels of each of these NS than NPTs. In the current study, 7αOHPREG was widely present in both human PA and NPT, with higher concentrations in PA. Corticotropinomas had the lowest presence at 33%, while at least 60% of other PA subgroups contained 7αOHPREG. The levels of 7αOHPREG were found to be the least in corticotropinomas and prolactinomas. NFA and somatotropinomas exhibited substantial quantities of 7αOHPREG. In animal studies, 7αOHPREG, derived from PREG, has been detected in the brain and is known to increase movement in vertebrates through dopamine system activation[22]. PRL plays a role in controlling the production of 7αOHPREG in the brain[22]. However, until this research, there has been a lack of information regarding the existence of 7αOHPREG in the pituitary gland. Further investigation is required to explore the role of 7αOHPREG, particularly in pituitary tissue, through dedicated research studies. PRG, the second product of PREG, was also more available in NPT. However, median levels in PA were 2 times more than the median levels in the NPT. In addition to PRG, 17OHP, which is a direct metabolite of PRG, appeared more often in NPT, but its concentrations were elevated in PA tissue. Certain studies indicate the contribution of PRG to the growth of PAs[23]. Another direct product of PREG; 17OHPREG, was available neither in PA nor in the NPT. Also, ALLO, a direct product of PRG, was present in only one NFA specimen.

Corticosterone and its metabolite aldosterone exhibited higher frequency of presence in NPT samples. Also, corticosterone levels were higher in NPT. In humans, the concentration of plasma corticosterone is 10–20 times lower than the primary glucocorticoid cortisol[24]. However, it can permeate BBB easily and the brain tissue displays a greater corticosterone to cortisol ratio than the plasma[25]. Its role in the CNS may be more critical than

A



B

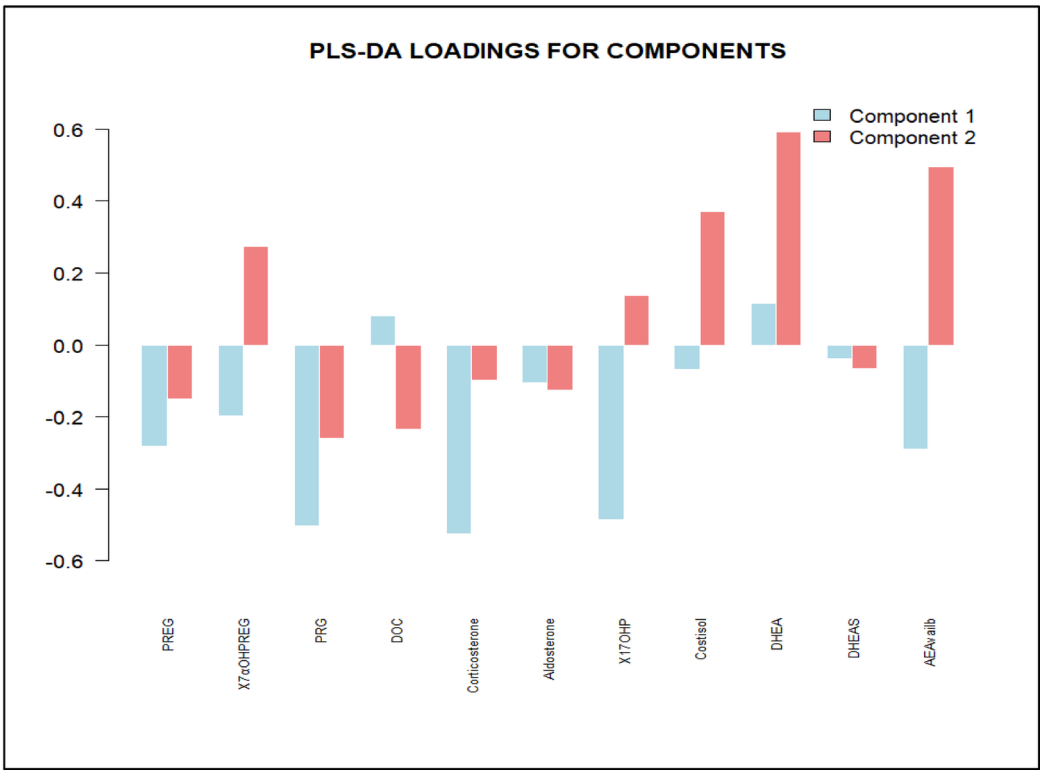


Fig. 2. PLS-DA based on availability of NS within the both tissue groups. (**A**) Component 1 explains 23% of the variance and Component 2 explains 10% of the variance. (**B**) The loadings for both components are presented together, visually demonstrating the contribution of each variable to both components.

	Comp1	Comp2
Metabolite		
PREG	-0.28	-0.15
7αOHPREG	-0.19	0.27
PRG	-0.50	-0.26
DOC	0.08	-0.23
Corticosterone	-0.52	-0.09
Aldosterone	-0.10	-0.12
17OHP	-0.48	0.14
Costisol	-0.07	0.37
DHEA	0.12	0.59
DHEAS	-0.04	-0.07
AE	-0.29	0.49
VIP Score		
PREG	0.94	0.91
7αOHPREG	0.65	0.67
PRG	1.67	1.62
DOC	0.27	0.34
Corticosterone	1.74	1.67
Aldosterone	0.35	0.35
17OHP	1.61	1.55
Costisol	0.22	0.41
DHEA	0.38	0.67
DHEAS	0.13	0.14
AE	0.96	1.03

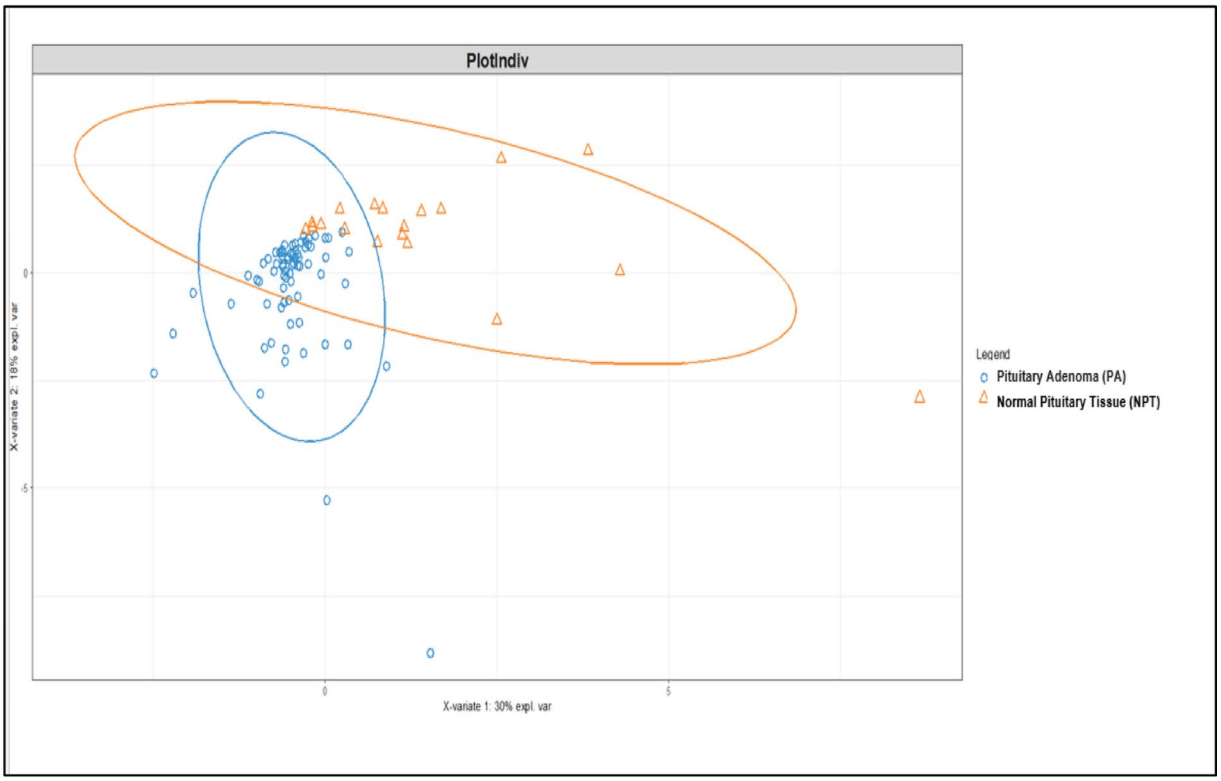
Table 4. The PLS-DA loading score and variable importance point (VIP) score of PLS-DA based on availability of neurosteroids within the tissues. *Comp* component.

on the periphery. Nevertheless, the scenario has shifted to favor cortisol dominance in the pituitary tissue in our research. Interestingly in NPT availability of both cortisol and corticosterone were 100%, with significantly higher cortisol levels. Cortisol was present in 96% and corticosterone was present 20% of PA samples, again with higher cortisol levels. To elucidate this discrepancy in pituitary tissue, investigating enzymatic activities would be useful. DOC, precursor of corticosterone, was present in 42% of NPT and 30% of PA, with similar concentrations within the two tissue types. Aldosterone was less available both in NPT and PA, again with similar concentrations in both groups. Of note, the two PA samples, in which aldosterone was detectable, were of NFA tissues. A previous research demonstrated that aldosterone levels were diminished in the brains of intact rats in contrast to their plasma levels, but in adrenalectomized rats, the levels were elevated in the brain compared to the plasma[26]. These findings might shed light on the reason for the low levels of aldosterone found in pituitary tissue in our investigation. Aldosterone synthesis could potentially occur in the brain, and it might be activated when required. As a strong mineralocorticoid and a precursor of aldosterone, the statement could also apply to DOC.

Of the NS that have androgenic actions; AE, T and DHT exhibited higher frequency of presence in NPT, while their levels being similar in both NPT and PA. In the PA subgroup analysis, T was found more commonly in NFA, while the levels were comparable across the hormonal subgroups. Of note, T was found in 50% of somatotropinomas, whereas only around 20% of corticotropinomas and prolactinomas had T detectable. The anterior pituitary has been recognized as a site where testosterone can be metabolized into its derivatives[27]. In this study, NPT had a significant presence of AE and androsterone, with DHT being less prominent and androsterone having the highest concentration. In PA, T, along with its metabolites AE, DHT, and androsterone, showed decreased presence, while androsterone levels were again higher in comparison to other androgens. AE acts as the precursor for both T and estrogens, being a less powerful androgen that gets converted into the more potent T in peripheral tissue. And, of the T metabolites, DHT is a powerful androgen, while androsterone is less potent. The results of this research indicate that T is frequently present in the pituitary gland, yet it might be converted into its less potent forms. E2 was present in none of the specimens obtained from PA and NPT. The estrogen receptor controls the transformation of cells into lactotroph adenoma in the pituitary gland[28]. Lactotroph adenomas harbor also testosterone receptors and furthermore, aromatization of testosterone may contribute in development of lactotroph adenomas[29]. Absence of E2 and presence of less active androgens in PA may be a protective mechanism. Yet, further evidence should be provided through more focused studies on this topic.

In essence, notable variations were identified in the presence and levels of NS between NPT and PA tissue. To investigate the contribution of each NS to tissue type predictions, we carried out a PLS-DA analysis. The identification of corticosterone, 17OHPRG, and PRG could imply a significant probability that the tissue is categorized as NPT. A further investigation conducted through PLS-DA, analyzing the levels of various NS

A



B

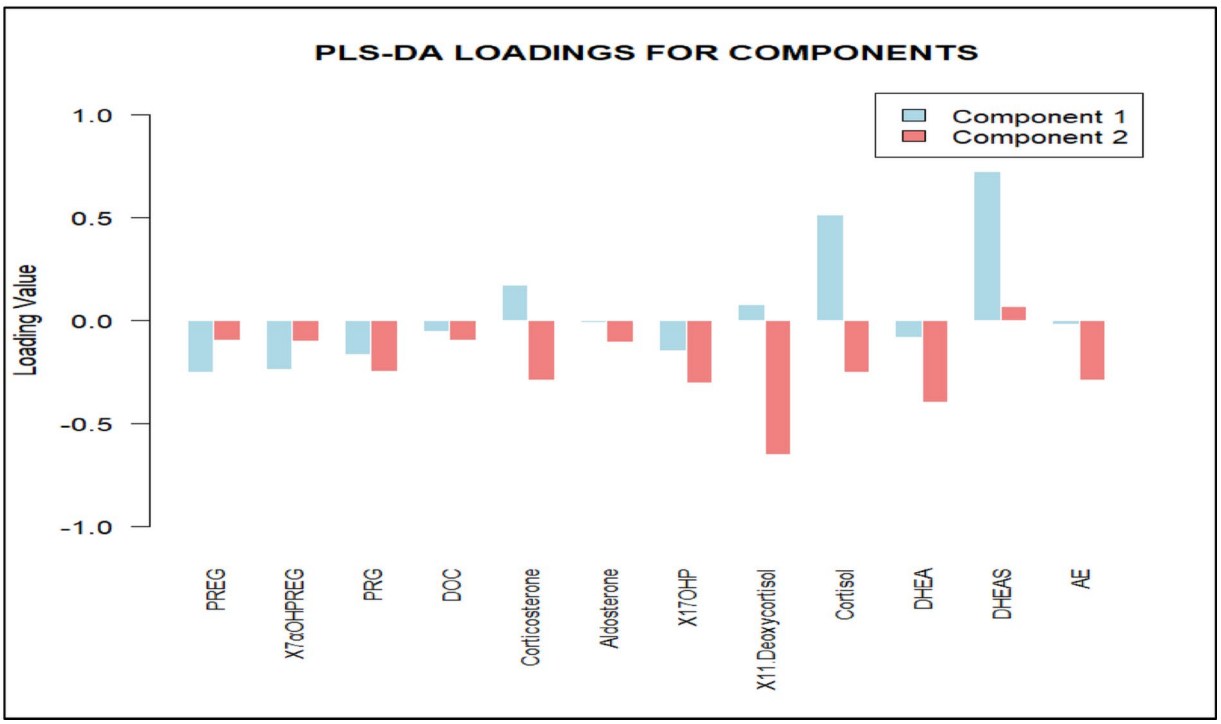


Fig. 3. PLS-DA based on the tissue levels of NS within the both tissue groups. (**A**) Component 1 explains 30% of the variance. Component 2 explains 18% of the variance. (**B**) The loadings for both components are presented together, visually demonstrating the contribution of each variable to both components.

	Comp1	Comp2
Metabolite		
PREG	-0.25	-0.09
7αOHPREG	-0.24	-0.09
PRG	-0.16	-0.25
DOC	-0.05	-0.09
Corticosterone	0.17	-0.29
Aldosterone	-0.01	-0.10
17OHP	-0.15	-0.30
11-Deoxycortisol	0.08	-0.65
Cortisol	0.51	-0.25
DHEA	-0.08	-0.39
DHEAS	0.72	0.07
AE	-0.02	-0.29
VIP Score		
PREG	0.86	0.77
7αOHPREG	0.83	0.74
PRG	0.56	0.64
DOC	0.18	0.22
Corticosterone	0.59	0.71
Aldosterone	0.03	0.17
17OHP	0.50	0.66
11-Deoxycortisol	0.27	1.09
Cortisol	1.78	1.62
DHEA	0.28	0.69
DHEAS	2.51	2.21
AE	0.06	0.48

Table 5. The PLS-DA loading score and variable importance point (VIP) score of PLS-DA based on the tissue levels of neurosteroids within the tissues. *Comp* component.

within the tissues, demonstrated that heightened levels of DHEAS and cortisol supported the classification of the tissue as an NPT.

Moreover, we assessed the potential link between NS levels in PA and preoperative blood levels, as NSs have the ability to cross the BBB. There was a tendency for tissue levels of 7αOHPREG to decline with increasing blood levels. This could imply that the existence of 7αOHPREG in pituitary tumors does not rely on crossing the BBB. There is still uncertainty regarding whether 7αOHPREG is actually produced in the pituitary, based on this data. Paracrine mechanisms could potentially be involved, meaning 7αOHPREG in another portion of the brain may be transmitted and act in the pituitary gland. Conversely, higher blood levels of DHEAS, 17OHPRG, and cortisol in patients with PA led to an increase in their tissue levels. These NSs could potentially cross the BBB and their concentrations in PA might be influenced by this transit. There was no relationship observed between the tissue and blood concentrations of other NSs. This could indicate that the origin might be located inside the CNS. However, it is difficult to say whether the origin in the CNS is pituitary or non-pituitary. Because the pituitary has an extensive blood supply network and is under the influence of hormones produced in the hypothalamus via the hypothalamo-hypophyseal portal circulation. Even if the source is non-pituitary, this does not negate the presence of NS in the pituitary. The involvement of NS in pituitary hormone interactions, whether paracrine or autocrine, cannot be ruled out.

Variances in sex may also have an impact on the presence and amounts of particular neurotransmitters identified in the pituitary gland. NPT samples collected from females had higher availability of both DOC and aldosterone compared to those from males. Concentrations of PREG, AE, PRG, 17OHP, and 11-Deoxycortisol were also raised in NPT samples from female cases. Sex-based variations were also observed in certain NSs within PA tissues. PA specimens from males had greater availability of PREG and T compared to specimens from females, while specimens from females tended to show higher concentrations of 11-Deoxycortisol. The impact of sex on blood NS levels had been previously documented[30]. Given the influence of certain NS such as T and E2, disparities in sex are to be expected, but differences in the levels and effects of other NSs are intriguing.

This study was not devoid of certain limitations. NS levels may change with estrogen status and also may show seasonal variations in brain. Selecting a precise menstrual phase for surgery in premenopausal women was

	NFA ($n=40$)	Somatotropinoma ($n=20$)	Corticotropinoma ($n=9$)	Prolactinoma ($n=5$)	p¶
PREG					
Availability*	34 (85%)	4 (20%)	2 (22%)	1 (20%)	**<0.001**
Level**	105.2 [54.9-214.4]	0.9 [0.2–2.9]	1.3 [-2.3]	21.3	**0.001**
7αOHPREG					
Availability*	31 (76%)	16 (80%)	3 (33%)	3 (60%)	**0.002**
Level**	47.8 [17.6-107.2]	36.6 [10.1-257.9]	32.9 [-45.4]	34.6 [-41.4]	0.05
DHEA					
Availability*	33 (83%)	16 (80%)	6 (67%)	4 (80%)	0.8
Level**	7.7 [4.8–16.8]	11.6 [7.8–24.8]	18 [11.5–50.3]	64.1 [30.7-449.2]	**0.006**
DHEAS					
Availability*	40 (100%)	20 (100%)	9 (100%)	5 (100%)	.
Level**	19.8 [7.2–50.5]	30.4 [15.6–42.9]	31 [16.6-106.7]	55.3 [23.5-202.9]	0.1
AE					
Availability*	25 (63%)	10 (50%)	3 (33%)	1 (20%)	0.2
Level**	0.9 [0.4–2.6]	0.8 [0.4–1.2]	1.7 [0.3-]	0.6	0.7
T					
Availability*	27 (68%)	10 (50%)	2 (22%)	1 (20%)	**0.03**
Level**	0.7 [0.5–1.5]	0.7 [0.5–0.9]	2.4 [2-]	0.9	0.2
DHT					
Availability*	2 (5%)	1 (5%)	1 (11%)	.	0.8
Level**	38.1 [0.5-]	99.7	3.3	.	0 0.4
Androsterone					
Availability*	22 (56%)	11 (55%)	4 (44%)	5 (100%)	0.2
Level**	9.5 [1.6–34]	17.3 [3.7–31.7]	12.4 [2.6-390.7]	19.1 [3.7-143.7]	0.7
PRG					
Availability*	4 (10%)	1 (5%)	2 (22%)	.	0.4
Level**	1.2 [0.4–5.9]	0.9	2.9 [0.5-]	.	0.9
ALLO					
Availability*	1 (1%)	.	.	.	.
Level**	148.5	.	.		.
DOC					
Availability*	13 (33%)	7 (35%)	2 (22%)	.	0.5
Level**	0.45 [0.39–0.57]	0.74 [0.29–1.25]	1.95 [0.4-4]	.	0.06
Corticosterone					
Availability*	8 (20%)	5 (25%)	1 (11%)	1 (20%)	0.9
Level**	0.9 [0.4–4.1]	0.99 [0.89–4.26]	9.7	0.8	0.3
Aldosterone					
Availability*	2 (5%)	.	.	.	.
Level**	0.7 [0.4-]	.	.	.	.
17OHPRG					
Availability*	6 (15%)	4 (20%)	2 (22%)	2 (40%)	0.6
Level**	0.8 [0.6–4.1]	0.7 [0.5–1.1]	1.7 [0.6-]	2.9 [0.3-]	0.9
11-Deoxycortisol					
Availability*	40 (100%)	20 (100%)	9 (100%)	5 (100%)	.
Level**	0.9 [0.7–1.5]	1.7 [0.9–3.3]	3.1 [1.5–9.4]	6 [2.4–13.2]	**<0.001**
Cortisol					
Availability*	39 (98%)	19 (95%)	8 (89%)	4 (80%)	0.1
Level**	5.1 [1.9–7.9]	6 [2.9–12.8]	12.1 [7.7–30.8]	10 [2.5–45]	**0.05**

Table 6. Comparison of neurosteroid availability and levels between subgroups of cases with pituitary adenoma. *NFA* nonfunctioning adenoma, *PREG* pregnenolone, *7αOHPREG* 7α-Hydroxypregnenolone, *17OHPREG* 17-Hydroxypregnenolone, *DHEA* dehydroepiandrosterone, *DHEAS* dehydroepiandrosterone sulfate, *AE-4* Androstene 3,17-dione, *T* testosterone, *DHT* dihydrotestosterone, *E2* estradiol, *PRG* progesterone, *ALLO* allopregnanolone, *DOC* 11deoxycorticosterone, *17OHPRG* 17Hydroxyprogesterone. ¶Statistically significant p values are shown in bold. * n (%). ** ng/g, Levels presented as median and interquartile range [IQR].

	Blood neurosteroid levels*
PREG	4 [1.9–13.7]
7αOHPREG	46.6 [9.9–67.3]
17OHPREG	1.4 [0.5–8.5]
DHEA	2.3 [1.1–4.4]
DHEAS	261.2 [105.7-556.2]
AE	0.2 [0.08–0.6]
T	0.1 [0.06–0.9]
DHT	1.1 [0.2–2.9]
E2	0.2 [0.1–0.5]
Androsterone	0.3 [0.1–0.7]
PRG	0.1 [0.05–0.3]
ALLO	0.4 [0.3–1.9]
DOC	0.08 [0.03–0.66]
Corticosterone	1.5 [0.5–6.6]
Aldosterone	0.07 [0.04–1.12]
17OHPRG	0.4 [0.2–1.1]
11 Deoxycortisol	0.4 [0.1–1.9]
Cortisol	60.6 [29.8-105.9]

Table 7. Blood levels of neurosteroid in cases with pituitary adenoma. *Levels (ng/ml) are presented as median and interquartile range [IQR]. *PREG* pregnenolone, *7αOHPREG* 7α-Hydroxypregnenolone, *17OHPREG* 17-Hydroxypregnenolone, *DHEA* dehydroepiandrosterone, *DHEAS* dehydroepiandrosterone sulfate, *AE-4* Androstene 3,17-dione, *T* Testosterone, *DHT* dihydrotestosterone, *E2* estradiol, *PRG* progesterone, *ALLO* allopregnanolone, *DOC* 11deoxycorticosterone, *17OHPRG* 17Hydroxyprogesterone.

unfeasible. And, the extensive duration of sample collection made it infeasible to condense them all into one season. Additionally, the probable influence of preoperative stress and anesthetics on NSs in PA could not be eliminated. These may have affected NS levels inevitably, but even so, the fact that NS are found in pituitary tissue cannot be disputed. Lastly, larger number of the subgroups, especially prolactinomas and corticotropinomas, would enhance the data. However, the number of operated cases with prolactinoma is restricted, since the first line treatment is medical therapy. And, the incidence of corticotropinoma is low to allow for a larger number of cases. Moreover, the research does not aim to determine if the pituitary gland functions as the origin or the recipient of the neurosecretory substances, which could present a potential limitation. Before exploring the source, target, and possible effects of NS in the pituitary tissue, it was crucial to verify the presence of NS in the pituitary gland, as human tissue evidence was lacking. At first, we carried out this study as an initial exploration to determine whether NS exist in our pituitary gland, and we are planning a follow-up research that will focus on enzymatic analysis. A significant constraint of this study is the dependence on normal pituitary tissue obtained from deceased individuals, which poses a considerable challenge to the research findings. Nevertheless, it remains ethically unfeasible to obtain viable pituitary tissue from a person who is still alive. To mitigate this constraint, we selected cadavers with a postmortem interval limited to 24 h.

In conclusion, both NPT and PA harbor NS. Moreover, variety and the levels of NS are affected by the tissue type and sex variences. Findings of NS in human pituitary and adenoma samples, alongside prior animal studies, may indicate possible further interactions in the synthesis and functions of pituitary hormones. Whether the pituitary is the source or only the target of these NS, can be elucidated by additional enzymatic investigations. NS may also be involved in pituitary cytogenesis and adenoma development, further elaboration of these effects may be useful in understanding adenoma pathogenesis.

	Normal pituitary tissue			Pituitary adenoma		
	Female ($n=8$)	Male ($n=11$)	p¶	Female ($n=40$)	Male ($n=34$)	p¶
PREG						
Availability*	8 (100%)	11 (100%)	-	17 (43%)	24 (71%)	**0.02**
Level**	16.6 [7.9–32.5]	3.4[2.6–6.1]	**0.001**	40.3 [3.4-143.3]	105.2 [49.6-206.7]	0.1
7αOHPREG						
Availability*	8 (100%)	11 (100%)	-	26 (65%)	27 (79%)	0.2
Level**	13.4 [7.6–22]	18.4 [7.2–29.9]	0.4	88.1 [15.2-197.1]	34.6 [14.2–79.4]	0.1
17OHPREG	-	-	-	-	-	-
DHEA						
Availability*	7 (88%)	7 (64%)	0.2	32 (80%)	27 (79%)	0.9
Level**	7.7 [6.2–14.5]	11.1 [3.1–15.2]	1	13.4 [7.5–32.8]	9.2 [4.9–23.4]	0.1
DHEAS						
Availability*	8 (100%)	11 (100%)	-	40 (100%)	33 (97%)	0.3
Level**	170 [95.3-367.8]	139.1 [70.4-210.8]	0.5	30.5 [13.4–52.4]	20.8 [6.9–54.6]	0.4
AE						
Availability*	8 (100%)	11 (100%)	-	18 (45%)	21 (62%)	0.2
Level**	1.9 [0.9–4.6]	0.6 [0.4–1.1]	**0.02**	0.9 [0.3–1.3]	0.9 [0.5–2.6]	0.4
T						
Availability*	8 (100%)	11 (100%)	-	14 (35%)	26 (77%)	**<0.001**
Level**	0.7 [0.4–1.4]	1.5 [0.8–1.8]	0.1	0.9 [0.5–1.6]	0.7 [0.5–1.3]	0.4
DHT						
Availability*	5 (63%)	5 (46%)	0.5	1 (3%)	3 (9%)	0.2
Level**	0.8 [0.7–0.9]	0.7 [0.4–1.1]	0.3	3.3	75.7 [0.5-]	1
E2	-	-	-	-	-	-
Androsterone						
Availability	8 (100%)	10 (91%)	0.4	22 (55%)	20 (61%)	0.6
Level**	4.1[2.5–5.7]	4.5 [2.3–5.9]	0.8	17.1 [4.8–44.3]	7.3 [1.7–31.7]	0.2
PRG						
Availability*	7 (88%)	8 (73%)	0.4	5 (13%)	2 (6%)	0.3
Level**	0.6 [0.5–1.4]	0.2 [0.1–0.3]	**0.001**	0.9 [0.4–6.3]	1.2 [0.4-]	0.9
ALLO	-	-	-	-	-	-
DOC						
Availability*	6 (75%)	2 (18%)	**0.01**	13 (33%)	9 (27%)	0.6
Level**	0.5 [0.4-1]	0.21 [0.2-]	0.07	0.6 [0.4–1.2]	0.4 [0.3–0.6]	0.3
Corticosterone						
Availability*	8 (100%)	11 (100%)	-	7 (18%)	8 (24%)	0.5
Level**	16.1[5.3–22.6]	2.1 [0.8–13.5]	0.06	0.9 [0.4–4.2]	0.4 [0.3–0.6]	1
Aldosterone						
Availability*	4 (50%)	1 (9%)	**0.05**	-	2 (6%)	0.1
Level**	0.6 [0.4–1.2]	0.23	0.4	-	0.7 [0.4-]	-
17OHPRG						
Availability*	6 (75%)	10 (91%)	0.3	8 (20%)	6 (18%)	0.8
Level**	1.4 [0.2-2]	0.2 [0.1–0.3]	**0.03**	0.8 [0.6–1.3]	0.9 [0.4–5.1]	0.9
11 Deoxycortisol						
Continued						

	Normal pituitary tissue			Pituitary adenoma		
	Female ($n=8$)	Male ($n=11$)	p¶	Female ($n=40$)	Male ($n=34$)	p¶
Availability*	8 (100%)	11 (100%)	-	40 (100%)	34 (100%)	-
Level**	2.6 [0.9–10.6]	0.8 [0.5–1.4]	**0.009**	1.5 [0.9–3.5]	1.1 [0.7–1.7]	**0.05**
Cortisol						
Availability*	8 (100%)	11 (100%)	0.4	37 (93%)	33 (97%)	0.1
Level**	82.9 [23.2–323]	23.9 [8-74.6]	0.1	6.7 [3.7–13.2]	3.9 [1.9–7.8]	0.07

Table 8. Comparison of neurosteroid availability and levels in normal pituitary tissues and pituitary adenoma tissues between female and male subjects. ¶Statistically significant p values are shown in bold. * n (%) ** ng/g, Levels presented as median and interquartile range [IQR]. *PA* pituitary adenoma, *NPT* normal pituitary tissue, *PREG* pregnenolone, -7α-Hydroxypregnenolone, *17OHPREG* 17-Hydroxypregnenolone, *DHEA* Dehydroepiandrosterone, *DHEAS* dehydroepiandrosterone sulfate, *AE-4* Androstene 3,17-dione, *T* Testosterone, *DHT* dihydrotestosterone, *E2* estradiol, *PRG* progesterone, *ALLO* allopregnanolone, *DOC* 11deoxycorticosterone, *17OHPRG* 17Hydroxyprogesterone.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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E.A; Writing - original draft, Methodology, Writing - review & editing, Project administration. B.E.; Project administration, Funding acquisition. S.Y; Formal analysis, Methodology. N.U; Methodology, Validation, Formal analysis. S.B; Software, Investigation. S.A; Methodology. O.G; Funding acquisition, Writing - review & editing. O.T; Writing - review & editing. M.N; Software, Validation. E.H; Writing - original draft, Writing - review & editing, Methodology, Project administration, Supervision.

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Declarations

Competing interests

The authors declare no competing interests.

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