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Research Article **Endocrinology**

Primary pigmented nodular adrenocortical disease (PPNAD) is a rare cause of ACTH-independent hypercortisolism. The disease is primarily caused by germline mutations of the protein kinase A (PKA) regulatory subunit 1A (*PRKAR1A*) gene, which induces constitutive activation of PKA in adrenocortical cells. Hypercortisolism is thought to result from PKA hyperactivity, but PPNAD tissues exhibit features of neuroendocrine differentiation, which may lead to stimulation of steroidogenesis by abnormally expressed neurotransmitters. We hypothesized that serotonin (5-HT) may participate in the pathophysiology of PPNAD-associated hypercortisolism. We show that PPNAD tissues overexpress the 5-HT synthesizing enzyme tryptophan hydroxylase type 2 (Tph2) and the serotonin receptors types 4, 6, and 7, leading to formation of an illicit stimulatory serotonergic loop whose pharmacological inhibition in vitro decreases cortisol production. In the human PPNAD cell line CAR47, the PKA inhibitor H-89 decreases 5-HT_4 and 5-HT_7 receptor expression. Moreover, in the human adrenocortical cell line H295R, inhibition of *PRKAR1A* expression increases the expression of Tph2 and 5-$HT_{4/6/7}$ receptors, an effect that is blocked by H-89. These findings show that the serotonergic process observed in PPNAD tissues results from PKA activation by *PRKAR1A* mutations. They also suggest that Tph inhibitors may represent efficient treatments of hypercortisolism in patients with PPNAD.

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RESEARCH ARTICLE

PKA regulatory subunit 1A inactivating mutation induces serotonin signaling in primary pigmented nodular adrenal disease

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Primary pigmented nodular adrenocortical disease (PPNAD) is a rare cause of ACTH-independent hypercortisolism. The disease is primarily caused by germline mutations of the protein kinase A (PKA) regulatory subunit 1A (*PRKAR1A*) gene, which induces constitutive activation of PKA in adrenocortical cells. Hypercortisolism is thought to result from PKA hyperactivity, but PPNAD tissues exhibit features of neuroendocrine differentiation, which may lead to stimulation of steroidogenesis by abnormally expressed neurotransmitters. We hypothesized that serotonin (5-HT) may participate in the pathophysiology of PPNAD-associated hypercortisolism. We show that PPNAD tissues overexpress the 5-HT synthesizing enzyme tryptophan hydroxylase type 2 (Tph2) and the serotonin receptors types 4, 6, and 7, leading to formation of an illicit stimulatory serotonergic loop whose pharmacological inhibition in vitro decreases cortisol production. In the human PPNAD cell line CAR47, the PKA inhibitor H-89 decreases 5-HT_4 and 5-HT_7 receptor expression. Moreover, in the human adrenocortical cell line H295R, inhibition of *PRKAR1A* expression increases the expression of Tph2 and 5-$HT_{4/6/7}$ receptors, an effect that is blocked by H-89. These findings show that the serotonergic process observed in PPNAD tissues results from PKA activation by *PRKAR1A* mutations. They also suggest that Tph inhibitors may represent efficient treatments of hypercortisolism in patients with PPNAD.

Introduction

Serotonin (5-hydroxytryptamine; 5-HT) and serotonin receptors (5-HTR) are key players in the regulation of a wide range of physiological and pathophysiological processes. In the CNS, 5-HT modulates multiple functions, such as mood control, sleep, anxiety, food intake, sexual behavior, blood pressure control, and thermoregulation (1). In addition, dysregulation of the serotonergic system has been involved in the pathogenesis of psychiatric disorders like depression and eating disorders (2). In peripheral organs, 5-HT is involved in the regulation of gastrointestinal mobility, cardiovascular functions, and bladder emptying (3–5). 5-HT is also able to act as an autocrine/paracrine factor to influence several physiological processes, including osteoclastogenesis (6), regulation of the pancreatic β cell mass during pregnancy (7), metabolism in adipose tissue (8, 9), and mammary gland development (10).

The adrenal gland is composed of functionally distinct layers, including the cortical zona glomerulosa and zona fasciculata. The zona glomerulosa contains steroidogenic cells producing aldosterone, a mineralocorticoid essential for sodium and potassium homeostasis, while the cells of the zona fasciculata

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secrete glucocorticoids, which control stress response, immune reaction, and glucose homeostasis. In the human adrenal, 5-HT — released by subcapsular mast cells — stimulates steroidogenesis through a paracrine mechanism involving 5-HT_4R (11–13). In the adrenal cortex, 5-HT_4R is mainly expressed by aldosterone-producing cells in the zona glomerulosa and, in a lesser extent, by cortisol-secreting cells in the zona fasciculata (14). Consequently, 5-HT is much more efficient to stimulate aldosterone than cortisol secretion in vitro (11–13). Accordingly, clinical studies have shown that, in healthy volunteers, 5-HT4R agonists stimulate aldosterone secretion without influencing plasma cortisol levels (15), whereas an elevation of plasma 5-HT concentrations favors an increase in cortisol secretion through a stimulatory action of the amine at the hypothalamo-pituitary level (16, 17).

Primary pigmented nodular adrenocortical disease (PPNAD) is a rare cause of ACTH-independent hypercortisolism responsible for central obesity, diabetes, and arterial hypertension (18). PPNAD is characterized by the presence of black micronodules in the adrenal cortex. It can be isolated or occur as part of the Carney complex, a genetic disorder that can also include spotty skin pigmentation, cardiac myxomas, schwannomas, breast adenomas, bone lesions, and endocrine disorders due to tumors of the pituitary and thyroid glands, the pancreas, and/or gonads (19). The Carney complex is primarily caused by germline mutations of the protein kinase A (PKA) regulatory subunit 1A (*PRKAR1A*) gene (20). In addition, mutations of the phosphodiesterase *PDE11A* and *PDE8* and the PKA catalytic subunit *PRKACA* genes have been described in patients with PPNAD (21–23). All these genetic events lead to constitutive activation of the cAMP/PKA pathway, which is thought to favor glucocorticoid hypersecretion. However, *PRKAR1A* mutation carriers with adrenal hyperplasia do not always present with hypercortisolism, suggesting that second-line molecular events may be necessary for cortisol overproduction (24, 25). It has been shown that PPNAD tissues abnormally express markers of neuroendocrine differentiation like synaptophysin, chromogranins, and catecholamine-synthesizing enzymes (26, 27). We have therefore hypothesized that PPNAD tissues may also aberrantly synthesize 5-HT and express serotonergic receptors that could be involved in the pathophysiology of hypercortisolism, as previously observed in bilateral macronodular adrenal hyperplasia (BMAH) (28, 29). To test this hypothesis, we have investigated abnormal expression of 5-HT–synthesizing enzyme and 5-HTRs in PPNAD tissues, and we examined in vitro the role of 5-HT in the control of cortisol production by adrenocortical cells from patients with PPNAD. Moreover, we have explored the potential link between the causative mutational defect and expression of the 5-HT signaling pathway by using adrenocortical cell lines. Our results demonstrate that, in PPNAD tissues, constitutive activation of the cAMP/PKA pathway results in formation of an autocrine/paracrine serotonergic regulatory loop that activates cortisol production and therefore participates in the pathogenesis of hypercortisolism.

Results

We examined 5-HT production in a series of 33 adrenal tissues removed from patients with PPNAD genotyped for *PRKAR1A*, *PRKACA*, *PDE11A*, and *PDE8* (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/jci.insight.87958DS1).

Mast cell localization in PPNAD tissues. Since mast cells represent the unique source of 5-HT in the normal adrenal (13), we have first localized mast cells in PPNAD tissues by IHC with antibodies against tryptase, a mast cell–specific protease. As expected, mast cells were detected in the subcapsular region of both normal adrenal and PPNAD tissues. Moreover, mast cells were abnormally visualized in the micronodules and internodular cortex of PPNAD tissues (Figure 1A). However, mast cell densities were similar in normal adrenal and PPNAD tissues (Figure 1B).

5-HT production by PPNAD tissues. We then explored the possibility that abnormal neuroendocrine differentiation may favor aberrant 5-HT synthesis in PPNAD cells. 5-HT is synthesized from tryptophan through hydroxylation and decarboxylation involving tryptophan hydroxylase (Tph) and aromatic L-amino acid decarboxylase. Two isoforms of Tph — Tph1 and Tph2 — encoded by 2 different genes (*TPH1* and *TPH2*) are expressed in peripheral tissues and the CNS, respectively (30). PPNAD tissues expressed *TPH1* at lower levels than normal adrenals (Figure 1C). In contrast, the *TPH2* mRNA, which was not detected in normal adrenals, was highly expressed in PPNAD specimens (Figure 1C). As predicted, immunohistochemical analysis of normal adrenals showed no staining for Tph2 but revealed the presence of subcapsular 5-HT–positive mast cells, which were labeled by nonselective Tph antibodies (Figure 1D). In PPNAD samples, mast cells were positive for Tph (Figure 1E). In addition, Tph2 immunoreactivity was observed in both adrenocortical micronodules and internodular tissue. Tph2-positive cells were also immunoreactive

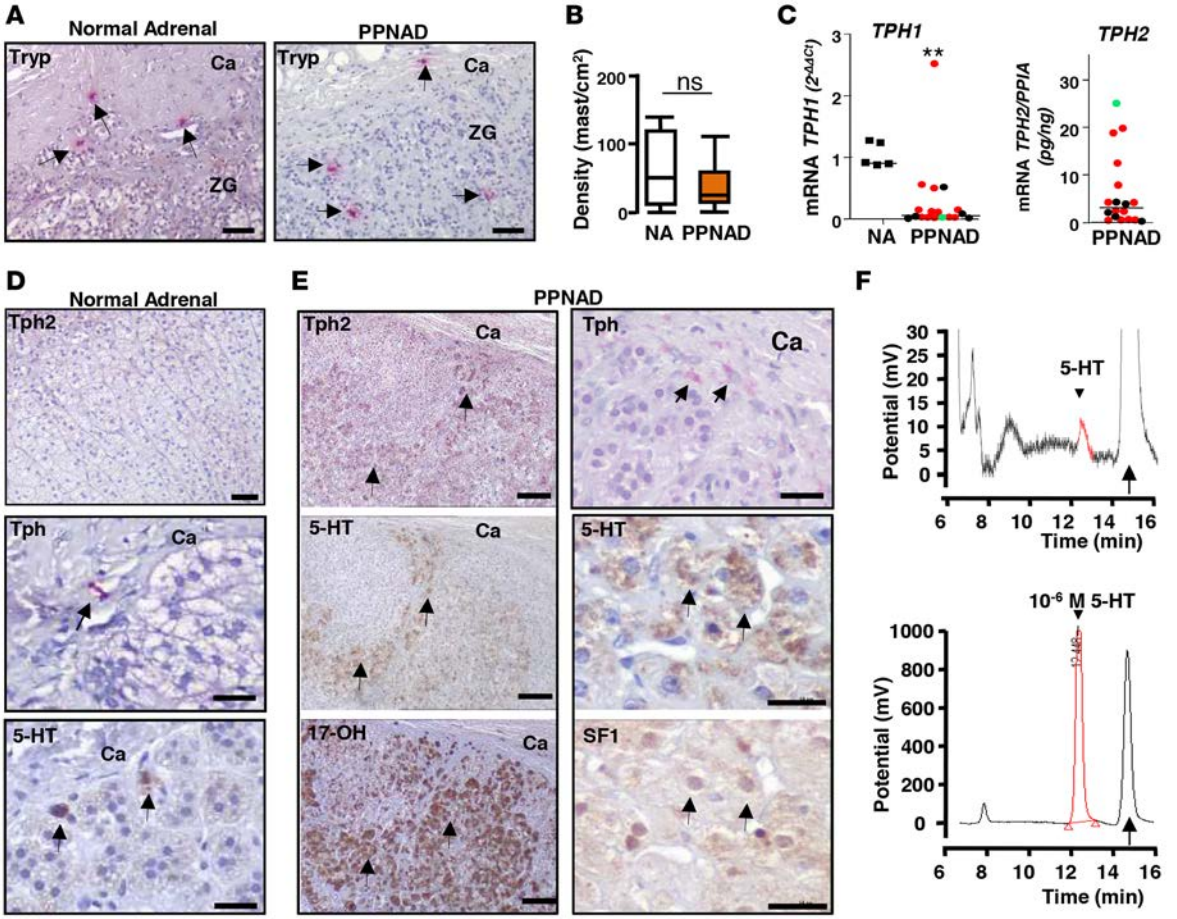

Figure 1. Serotonin (5-HT) production by PPNAD tissues. (**A**) Tryptase immunoreactivity (Tryp) in normal adrenal and PPNAD tissue from patient 13 (P13). Mast cells are designated by arrows. (**B**) Mast cell density in PPNAD tissues (n = 19) compared with normal adrenals (NA) (n = 7). (**C**) *TPH1* and *TPH2* mRNA levels in explants of NA and PPNAD tissues. PPNAD with *PRKAR1A* (red), *PDE11A* (green), and no (black) mutations. mRNA expression levels were normalized to *PPIA*. (**D**) Tph2, Tph, and 5-HT immunoreactivities in normal adrenals. Immunoreactive cells are designated by arrows. (**E**) Tph2, Tph, 5-HT, 17α-hydroxylase (17-OH), and steroidogenic factor 1 (SF1) immunoreactivities in PPNAD tissues on a section from P13 (Tph) and consecutive sections from P13 (Tph2, 5-HT, and 17-OH; left panels) and P30 (5-HT and SF1; right panels). In the left column, arrows designate groups of cells coexpressing Tph2, Tph, and 17-OH. In the right column, arrows designate immunopositive cells for Tph, 5-HT, or SF1. (**F**) HPLC detection of 5-HT in incubation medium of PPNAD explants from P32 (higher panel) and culture medium added with synthetic 5-HT (10^{-6} M; lower panel). Retention time of synthetic 5-HT (▼). N-methylserotonin was used as an internal standard (arrow). Data were analyzed by using Mann-Whitney U test. $^{**}P < 0.01$. Ca, capsule; ZG, zona glomerulosa. Scale bars: 50 μm.

for 5-HT, the steroidogenic enzyme 17α-hydroxylase, and the steroidogenic factor 1 (SF1), indicating that 5-HT is synthesized by Tph2 in adrenocortical steroidogenic cells (Figure 1E).

The presence of 5-HT in PPNAD cells could also theoretically result from uptake of the amine from the plasma. However, the *SLC6A4* mRNA encoding the serotonin transporter (SERT) responsible for the entry of 5-HT into cells is expressed at lower levels in PPNAD tissues than in the normal adrenals (Supplemental Figure 1), indicating that the ectopic occurrence of 5-HT in PPNAD adrenocortical cells is not due to an increase in 5-HT uptake.

Table 1. Maximum efficacy (Emax) and potency (EC_{50}) of 5-HT to stimulate cortisol production from cultured PPNAD cells.

	NA	P14	P15	P21	P28	P29	P32	P33
Emax (% BL)	193 ± 4	415 ± 2[A]	238 ± 5[A]	634 ± 25[A]	93 ± 3[B]	610 ± 9[A]	322 ± 9[A]	268 ± 9[A]
EC_{50} (nM)	44	1.1	0.8	84.6	7,000	0.5	1.2	1.5

[A]$P<0.001$; [B]$P<0.01$. NA, normal adrenal; BL, basal level; EC_{50}, half maximal effective concentration.

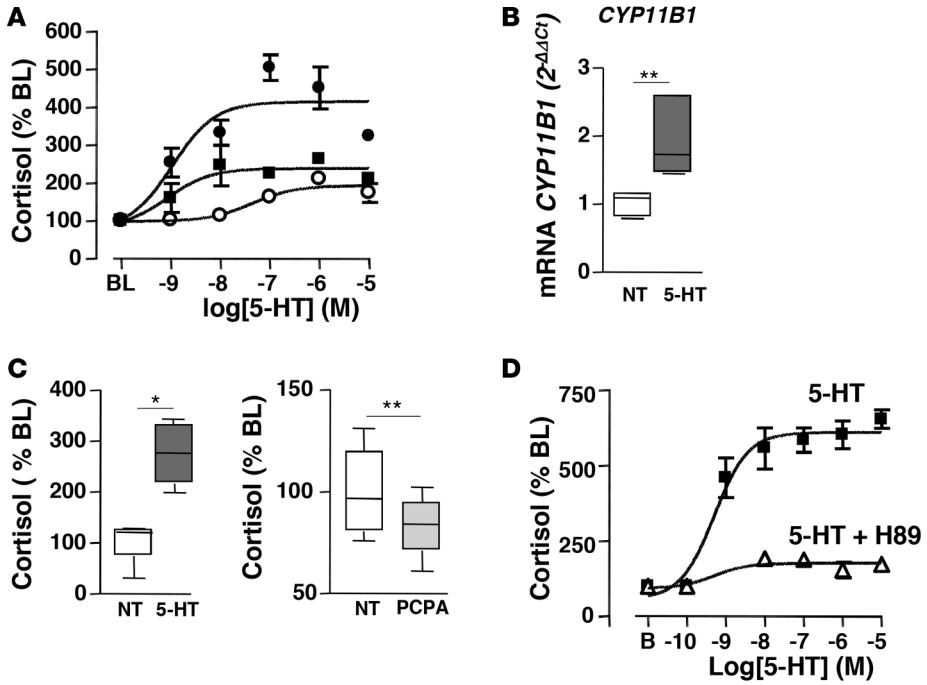

Figure 2. Effect of 5-HT on PPNAD tissues. (**A**) Effects of graded concentrations of 5-HT (10^{-9} to 10^{-5} M) on cortisol secretion by cultured normal adrenal cells (○) and PPNAD cells derived from P14 (●) and P15 (■). (**B**) Effect of 5-HT (10^{-7} M; 6h) on *CYP11B1* mRNA levels in cultured PPNAD cells from P32 (NT: not treated). (**C**) Effects of 5-HT (10^{-7} M) and the Tph inhibitor p-chlorophenylalanine (PCPA; 10^{-5} M; 2h) on cortisol production by PPNAD explants from P32. (**D**) Effect of the PKA inhibitor H-89 (10^{-5} M) on 5-HT–induced cortisol response of cultured PPNAD cells derived from P29 (5-HT, ■; 5-HT+H-89, Δ). BL, basal level; B, base; NT, not treated. Data are presented as mean ±SEM or box plots from at least 3 values. Data were analyzed by using Mann-Whitney *U* test. $*P < 0.05$; $**P < 0.01$.

To verify whether 5-HT could be released by PPNAD cells, we have carried out HPLC analysis of culture medium after incubation with PPNAD explants. HPLC coupled with electrochemical detection allowed detection of 5-HT in incubation medium of PPNAD explants (Figure 1F). Collectively, these results indicate that 5-HT is produced by a subpopulation of steroidogenic cells in PPNAD tissues.

Effect of 5-HT on cortisol secretion from PPNAD tissues. We have examined whether 5-HT could activate cortisol secretion from cultured PPNAD cells derived from 7 patients (Supplemental Table 2). Graded concentrations of 5-HT (10^{-9} to 10^{-5} M) stimulated cortisol synthesis by PPNAD cells with higher efficacy and/or potency than in normal adrenal cells, except for P28 tissue, which appeared less sensitive to the action of 5-HT than normal adrenals (Figure 2A and Table 1). 5-HT (10^{-7} M) also increased expression of *CYP11B1* mRNA encoding 11β-hydroxylase, a key enzyme in cortisol synthesis (Figure 2B). To verify whether intraadrenal 5-HT could exert a stimulatory tone on glucocorticoid secretion in PPNAD tissues, we have measured cortisol levels in the incubation medium of PPNAD explants exposed to the Tph inhibitor p-chlorophenylalanine. Incubation with p-chlorophenylalanine (10^{-5} M) decreased cortisol production by PPNAD explants (Figure 2C), demonstrating that locally produced 5-HT exerts a stimulatory tone on glucocorticoid secretion.

Inhibition of the cAMP/PKA pathway has been reported to decrease the cortisol response to 5-HT in BMAH, causing hypercortisolism (29). We have thus examined whether the stimulatory action of 5-HT on PPNAD cells could be mediated by PKA, whose activity is enhanced in PPNAD tissues. The PKA inhibitor H-89 abolished the cortisol response of PPNAD cells to 5-HT (Figure 2D), indicating that 5-HT–induced glucocorticoid secretion involves 5-HTRs positively coupled to the PKA pathway, i.e., the 5-HT_4R, 5-HT_6R, and 5-HT_7R.

Serotonergic receptor expression in PPNAD tissues. PPNAD tissues overexpressed *HTR4* mRNAs versus normal adrenals (Figure 3A). It has been demonstrated that alternative splicing of *HTR4* transcripts has the potential to generate several receptor isoforms (a, b, d, g, and i) that differ in the structure of their C-terminal tail and their pharmacological properties (31). Reverse transcriptase PCR (RT-PCR) analysis showed that the expression pattern of 5-HT_4R isoforms is distinct in PPNAD tissues from normal adrenals (Figure

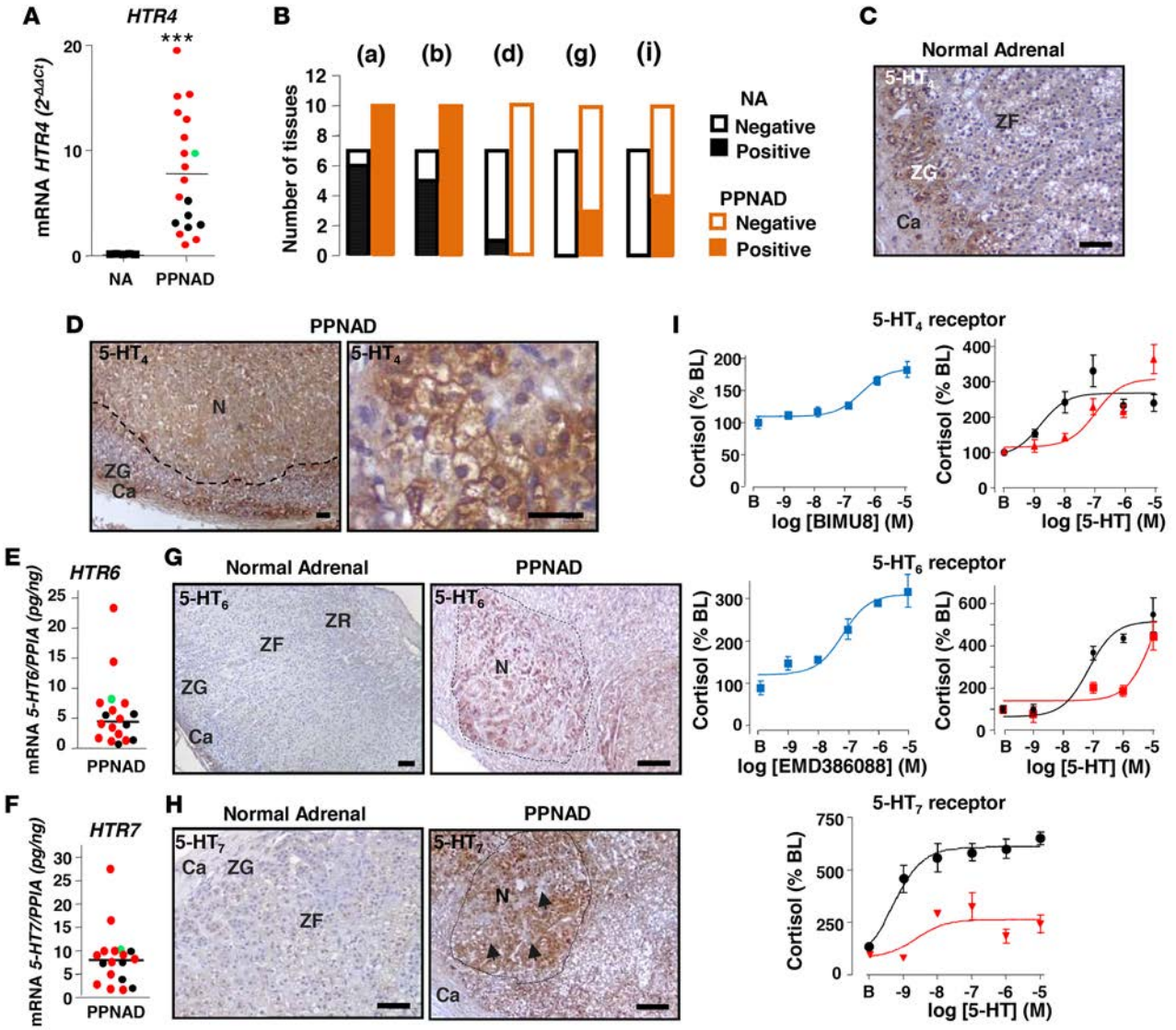

Figure 3. Identification of 5-HT receptors in PPNAD tissues. (**A**) *HTR4* mRNA levels in explants of normal adrenals (NA) and PPNAD tissues. PPNAD with *PRKAR1A* (red), *PDE11A* (green), and no (black) mutations. (**B**) Number of patients expressing the (a), (b), (d), (g), and (i) isoform *HTR4* mRNAs in 10 PPNAD tissues compared with normal adrenals (n = 7). Positive tissues, filled histograms; negative tissues, empty histograms. (**C**) 5-HT$_4$ receptor immunoreactivity in zona glomerulosa from normal adrenal. (**D**) 5-HT$_4$ receptor immunoreactivity in zona glomerulosa and hyperplastic nodules from PPNAD tissues at low (P17; left panel) and high (P20; right panel) magnifications. (**E** and **F**) *HTR6* (**E**) and *HTR7* (**F**) mRNA levels in explants of PPNAD tissues. (**G** and **H**) 5-HT$_6$ (**G**) and 5-HT$_7$ (**H**) receptor immunoreactivities were present in nodules (dotted line) from P21 and P7 PPNAD tissues (right) but absent in normal adrenal cortex (control; left). (**I**) Pharmacological profiles of 5-HT receptors expressed in PPNAD cells. Effects of graded concentrations (10^{-9} to 10^{-5} M) of the 5-HT$_4$ receptor agonist BIMU8 (left, higher panel) and the 5-HT$_6$ receptor agonist EMD386088 (left, middle panel) on cortisol secretion by cultured cells derived from PPNAD P21. Effects of graded concentrations of 5-HT (10^{-9} to 10^{-5} M) on cortisol secretion by cultured cells derived from PPNAD P33 (right, higher panel), P21 (right, middle panel) and P29 (lower panel) in the absence (●) or presence of antagonists (10^{-7}M, red lines) of the 5-HT$_4$ (GR113808, ▲), the 5-HT$_6$ (SB258585, ■), or the 5-HT$_7$ (SB269970, ▼) receptors. Data represents mean ±SEM. BL, basal level; B, base. mRNA expression levels were normalized to *PPIA*. Data were analyzed by using Mann-Whitney *U* test. ***P < 0.001. Ca, capsule; ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis; N, nodule. Scale bars: 50 μm.

3B, Supplemental Figure 2, and Supplemental Table 3). Isoforms (a) and (b) were expressed in most normal adrenals and all PPNAD tissues. Isoform (d), which has rarely been found in normal adrenals, was absent in PPNAD tissues. In contrast, isoforms (g) and (i) were only expressed in some PPNAD tissues. Immunohistochemical studies revealed the presence of 5-HT$_4$R immunoreactivity in the subcapsular region of PPNAD, as in normal adrenals (Figure 3, C and D). Moreover, PPNAD samples contained 5-HT$_4$–positive cells in both micronodules and internodular tissue. At variance with normal adrenals, PPNAD tissues were found to express mRNAs encoding the 5-HT$_6$R and 5-HT$_7$R (Figure 3, E and F). In addition, intense 5-HT$_6$R and 5-HT$_7$R immunoreactivities were observed in adrenocortical micronodules but were

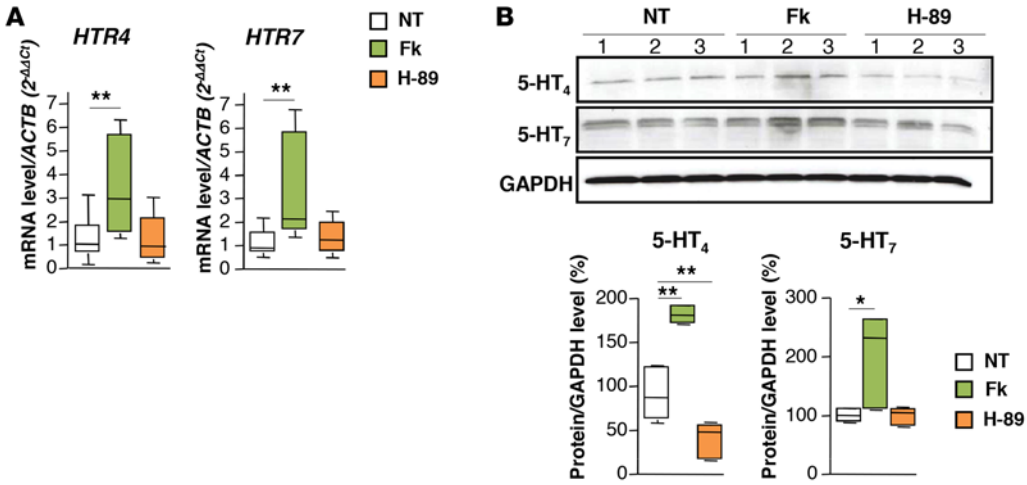

Figure 4. Regulation of the expression of 5-HT$_4$ and 5-HT$_7$ receptors in the CAR47 cell line. (**A** and **B**) Expression levels of *HTR4* and *HTR7* mRNAs normalized to *ACTB* (**A**) and Western blot analysis of 5-HT$_4$ and 5-HT$_7$ receptors normalized to GAPDH (**B**) in CAR47 cells cultured in basal conditions (not treated; NT) and after treatment with either forskolin (Fk, 10^{-5} M) or H-89 (10^{-5} M). Data illustrates results of 3 cell batches (1, 2, 3). Data of at least 4 determinations are presented as box plots. Data were analyzed by using Mann-Whitney *U* test. $^*P < 0.05$; $^{**}P < 0.01$.

not detected in normal adrenals (Figure 3, G and H). The distribution of the 5-HT$_4$R, 5-HT$_6$R, and 5-HT$_7$R was globally heterogeneous, explaining the variability in the tissues responsiveness to 5-HT.

Involvement of 5-HT$_{4/6/7}$ receptors in the stimulatory effect of 5-HT on PPNAD tissues. We have then investigated whether the overexpressed 5-HT$_{4/6/7}$ receptors could mediate the effect of 5-HT on PPNAD tissues by using various 5-HTR ligands (Figure 3I). The 5-HT$_4$R agonist BIMU8 and 5-HT$_6$R agonist EMD-386088 increased cortisol production in cultured PPNAD cells, whereas the partial 5-HT$_4$R agonists metoclopramide and cisapride triggered weak or nonsignificant cortisol responses (Figure 3I and Supplemental Figure 3). In contrast, the 5-HT$_4$R antagonist GR-113808, the 5-HT$_6$R antagonist SB-258585, and the 5-HT$_7$R antagonist SB-269970 decreased the cortisol response to 5-HT in cultured PPNAD cells (Figure 3I). These data indicate that the stimulatory action of 5-HT on cortisol release is mediated by both overexpressed eutopic 5-HT$_4$R and ectopic 5-HT$_6$R and/or 5-HT$_7$R, explaining the increased efficacy and potency of 5-HT to activate glucocorticoid production by PPNAD cells.

Regulation of 5-HTR expression in the PPNAD cell line CAR47. We have investigated the regulation of 5-HTR expression in PPNAD by using the *PRKAR1A*-mutated PPNAD CAR47 cell line (32), which expressed *HTR4* and *HTR7* mRNAs but not the *HTR6* mRNA (Figure 4, A and B). Activation of the cAMP/PKA pathway by forskolin (10^{-5} M) enhanced expression of 5-HT$_4$R and 5-HT$_7$R at both mRNA and protein levels. By contrast, the PKA inhibitor H-89 (10^{-5} M) reduced the level of the *HTR7* mRNA and the 5-HT$_4$R protein. These data indicated that expression of 5-HT$_{4/7}$ receptors is positively controlled by the cAMP/PKA pathway in CAR47 cells.

Inhibition of PRKAR1A expression triggers expression of a serotonergic loop in the human adrenocortical cell line H295R. Previous investigations have shown that *PRKAR1A* silencing increases total PKA activity in adrenocortical H295R cell lines (33), as observed in PPNAD cells. In order to confirm that activation of the cAMP/PKA pathway upregulates *TPH2* and *HTR4/6/7* gene expression, we have used the human adrenocortical cell line H295R to generate cell clones expressing a doxycyclin-inducible (Dox-inducible) shRNA targeted to *PRKAR1A* mRNA. Treatment of these clones with Dox leads to inhibition of *PRKAR1A* mRNA expression in a similar way as the pathological process occurring in PPNAD adrenocortical cells (Supplemental Figure 4). After shRNA-PRKAR1A induction by Dox, expression of Tph2 and 5-HT$_4$R, 5-HT$_6$R, and 5-HT$_7$R significantly increased (Figure 5, A–C) without variation of *TPH1* mRNA level (Supplemental Figure 5). Interestingly, forskolin increased the expression of *TPH2* and *HTR4/6/7* mRNAs in H295R shPRKAR1A cells (Figure 5D). Moreover, upregulation of *TPH2* and *HTR4/6/7* mRNAs induced by forskolin or Dox in H295R shPRKAR1A cells was reduced by H-89 pretreatment. Taken together, our results indicated that activation of PKA activity by *PRKAR1A* mutations upregulates the key enzyme in

5-HT synthesis Tph2 and the cAMP/PKA coupled 5-$HT_{4/6/7}$R in adrenocortical cells.

In order to investigate whether *PRKAR1A* downregulation is actually responsible for cortisol overproduction and 5-HT hypersensitivity, we have quantified cortisol production by H295R shPRKAR1A cells (Figure 5E). As expected, treatment with Dox increased cortisol secretion. 5-HT (10^{-8} M, a value close to the mean half-maximal effective concentration (EC_{50}) of 5-HT observed in PPNAD cell culture experiments) had no significant effect on cortisol release by shPRKAR1A cells but stimulated glucocorticoid synthesis after preincubation of the cells by Dox. In addition, cortisol secretion elicited by cotreatment with Dox and 5-HT was totally blocked by H-89 (10^{-5} M).

Discussion

There is growing evidence that 5-HT acts as an autocrine/paracrine factor modulating important physiological processes in various peripheral organs (6–9, 13, 34). In the present study, we report the occurrence of an aberrant serotonergic regulatory loop in PPNAD tissues from patients with *PRKAR1A* mutations.

In the normal human adrenal tissue, 5-HT is released by subcapsular mast cells, which are thus in the immediate vicinity of zona glomerulosa cells (35), explaining why 5-HT more efficiently stimulates aldosterone than cortisol secretion (11, 13, 15). Although mast cell densities were similar in normal adrenal and PPNAD tissues, PPNAD mast cells were found to be localized in adrenocortical micronodules and internodular tissue, suggesting that they may be able to release 5-HT close to cortisol-producing cells and therefore influence their secretory activity. In addition, we have explored the possibility that adrenocortical cells may abnormally synthesize 5-HT consecutively to the neuroendocrine differentiation of PPNAD tissues (26, 27). It is well established that 5-HT is synthesized from tryptophan through hydroxylation and decarboxylation, involving the limiting enzyme Tph and the ubiquitous and nonspecific enzyme aromatic L-amino acid decarboxylase, respectively. Aromatic L-amino acid decarboxylase is known to be expressed in the normal adrenal and PPNAD tissues (26, 36, 37). Interestingly, we found that Tph2, normally present in the brain, was expressed in PPNAD tissues, leading to 5-HT synthesis by a subpopulation of steroidogenic cells. We further examined whether 5-HT produced by adrenocortical cells could exert an intraadrenal stimulatory tone on cortisol secretion by PPNAD cells. Using the Tph inhibitor p-chlorophenylalanine, we noticed a significant decrease in cortisol production by PPNAD samples in vitro, whereas p-chlorophenylalanine had no effect on glucocorticoid secretion by normal adrenal slices (data not shown). Because PPNAD tissues are independent of circulating adrenocorticotropin (ACTH) in vivo, this finding is particularly important in terms of clinical implications, since it strongly suggests that 5-HT synthesis inhibitors may be as effective to reduce cortisol hypersecretion in patients with PPNAD as they are to inhibit in vitro glucocorticoid release from PPNAD explants.

In PPNAD cultured cells, 5-HT stimulated cortisol production with higher potency and/or efficacy than in normal adrenal cells, suggesting overexpression of 5-HTRs and/or abnormal receptor coupling. The observation that the stimulatory effect of 5-HT was blocked by the PKA activity inhibitor H-89 indicated that, in PPNAD like in all adrenal tissues investigated so far (11, 12, 29, 38), 5-HT activates corticosteroidogenesis through receptors positively coupled to the cAMP/PKA pathway. In agreement with these observations, we found that PPNAD tissues abnormally express the 5-$HT_{4/6/7}$ receptors, which were involved in various degrees in their response to 5-HT. At variance with the full 5-HT_4R agonist BIMU8, the partial 5HT_4R agonists metoclopramide and cisapride, which are known to in vivo and in vitro stimulate corticosteroidogenesis from BMAH overexpressing the 5-HT_4R (28, 29), had no action on glucocorticoid production by PPNAD explants in accordance with previous clinical reports (39). This intriguing observation probably results from the fact that the pattern of expression of 5-HT_4R isoforms found in PPNAD seems to be different from those observed in both normal adrenals and BMAHs. In fact, the combination of isoforms (a), (b), (g), and (i) is not found in BMAH tissues (40, 41), and it is now well established that differences in the expression profiles of *HTR4* splice variants induces functional variability in tissue responses mediated by the 5-HT_4R (31, 42). It is conceivable that the altered expression profile of 5-HT_4R isoforms in PPNAD tissues in comparison with normal adrenals may result from chronic activation of PKA by the causative genetic events. Indeed, it has recently been shown that ACTH, which exerts its biological effects in adrenocortical cells via activation of the cAMP/PKA pathway, regulates alternative splicing of numerous transcripts in the murine Y1 adrenocortical cell line (43). Taken together, our results show for the first time to our knowledge the presence of an aberrant serotonergic regulatory loop in PPNAD tissues associated with hypercortisolism, resulting from abnormal expression of both the 5-HT synthesizing enzyme Tph2 and the 5-HT_4R, 5-HT_6R, and 5-HT_7R.

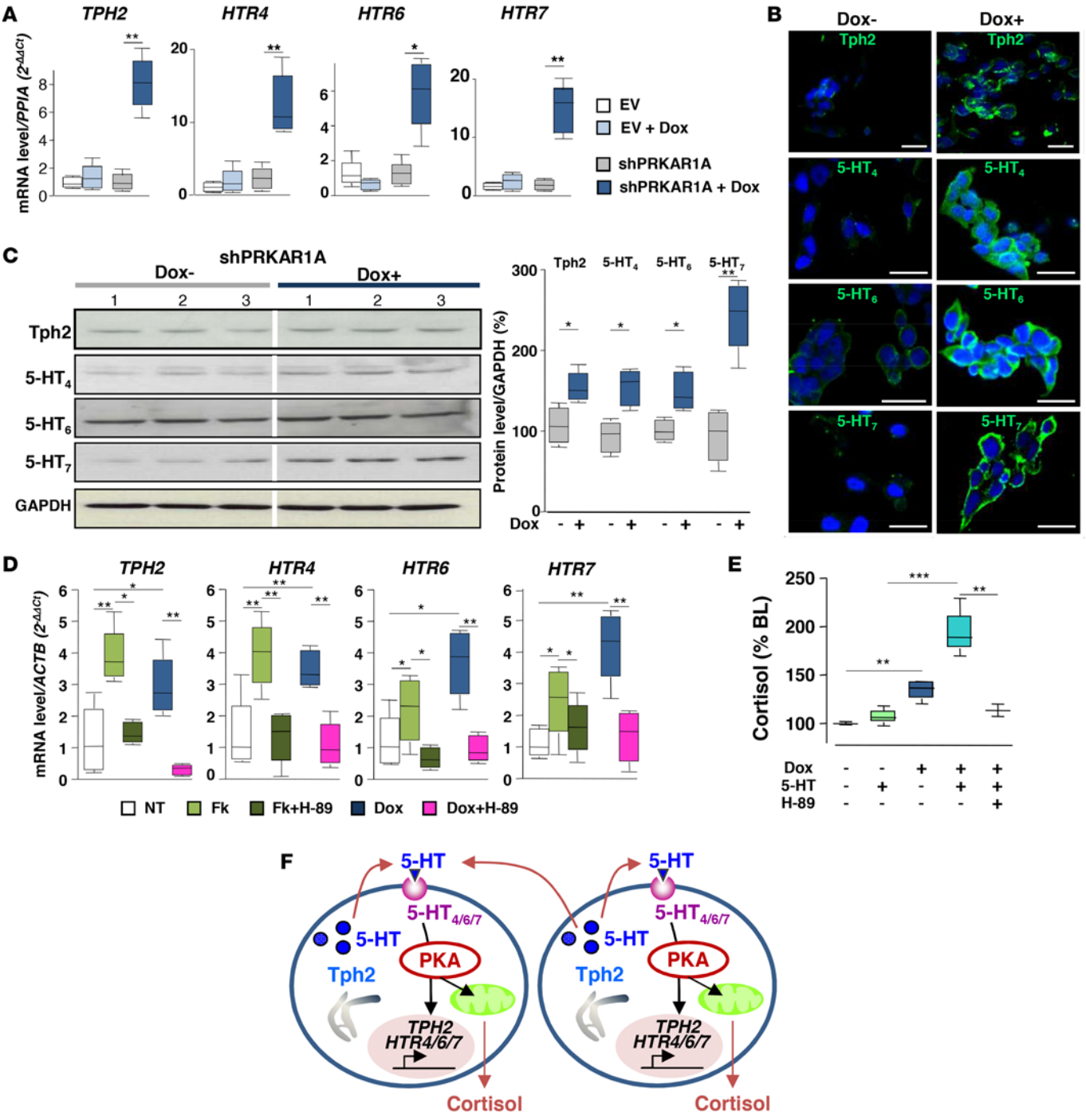

Figure 5. Regulation of the expression of Tph2 and 5-HT$_{4/6/7}$ receptors in the shPRKAR1A H295R cell line. (**A**) Expression levels of *TPH2* and *HTR4/6/7* mRNAs normalized to *PPIA* in transfected H295R cells with either the empty vector (EV) or the doxycyclin-sensitive shPRKAR1A–expressing vector in the absence or presence of doxycyclin (Dox). (**B**) Tph2 and 5-HT$_{4/6/7}$ receptor immunoreactivity in shPRKAR1A H295R cells cultured in the absence (Dox-) or presence of doxycyclin (Dox+). Scale bars: 20 μm. (**C**) Western blot analysis of Tph2 and 5-HT$_{4/6/7}$ receptors in shPRKAR1A H295R cells cultured in the absence (Dox-) or presence of doxycyclin (Dox+). Data illustrates results of 3 cell batches (1, 2, 3). (**D**) Expression levels of *TPH2* and *HTR4/6/7* mRNAs in basal conditions and after treatment of shPRKAR1A H295R cells with forskolin (Fk; 10^{-5} M), forskolin and H-89 (Fk+H-89; 10^{-5} M), doxycyclin (0.5 μg/ml), and doxycyclin and H-89 (Dox+H-89) for 24h. (**E**) Cortisol secretion by shPRKAR1A H295R cells incubated for 24h with 5-HT (10^{-8} M) and H-89 (10^{-5} M) in the absence or presence of doxycyclin. BL, basal level. Data of at least 4 determinations are presented as box plot. (**F**) Schematic representation of the abnormal serotonergic regulatory loop controlling cortisol secretion in patients with *PRKAR1A* mutation. Activation of PKA due to *PRKAR1A* inactive mutation stimulates abnormal expression of the limiting enzyme for 5-HT synthesis tryptophan hydroxylase type 2 (Tph2), as well as functional 5-HT receptors – i.e., the 5-HT$_4$, 5-HT$_6$, and 5-HT$_7$ receptors – in adrenocortical cells. Release of locally produced 5-HT stimulates cortisol secretion through autocrine and paracrine mechanisms involving the 3 receptor types positively coupled to the cAMP/PKA pathway. Data were analyzed by using Mann-Whitney *U* test and Bonferroni's test after one-way ANOVA. **P* < 0.05; ***P* < 0.01; or ****P* < 0.001.

A large majority of PPNAD cases are related to mutations of the RIα subunit gene (*PKRAR1A*) (44, 45). Most of them lead to synthesis of an unstable mRNA, which is rapidly catabolized through a nonsense-mediated decay process (46). The resulting decrease in cytosolic RIα subunit concentration favors an elevation of the level of free catalytic subunits in adrenocortical cells and, subsequently, an increase in PKA activity (20, 47, 48). We have then postulated that formation of the abnormal intraadrenal serotonergic loop in PPNAD tissues may be a consequence of the constitutive hyperactivity of PKA in adrenocortical cells.

We first used the immortalized PPNAD CAR47 cell line to investigate the potential role of the cAMP/PKA pathway in the regulation of 5-HT_4R and 5-HT_7R expression, the 5-HT_6R being not expressed in this cell line. Forskolin, an activator of the PKA pathway, was found to enhance expression of *HTR4* and *HTR7* mRNAs, whereas H-89 had the opposite effect on 5-HT_7R transcript and protein levels. These results indicated that expression of *HTR4* and *HTR7* genes can be upregulated by the cAMP/PKA pathway in PPNAD cells. The molecular process involved in this mode of regulation likely involves the PKA regulatory subunits not affected by the causative mutation of the disease, such as RIIβ, which is overexpressed in PPNAD tissues to partially compensate RIα subunit inactivation (49, 50).

Previous investigations had shown that *PRKAR1A* silencing increases total PKA pathway activity in adrenocortical H295R (33) and HEK293 (51) cell lines, a finding that is similar to what naturally occurs in patients with inactivating mutations of the *PRKAR1A* gene (20). In the present study, we have used a stable H295R cell line model expressing a Dox-inducible shRNA for *PRKAR1A*. In this model, *PRKAR1A* mRNA silencing resulted in an increase in Tph2, as well as 5-HT_4R, 5-HT_6R, and 5-HT_7R expression. The elevation of 5-HTR expression was associated with an enhancement of the cortisol response to 5-HT. *PRKAR1A* shRNA–induced upregulation of proteins of the serotonergic signaling pathway was the consequence of PKA activation, as shown by the inhibitory effect of H-89 on the action of Dox. The formation of the autocrine/paracrine serotonergic stimulatory loop in PPNAD cells may exclusively be regarded as a pathological process randomly resulting from the genetic events that cause the disease. However, it has recently been reported that prolonged stress in rats induces both activation of the hypothalamo-pituitary corticotropic axis and abnormal 5-HT synthesis and 5-HT_7R overexpression in adrenal cortex (52). Because the action of stress on the adrenal cortex is principally mediated by ACTH and its second messenger cAMP (53), it seems that our findings in PPNAD cells could rather be considered as a physiological process abnormally reactivated by PKA hyperactivity.

In conclusion, our results show that activation of the PKA pathway caused by *PRKAR1A* gene mutations triggers formation of an illicit serotonergic stimulatory loop in PPNAD tissues, which resemble the autocrine/paracrine regulation of pancreatic β cell mass by 5-HT during pregnancy (7) (Figure 5F). This new pathogenic mechanism may also participate in the other clinical manifestations of the Carney complex. Our data also indicate that this autocrine/paracrine mechanism is involved in cortisol hypersecretion and may therefore be regarded as a promising therapeutic target in patients with PPNAD. For instance, Tph inhibitors, which are currently developed for the clinical management of carcinoid syndrome (54, 55), may represent efficient new pharmacological treatments of PPNAD-associated hypercortisolism and could constitute a valuable alternative to adrenal surgery, which is presently recommended in this condition.

Methods

Patients and tissue collection. Adrenal tissues were collected at surgery from 33 patients with PPNAD (Supplemental Table 1). The diagnosis of PPNAD had been performed in accordance with the criteria previously published (24). For DNA/mRNA extractions, tissues were immediately snap frozen in liquid nitrogen after careful dissection of periadrenal fat and fibrous tissues by the pathologist and kept at –80°C. DNA extracted from the tissues was screened for the presence of mutations in the *PRKAR1A*, *PRKACA*, *PDE11A*, and *PDE8* genes, as previously published (21–23, 56). All patients except patient P26 were investigated for *PRKAR1A*, *PDE11A*, and *PDE8* mutations. In addition, *PRKACA* mutations were searched for in patients P1–P12 and P16–P20. Normal adrenal tissues were removed from 7 patients with renal carcinoma and used as controls.

PPNAD primary cell cultures. Tissues from 7 patients (Supplemental Table 2) were obtained at surgery and immediately immersed in culture medium until cell dissociation. Briefly, adrenocortical explants were enzymatically dispersed as previously described (57). Adrenocortical cells were cultured at 37°C in 5% CO_2. Incubation experiments of cells were conducted for 24 hours after 2 days in culture with fresh DMEM (control experiments) or DMEM with test substances. H-89, 5-HT, BIMU8, metoclopramide, cisapride, GR113808, SB269970, and SB258585 were obtained from Sigma-Aldrich. EMD386088 was provided by Tocris. Test substances were applied for 24 hours at 37°C.

For p-chlorophenylalanine (4-chloro-DL-phenylalanine) treatment experiments, PPNAD and normal adrenal explants were cut into 4 fragments of equal size and weight. The explants were immediately placed in fresh DMEM for 4 hours of stabilization. PPNAD and normal adrenal explants were successively incubated with DMEM for 2 hours (not-treated cells, NT) then with DMEM + p-chlorophenylalanine (10^{-7}M) for 2 hours. All culture media were collected for cortisol level measurement.

CAR47 cell culture. The immortalized adrenocortical CAR47 cell line, derived from a patient with PPNAD, was cultured under the normal growth conditions previously described (32). Cells were incubated 6 hours and 12 hours with forskolin (10^{-5}M; Sigma-Aldrich) or H-89 (10^{-5}M) to investigate expression of mRNAs and proteins.

H295R cell culture and generation of H295R shPRKAR1A cell clones. The adrenocortical carcinoma cell line H295R stably transfected with the Tet repressor (H295R/TR) (58) was provided by Enzo Lalli (Institute of Molecular and Cellular Pharmacology, Sophia Antipolis, Valbonne, France). Cells were grown as previously described (35). pSuperior.puro vector (Oligoengine), which can express a Dox-inducible shRNA, was used to created the H295R/TR/shRNA-*PRKAR1A* clones. Silencing hairpin-targeted *PRKAR1A* mRNA (targeted sequence: TGAATGGGCAACCAGTGTT) was cloned into pSuperior.puro vector following the manufacturer's instructions to generate the pSuperior.puro/shRNA-*PRKAR1A* vector. H295R/TR cells were further transfected with the pSUPERIOR.puro/shRNA-*PRKAR1A* vector, and clones were selected with puromycin (5 μg/ml; Sigma-Aldrich). Three shRNA-*PRKAR1A* clones were isolated in which *PRKAR1A* expression was downregulated at least 0.5-fold by Dox (0.2 mg/ml; Sigma-Aldrich) in comparison with control clone (empty vector, EV) transfected with pSuperior.puro vector. All cell clones were investigated for their ability to express *TPH1/2, HTR4/6/7* genes after treatment with Dox (0.5 μg/ml), forskolin (10^{-5}M), and/or H-89 (10^{-5}M) for 6 hours or 12 hours. Cortisol secretion was assayed in cell culture medium after incubation with Dox (0.5 μg/ml), 5-HT (10^{-8} M), and/or H-89 (10^{-5}M) for 24 hours.

mRNA extraction and real-time PCR. Total RNA was extracted and converted into cDNA as previously reported (59). PCR amplification was performed as formerly described (59) by using specific primers (Supplemental Table 4). The amount of cDNA in each sample was calculated by using both standard curve and ΔCt methods. Standard curves were established by using dilution series of cDNAs derived from control tissues known to express the studied genes. Gene expression was also normalized using the average ΔCt value of the normal adrenal cortex (ΔΔCt). The final fold expression changes were calculated using the equation $2^{-\Delta\Delta Ct}$ using *PPIA* or *ACTB* mRNA as an internal control. All experiments were performed at least 3 times.

Characterization of HTR4 isoforms by conventional RT-PCR. Amplification of the cDNAs encoding the different 5-HT_4R C-terminal splice variants was performed by PCR using primer that hybridizes to all *HTR4* mRNAs, as well as splice variant-specific reverse primers (Supplemental Figure 2 and Supplemental Table 5). All procedures were performed as already described (14). The PCR products were analyzed in 2% agarose gels, blotted on a nylon membrane, and hybridized with the [^{32}P]ATP-labeled oligonucleotide probe. Membranes were visualized on a Molecular Dynamics Storm 860 (Molecular Devices). Expression of 5-HT_4R isoforms was evaluated by densitometry using ImageJ software and was considered positive for signal density higher than 3-fold signal/noise ratio.

HPLC characterization of 5-HT. 5-HT present in incubation medium, as well as in DMEM and DMEM added with synthetic 5-HT (10^{-6}M), was detected by using HPLC system equipped with 515 HPLC pumps and 2465 electrochemical detector (Waters, Saint-Quentin-en-Yvelines, France) according to the previously described procedure (13).

Cortisol secretion. Cortisol concentrations in culture medium were measured using a radioimmunoassay procedure (Sigma-Aldrich). Cross-reactivity of cortisol antibodies with test compounds was less than 0.01%. Results are expressed as mean ±SEM.

IHC. Sections from formalin-fixed and paraffin-embedded adrenal tissues were immunostained using antibodies (Supplemental Table 6) diluted in Zymed (Invitrogen). The immunohistochemical procedures were performed as previously described (28). Mast cell densities in the tissues were evaluated by using the Mercator image analysis software (Explora Nova), as previously described (35). Briefly, nodular and adrenal regions adjacent to nodules were defined at low magnification to establish the section maps and quantify surface areas (35). Immunopositive cells were then counted at high magnification. Data were expressed as density of positive cells per area unit (cm^2). All images were obtained on PRIMACEN, the Cell Imaging Platform of Normandy, University of Rouen Normandie.

Immunohistofluorescence. CAR47 and shPRKAR1A H295 cell lines were washed twice with ice-cold

PBS, fixed in a paraformaldehyde solution (4% w/v) for 15 minutes, and then permeabilized with a Triton X-100 solution (Sigma-Aldrich; 0.1% Triton X-100 in PBS) for 20 minutes. After a saturation step of 1 hour in BSA at 1 mg/ml in PBS, cells were incubated overnight at 4°C with primary antibodies (Supplemental Table 6). Immunoreactivities were revealed with FITC-conjugated goat anti-rabbit IgG (1:300), Alexa 488–conjugated donkey anti-rabbit IgG, and Alexa 594–conjugated donkey anti-mouse at 1:300 (all from Invitrogen). Nuclei were visualized by DAPI staining (1 pg/ml; Sigma-Aldrich). Fluorescence was examined on a TCS SP2 confocal microscope (Leica). Specificity controls of immunoreactions were performed by substituting the primary or secondary antibodies with PBS. For each staining analysis, 3 fields were randomly selected. For all images of the same experiment, the acquisition settings were kept unchanged except the z parameters.

Western blot analysis. CAR47 or shPRKAR1A H295R cells cultured in 6-well plates (10^5 cells/well) were lysed in a buffer containing 10 mM Tris-HCl, pH 7.4, 0.05% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation (12,000 *g*; 15 minutes), the whole protein precipitate was dissolved in electrophoresis-denaturing buffer. Proteins were quantified by the Bradford assay (Bio-Rad, Life Science). Extracts were separated by SDS-polyacrylamide gel electrophoresis followed by electrotransfer onto nitrocellulose membranes (Amersham Biosciences). The proteins were detected by using polyclonal antibodies (Supplemental Table 7) followed by horseradish peroxidase–conjugated secondary antibodies (1:5,000). The antigen-antibody complexes were visualized by the chemiluminescence ECL Western blotting analysis system (Amersham Biosciences) and exposure to Kodak X-OMAT films (Sigma-Aldrich). Signals were quantified using the ImageJ software developed by Wayne Rasband (NIH).

Statistics. Statistical analysis of the data was performed by use of the nonparametric Mann-Whitney *U* test and Bonferroni's test after one-way ANOVA (Prism, GraphPad Software). Probability values less than 0.05 were considered significant.

Study approval. The protocol was reviewed by Cochin Institute and NIH IRBs, and written informed consent was obtained from each patient prior to participation in the study.

Author contributions

ZB, EL, BR, and HL designed research; ZB, EL, BR, SR, JW, CD, IB, and MRR performed research; RL, JY, DC, MCV, ES, CAS, JB, and HL collected clinical, biological, histological, and genetic data for patients; ZB, EL, AM, and HL analyzed data; and ZB, EL, and HL wrote the paper.

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