

Supplemental figures

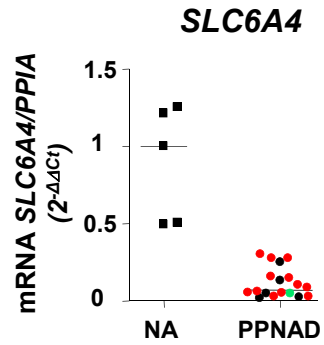


Figure 1. *SLC6A4* mRNAs encoding the 5-HT transporter SERT in adrenocortical explants of normal adrenals (NA) and PPNAD tissues. PPNAD with *PRKAR1A* (red), *PDE11A* (green) and no (black) mutations. mRNA expression levels were normalized to *PPIA*.

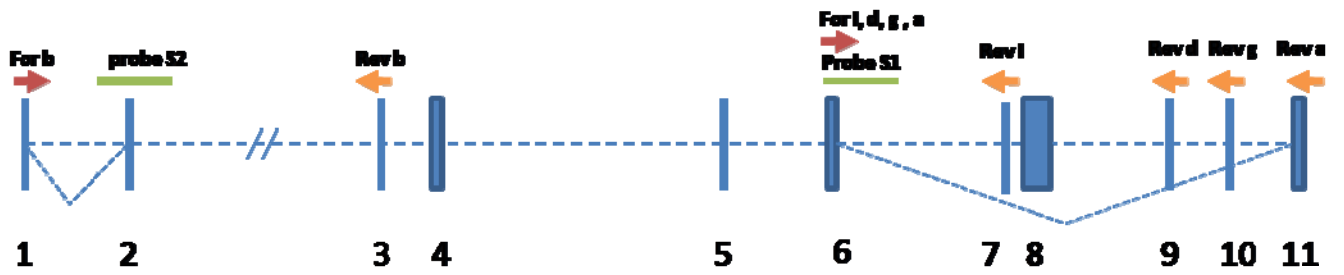


Figure 2. Schematic representation of the human *HTR4* gene showing the position of the primers used to amplify the *HTR4* isoform mRNAs.

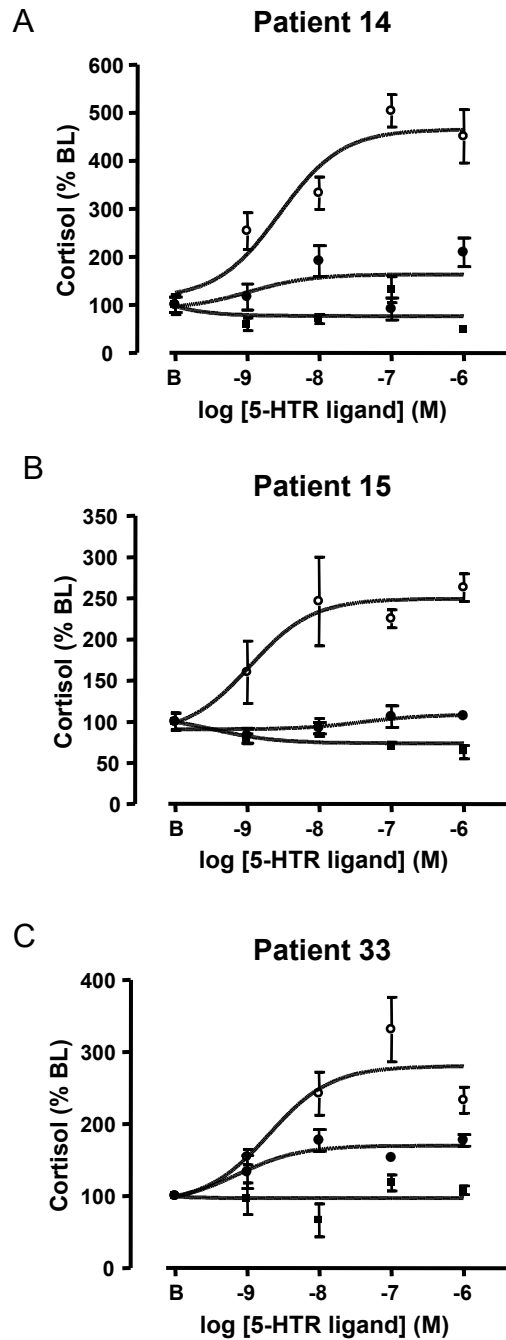


Figure 3. Sensitivity of PPNAD cells to 5-HT₄ receptor agonists. (A-C) Effects of graded concentrations (10⁻⁹ to 10⁻⁶ M) of 5-HT and the partial 5-HT₄ receptor agonists cisapride (■) and metoclopramide (●) on cortisol secretion by cultured cells derived from PPNAD patients P14 (A), P15 (B) and P33 (C). Data represents mean ± sem,

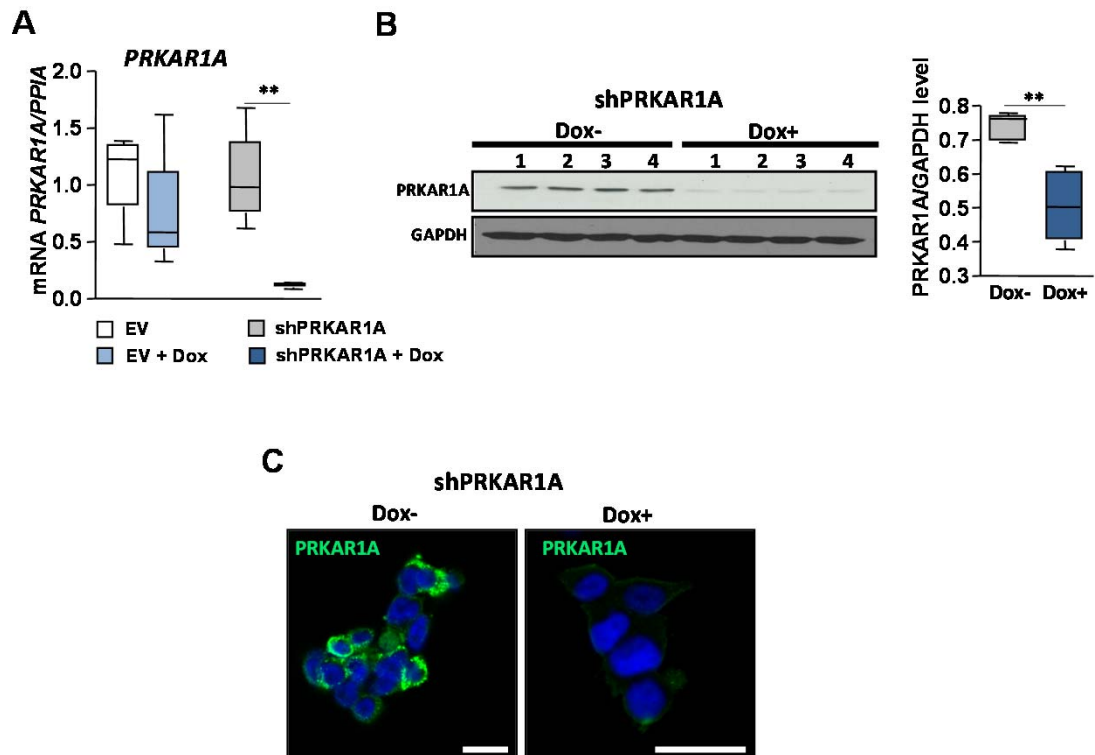


Figure 4. *PRKAR1A* silencing in shPRKAR1A H295R cells. (A) Expression levels of *PRKAR1A* mRNAs normalized to *PPIA* in H295R cells transfected with either the empty vector (EV) or the doxycycline sensitive shPRKAR1A expressing vector and cultured in the absence or presence of doxycycline (Dox). (B) Western blot analysis of PRKAR1A in shPRKAR1A H295R cells cultured with or without doxycycline. (C) Immunocytofluorescence staining for PRKAR1A in shPRKAR1A H295R cells cultured with or without doxycycline. Cells were treated with doxycycline to induce shPRKAR1A expression. Data of 4 determinations are presented as box plot. Data were analyzed by using Mann-Whitney t-test. **, $P < 0.01$; *, $P < 0.05$. Scale bars: 30 μm .

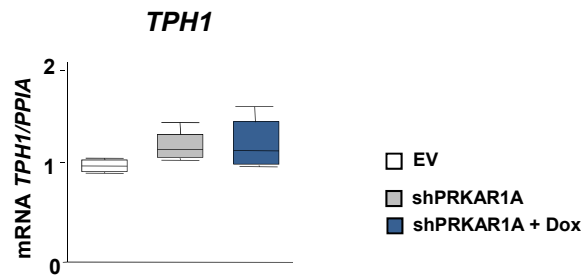


Figure 5. Expression levels of *TPH1* mRNAs normalized to *PPIA* in H295R cells transfected with either the empty vector (EV) or the doxycycline sensitive shPRKAR1A expressing vector and cultured in the absence or presence of doxycycline (Dox). Data are presented as box plot.

Supplemental Table 1: Clinical and genetic characteristics of the patients

Patients	Gender	Age (Yrs)	Disease	Gene	Mutation	In vitro investigations		
						Cell cultures	RT-PCR	IHC
P1	Female	42	PPNAD	PRKAR1A	c.439A>G/p.Ser147Gly	-	X	-
P2	Female	5	PPNAD	PRKAR1A	c.865G>T /p.G289W	-	X	-
P3	Female	4	iMAD	-	none	-	X	-
P4	Female	21	PPNAD	PRKAR1A	c.709(-5-107)del	-	X	-
P5	Female	7	iMAD	PDE11A	171delTfs41X	-	X	-
P6	Female	5	iMAD/iPPNAD	-	none	-	X	-
P7	Male	53	PPNAD	PRKAR1A	c.891+3A>G/	-	X	X
P8	Female	14	PPNAD	PRKAR1A	c.101_105del/p.S34fsX9	-	X	X
P9	Female	7	iMAD	-	none	-	X	-
P10	Female	27	PPNAD	PRKAR1A	c.177+1G>A	-	X	-
P11	Female	16	PPNAD	PRKAR1A	c.43_58del/p.L15SfsX104	-	X	X
P12	Female	22	PPNAD	PRKAR1A	c.491_492del/ p.V164DfsX4	-	X	-
P13	Female	27	PPNAD	PRKAR1A	C 709 (-7-2)del 6	-	-	X
P14	Female	56	PPNAD	PRKAR1A	C 709 (-7-2)del 6	X	-	X
P15	Female	35	PPNAD	PRKAR1A	C 709 (-7-2)del 6	X	-	X
P16	Female	43	PPNAD	-	none	-	X	X
P17	Female	16	PPNAD	PRKAR1A	c.440+1insG	-	X	X
P18	Male	19	PPNAD	-	none	-	-	X
P19	Female	33	PPNAD	PRKAR1A	c.178-22A>G	-	-	X
P20	Female	45	PPNAD	PRKAR1A	c.353_365del/p.I118TfsX6	-	X	X
P21	Female	14	PPNAD	PRKAR1A	c.-7+5delG	X	X	X
P22	Female	49	PPNAD	PRKAR1A	C 709 (-7-2)del 6	-	-	X
P23	Female	25	PPNAD	PRKAR1A	C 709 (-7-2)del 6	-	-	X
P24	Female	56	PPNAD	PRKAR1A	none	-	-	X
P25	Female	38	PPNAD	PDE11A	1655_1657delTCT/insCCfs15X	-	-	X
P26	Female	16	PPNAD	-	-	-	-	X
P27	Female	14	PPNAD	PRKAR1A	none	-	-	X
P28	Female	49	PPNAD	PRKAR1A	C 709 (-7-2)del 6	X	-	X
P29	Female	12	PPNAD	PRKAR1A	C 709 (-7-2)del 6	X	-	X
P30	Female	16	PPNAD	-	none	-	-	X
P31	Female	47	PPNAD	PRKAR1A	c.763-764 del AT	-	X	X
P32	Female	27	PPNAD	PRKAR1A	none	X	X	-
P33	Male	28	PPNAD	PRKAR1A	none	X	-	-
Total						7/33	18/33	22/33

IHC, immunohistochemistry

Supplemental Table 2: Biological and radiological characteristics of PPNAD patients enrolled for adrenocortical cell culture experiments

Patients	P14	P15	P21	P28	P29	P32	P33
Urinary Free Cortisol (UCF) (µg/24h) (N<80)	76	200	328	110	303	161	447
Plasma Cortisol at 08H00 (µg/l) (N: 90-308)	155	194	-	217	3.9 saliva	180	168
Plasma Cortisol at 00h00 (µg/l) (N< 75)	138	180	230	226	5.7 saliva	204	160
ACTH Plasma at 08H00 (pg/ml)	<5	<5	<5	<5	<5	3	<5
Paradoxical plasma cortisol response to dexamethasone ¹	Yes	No	Yes	Yes	Yes	Yes	Yes
Presentation of adrenal glands at CT Scan	MicroN	MicroN	MicroN	MicroN	MicroN	MicroN	MicroN

CT, computerized tomography; MicroN, micronodular

1. Stratakis, C. A. *et al.* Paradoxical response to dexamethasone in the diagnosis of primary pigmented nodular adrenocortical disease. *Ann. Intern. Med.* **131**, 585–591 (1999).

Supplemental Table 3: Expression of *HTR4* splicing variants in PPNAD

Patients	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
(a)	+	+	+	+	+	+	+	+	+	+
(b)	+	+	+	+	+	+	+	+	+	+
(d)	-	-	-	-	-	-	-	-	-	-
(g)	+	-	-	-	+	-	-	-	-	+
(i)	+	-	+	+	-	-	-	+	-	-

+, isoform detected; -, isoform not detected

Supplemental Table 4: Sequences and GenBank accession numbers of the primers used for Real-Time PCR studies

Gene name	Accession number	Primer	Sequence primer	Product size (bp)
TPH1	NM_004179.2	Forward	5'-TGCAAAGGAGAAGATGAGAGAATTTAC-3'	114
		Reverse	5'-CTGTTATGCTCTTGGTGTCTTTC-3'	
TPH2	NM_173353.3	Forward	5'-GCCTTCTCTGTGTTATTAG-3'	186
		Reverse	5'-TCATCCAACTGCTGTGTTACC-3'	
SLC6A4	NM_001045.5	Forward	5'-TGGTTCTATGGCATCACTCAGTTC-3'	148
		Reverse	5'-GTTGTGGCGGGCTCATCAG-3'	
HTR4	NM_001040169.2 NM_000870.6 NM_001286410.1 NM_001040172.2 NM_199453.3 NM_001040173.2	Forward	5'-CTGGTGGCCTTCTACATCCC-3'	199
		Reverse	5'-ATGCACAGGGTCTTGCTGC-3'	
HTR6	NM_000871.2	Forward	5'-GGGTGCCATATGCTTACCT -3'	150
		Reverse	5'-TCAGCAGACTCCACCCTG -3'	
HTR7	NM_000872.4 NM_019860.3 NM_019859.3	Forward	5'-AAAACATCTCCATCTTTAAGCGAGAA-3'	70
		Reverse	5'-GTAAGGCCCGACGATGAT-3'	
PRKAR1A	NM_002734.4 NM_212471.2 NM_212472.2 NM_001276289.1 NM_001276290.1 NM_001278433.1	Forward	5'-AATGGCCGCTTTAGCCAAAGCC-3'	235
		Reverse	5'-TTCTCAAAGCTCCCTCCTTC-3'	
ACTB	NM_001101.3	Forward	5'-ACCTGTACGCCAACACAGTG-3'	144
		Reverse	5'-ACACGGAGTACTTGCGCTCA-3'	
PPIA	NM_001300981.1 NM_021130.4	Forward	5'-ATGCACTGGTGGCAAGTCC-3'	241
		Reverse	5'-TTGCCATTCTGGACCCAAA-3'	
CYP11B1	NM_000497.3	Forward	5'-AGGAGACCTTGCGGCTTACC-3'	110
		Reverse	5'-GAACACGCGACCAATGTC-3'	

Supplemental Table 5: Sequences and GenBank accession numbers of the primers used for PCR amplification of *HTR4* splicing variants.

HTR4 isoforms	Accession number	Primer	Sequence primer	Product size
(a)	NM_001040169.2	Forward	5'-CGGGCAGGAGCCTCCTCCGAGAG-3'	439
		Reverse	5'-GTATGGGCAGTTTCTCGAGTTCCTGATGATG-3'	
(b)	NM_000870.6	Forward	5'-GAATCTGCCAGGGCACCTCGCT-3'	207
		Reverse	5'-ACCAGCAGGTTCCCAAGAT-3'	
(g)	NM_199453.3	Forward	5'-CGGGCAGGAGCCTCCTCCGAGAG-3'	398
		Reverse	5'-CAGAAGAGCAGGAGGAAGCTGGAGACAG-3'	
(d)	NM_001040172.2	Forward	5'-CGGGCAGGAGCCTCCTCCGAGAG-3'	494
		Reverse	5'-CATCCAATGAATTTATTTGATAACTTCAG-3'	
(i)	NM_001040173.2	Forward	5'-CGGGCAGGAGCCTCCTCCGAGAG-3'	455
		Reverse	5'-AATGGACCCGCTCTGGCAGGCTTT-3'	
Probes used for southern blot analysis		S1	5'-CCCTGGGCAGGTGTGGACTGC-3'	-
		S2	5'- TGGACAAACTTGATGCTAA-3'	-

Supplemental Table 6: Antibodies used for immunohistochemical and/or immunohistofluorescence studies

Antibodies	Source	Host	Antigen retrieval	Dilution
5-HT	AB serotec (AHP 522)	Polyclonal rabbit	Citrate buffer pH=6 95°C – 20 min	1:20
5-HT ₄ R	MBL (LS-A655)	Ployclonal rabbit	Citrate buffer pH=6 95°C – 20 min	1:100
5-HT ₆ R	Abcam (ab101911)	Polyclonal rabbit	-	1:1200
5-HT ₇ R	Sigma (S0320)	Polyclonal rabbit	Citrate buffer pH=6 95°C – 20 min	1:500
Tryptophan hydroxylase	Sigma (T0678)	Monoclonal mouse	-	1:500
Tryptophan hydroxylase type 2 (Tph2)	Everest BIOTECH (EB07050)	Polyclonal goat	-	1:500
Steroidogenic factor 1 (SF1)	R&D systems (PP-N1665-00)	Monoclonal mouse	Citrate buffer pH=6 95°C – 20 min	1:100
Tryptase	AB serotec (MCA 1438T)	Monoclonal mouse	-	1:20
17 α -Hydroxylase (17-OH)	Drs. V. Luu the and G. Pelletier, Laval University Medical Center	Polyclonal rabbit	Citrate buffer pH=6 95°C – 20 min	1:2000

Supplemental Table 7: Antibodies used for western blot analyses

Antibodies	Source	Host	Predicted molecular weight	Dilution
Tph2	Abcam (ab111828)	Polyclonal rabbit	56 kDa	1:1000
5-HT₄R	Abcam (ab87337)	Polyclonal rabbit	43 kDa	1:400
5-HT₆R	Abcam (ab101911)	Polyclonal rabbit	47 kDa	1:1000
5-HT₇R	Abcam (ab128892)	Monoclonal rabbit	54 kDa	1:1000
GAPDH	Cell signaling (14C10)	Polyclonal rabbit	37 kDa	1:1000