G-protein-coupled receptors in aldosterone-producing adenomas: a potential cause of hyperaldosteronism

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Abstract

The source of aldosterone in 30–40% of patients with primary hyperaldosteronism (PA) is unilateral aldosterone-producing adenoma (APA). The mechanisms causing elevated aldosterone production in APA are unknown. Herein, we examined the expression of G-protein-coupled receptors (GPCRs) in APA and demonstrated that when compared with normal adrenals, there is a general elevation of certain GPCR in many APA and/or ectopic expression of GPCR in others. RNA samples from normal adrenals ($n=5$), APAs ($n=10$), and cortisol–producing adenomas (CPAs; $n=13$) were used on 15 genomic expression arrays, each of which included 223 GPCR transcripts presented in at least 1 out of 15 of the independent microarrays. The array results were confirmed using real-time RT-PCR (qPCR). Four GPCR transcripts exhibited a statistically significant increase that was greater than threefold when compared with normal adrenals, suggesting a general increase in expression when compared with normal adrenal glands. Four GPCR transcripts exhibited a >15-fold increase of expression in one or more of the APA samples when compared with normal adrenals. qPCR analysis confirmed array data and found the receptors with the highest fold increase in APA expression to be LH receptor, serotonin receptor 4, GnRH receptor, glutamate receptor metabotropic 3, endothelin receptor type B-like protein, and ACTH receptor. There are also sporadic increased expressions of these genes in the CPAs. Together, these findings suggest a potential role of altered GPCR expression in many cases of PA and provide candidate GPCR for further study.

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Introduction

It is well known that aldosterone is closely associated with hypertension, as well as cardiac and renal injury (Funder 2001, Anand *et al.* 2006, Rossi *et al.* 2006c). The pathophysiological changes in the cardiovascular system caused by aldosterone excess are either from genetic causes or due to primary hyperaldosteronism (PA). PA is characterized by hypertension, hypokalemia, elevated plasma aldosterone levels, and suppressed plasma renin activity, which leads to renal, cardiovascular, and other pathologic complications. Considerable evidence now suggests that PA may be a common cause of secondary hypertension and is likely to affect almost 10% of all hypertensive patients (Lim *et al.* 2000, Gordon 2004, Rossi *et al.* 2006d). In almost half of these cases, the cause is an adrenal aldosterone-producing adenoma (APA; Koshiyama *et al.* 2003, Gordon 2004, Plouin *et al.* 2004, Rossi *et al.* 2006d). These adenomas have in common elevated expression of the enzyme aldosterone synthase (CYP11B2), which is responsible for the final step in aldosterone production and is normally found only in the adrenal glomerulosa (Fallo *et al.* 2002, Saner-Amigh *et al.* 2006). Expression of this enzyme is normally regulated by the

renin–angiotensin II (Ang II) system. While the renin–Ang II–aldosterone axis has a tight feedback regulatory system, the production of aldosterone in APA has lost normal regulation and continues to produce aldosterone under low-renin conditions (Bassett *et al.* 2005).

The mechanisms causing aldosterone production in APA remain poorly defined. Definition of the molecular mechanisms that cause adenoma expression of CYP11B2 would contribute significantly to our understanding of PA. There are compelling evidence to support elevated expression of certain G-protein-coupled receptors (GPCRs) as a cause of some cases of adrenocorticotrophin (ACTH)-independent Cushing's syndrome (Bugalho *et al.* 2000, Dall'Asta *et al.* 2004, Lampron *et al.* 2006, Mazzuco *et al.* 2006b). We have recently noted elevated expression of the GPCR luteinizing hormone receptor (LH-R) in certain cases of APA (Saner-Amigh *et al.* 2006). Herein, we used microarray analysis and real-time RT-PCR confirmation that certain GPCRs exhibit elevated expression in APA. Together, these findings suggest a potential role for elevated expression of GPCR in many cases of PA and provide candidate GPCR for further clinical study.

Materials and Methods

Subjects and tissues

Normal human adult adrenals were obtained through the Cooperative Human Tissue Network (Philadelphia, PA, USA) and Clontech. All the normal adrenal samples came from patients who underwent adrenalectomy along with a renalectomy due to renal carcinoma. The adrenal samples acquired from autopsies were obtained no more than 6 h after death, and it was confirmed that the causes of death were unrelated to adrenal function. Real-time quantitative RT-PCR was used to check the legitimacy of the normal control adrenal samples. Examination of tissue sections from each adrenal gland also suggested normal histology.

The APA samples were obtained from the Department of Medicine at UT Southwestern and Division of Endocrinology at the University of Padua. All APA samples were from Conn's syndrome patients with significantly elevated circulating aldosterone levels that were lowered to the normal range after surgical removal of the tumor. Thirty-two adenomas were collected from patients with PA. Twenty-eight of these adenomas had levels of CYP11B2 that were two s.d.s greater than seen in normal adrenal glands and these samples were used for analyses. It is currently not clear if the adenomas with low CYP11B2 expression are the source of aldosterone in these patients. Other studies suggest that subcapsular micronodules have varying expression of steroidogenic enzymes – some with aldosterone synthase and some without (Shigematsu *et al.* 2006). Future studies are required to determine whether some tumor-bearing adrenals from PA patients also have small CYP11B2-positive micronodules as the source of aldosterone production.

The cortisol-producing adenoma (CPA) tissues were obtained from Division of Endocrinology at the University of Padua from patients with Cushing's syndrome. Each of the patients was cured following surgical removal of the adenoma. The use of these tissues was approved by the Institutional Review Boards of the University of Texas Southwestern Medical Center (Dallas, TX, USA), the Medical College of Georgia (Augusta, GA, USA), and the University of Padua (Padua, Italy). In addition, this study was approved by the ethical committee at the University of Padua, and informed consent was obtained from every patient.

Microarray analysis

Total RNA isolated from normal adult adrenal glands ($n=5$) and APA ($n=10$) were used on 15 genomic expression arrays. In brief, RNA was hybridized to an Affymetrix human HG-U133+2 oligonucleotide microarray set containing 54 675 probe sets representing ∼40 500 independent human genes. The arrays were scanned at high resolution using an Affymetrix GeneChip Scanner 3000 located at the Medical College of Georgia Microarray Core Facility (Augusta, GA, USA). Results were analyzed using GeneSpring GX 7.3.1 software (Silicon Genetics, Redwood City, CA, USA) to identify differences in expression of GPCR between normal adult adrenal and APA.

RNA extraction

The tissue was pulverized in liquid nitrogen to a powder. Total RNA was extracted using TRIzol Reagent (Invitrogen). The purity and integrity of the RNA were assessed by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and its quantity was determined by NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

cDNA synthesis

Total RNA of 2 µg was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's recommendations and incubated at 25 °C for 10 min and 37 °C for 2 h. The synthesized cDNA was subjected to 1:10 dilution and stored at −20 °C.

Real-time quantitative PCR

Primers and probes for the amplification of the selected GPCR sequences were performed using the TaqMan Gene Expression Assays (Applied Biosystems) based on published sequences for genes encoding the respective human GPCR. The gene symbols and AB assay numbers are listed in Table 1.

The primer and probe set for human CYP11B2 was designed using Primer Express 3.0 (Applied Biosystems) and purchased from IDT (Integrated DNA Technologies Inc., Coralville IA, USA) as published previously (Saner-Amigh *et al.* 2006). In brief,

Forward: 5′-GGCAGAGGCAGAGATGCTG-3′,
Reverse: 5′-CTTGAGTTAGTGTCTCCACCAGGA-3′,
Probe: 5′-CTGCACCACGTGCTGAAGCACT-3′.

PCRs were performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) with a total volume of 20 µl per reaction following the reaction parameters recommended by the manufacturer, which include denaturation at 95 °C for 20 s followed by amplification for 40 cycles (95 °C at 3 s, 60 °C at 30 s, fluorescence measurement). For each GPCR, the 20 µl total volume consisted of 10 µl TaqMan Fast Universal PCR Master Mix (2×; Applied Biosystems), 900 nM of each primer, 400 nM probe, and 5 µl of each first-strand cDNA sample. CYP11B2 reaction mix consisted of 10 µl TaqMan PCR Master Mix (2×; Applied Biosystems), 100 nM primer/probe mix, and 5 µl of each first-strand cDNA sample. The 18S rRNA was detected and quantified using the TaqMan Ribosomal RNA Control Reagents (Vic Probe; Applied Biosystems). Each reaction included 10 µl TaqMan PCR

Table 1 G-protein-coupled receptor (GPCR) transcripts with a mean increase of greater than or equal to threefold based on ten separate APA and five normal adrenal microarrays. The microarray results are presented as pooled data from the applicable microarray experiments. The mean values represent the fold-induction of APA over normal adrenal. These data are presented as the mean and s.e.m. with *P* value between the APA and normal adrenal provided. The microarray data are provided for GPCR with a greater than 3-fold increase or decrease in expression.

Gene name	Gene symbol	Affymetrix probe ID	Accession no.	Mean ± s.e.m.	*P* value
GPCR transcripts with a mean increase of greater than or equal to threefold based on ten separate APA and five normal adrenal microarrays					
Putative endothelin receptor type B-like protein	*GPR37*	209631_s_at	U87460	14·43 ± 5·20	0·10
Glutamate receptor, metabotropic 3	*GRM3*	205814_at	NM_000840	5·76 ± 2·47	0·21
5-Hydroxytryptamine (serotonin) receptor 4	*HTR4*	207577_at	AJ131724	4·98 ± 1·01	0·02
Luteinizing hormone/choriogonadotropin receptor	*LH-R*	207240_s_at	NM_000233	4·87 ± 3·49	0·46
Melanocortin 2 receptor (adrenocorticotropic hormone)	*MC2R*	217434_at	Y10259	4·24 ± 0·74	0·01
Interleukin 8 receptor, beta	*IL8RB*	207008_at	NM_001557	3·83 ± 1·32	0·16
Olfactory receptor, family 2, subfamily J, member 2	*OR2J2*	208508_s_at	NM_030905	3·82 ± 1·04	0·10
5-Hydroxytryptamine (serotonin) receptor 7 (adenylate cyclase coupled)	*HTR7*	236281_x_at	R44298	3·45 ± 0·75	0·04
G-protein-coupled receptor 23	*GPR23*	206960_at	NM_005296	3·43 ± 0·75	0·05
Olfactory receptor, family 2, subfamily W, member 1	*OR2W1*	221451_s_at	NM_030903	3·00 ± 0·65	0·06
GPCR transcripts with a mean decrease of greater than threefold based on ten separate APA and five normal adrenal microarrays					
Duffy blood group	*FY*	208335_s_at	NM_002036	−3·70 ± 0·09	0·01
Arginine vasopressin receptor 1A	*AVPR1A*	206251_s_at	NM_000706	−4·17 ± 0·06	<0·01
Prostaglandin F receptor (FP)	*PTGFR*	207177_at	NM_000959	−6·35 ± 0·04	<0·01

Samples of total RNA from 10 aldosterone producing adenomas (APA) and 5 normal adrenal glands (AN) were used for 15 independent microarray assays and analyzed using GeneSpring software.

Master Mix (2×; Applied Biosystems), 100 nM probe, and 50 nM primers. Negative controls contained water instead of first-strand cDNA. Quantitative normalization of cDNA in each tissue-derived sample was performed using expression of 18S rRNA as an internal control. The generated C_t value of each gene was normalized by its respected C_t value of 18S rRNA (ΔC_t). Then each gene was further normalized using the average ΔC_t value of the normal adult adrenal ($\Delta\Delta C_t$). The final fold expression changes were calculated using the equation $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen 2001).

Statistical analysis

Data were analyzed and compared with control values (mean of normal adrenal samples) using the Mann–Whitney Rank Sum test with the SigmaStat 3.0 software package (SPSS, Chicago, IL, USA). The results were considered significantly different when *P* value was ≤0·05.

Results

A total of 544 independent GPCRs were present on the Affymetrix human HG-U133+2 oligonucleotide microarray. Of the 544 GPCR on the array, 223 transcripts were determined to be present (with regard to signal intensity) in at least 1 out of

the 15 microarrays (ten APA and five normal adrenal RNA samples). The vast majority of GPCR (95%) did not show a threefold difference between normal adrenal and APA (Fig. 1). However, 12 GPCR had a mean increased or decreased expression level by greater than threefold in APA versus normal adrenal glands (Fig. 1 and Table 1). Four transcripts (5--hydroxytryptamine (serotonin) receptor 4 (HTR4), melano-cortin 2 receptor (ACTH; MC2R), HTR7, and GPR23, defined in Table 1) were expressed at statistically higher levels in APA than in normal adrenal glands (Table 1). Four of the ten transcripts (HTR4, LH-R, MC2R, and HTR7) have previously been studied and exhibited higher expression levels in APA samples (Contesse *et al.* 1999, Schubert *et al.* 2001, Lefebvre *et al.* 2002, Cartier *et al.* 2005, Saner-Amigh *et al.* 2006). The remaining six transcripts have not been studied in APA as potential regulators of adrenal function.

Because sporadic expression of GPCR have been shown in cortisol-producing tumors, we examined GPCR that had >15-fold higher levels in at least 1 out of the 15 microarrays. Four transcripts out of the 223 GPCR analyzed (GPR37, GRM3, LH-R, and gonadotropin–releasing hormone receptor (GnRHR), defined in Table 2) had a >15-fold increase in the APA microarrays. Only two receptor transcripts were found that significantly varied in APA. One of the two GPCR transcripts (prostaglandin F receptor) that decreased in expression is known to be expressed in the
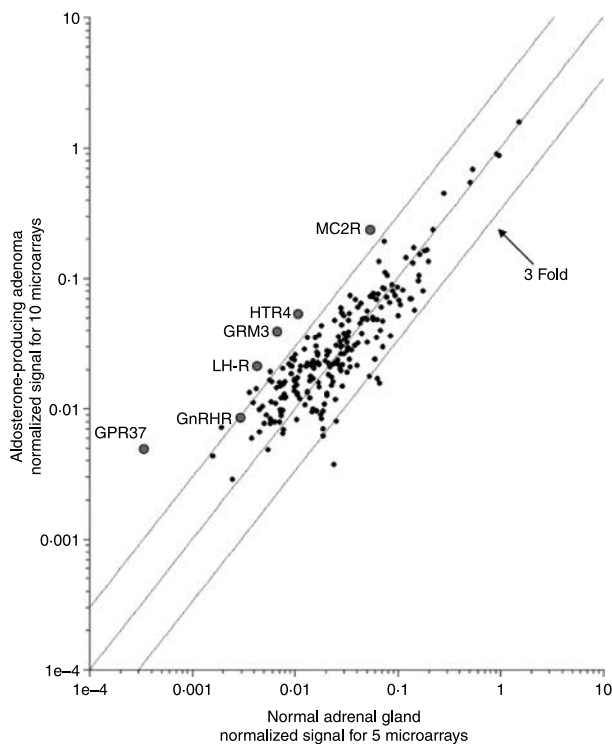
Figure 1 Microarray scatterplot for G-protein-coupled receptors (GPCRs). Data represent average values from ten independent microarrays done using aldosterone-producing adenoma (APA) versus five independent normal adrenal gland samples. Each spot represents a unique sequence with a total of 223 independent GPCR transcripts based on normalized microarray data. Dots within the parallel lines represent mRNAs with less than threefold differences in expression. The five transcripts with the highest mean increased expression as well as GnRHR are labeled on the plot.

adrenal medulla and may therefore result from medullary component of the whole adrenals used as controls (Table 1).

To better examine the incidence of increased GPCR expression, confirmation studies of focus were placed on the

five transcripts with the highest mean increase in expression relative to normal adrenal, as well as the GnRHR (Fig. 1, Tables 1 and 2). First, microarray data were confirmed in a bank of 28 independent APA samples using qPCR. Thirteen cases of CPA samples were also included and compared with the receptor expression in Cushing's syndrome. In addition, we separated the normal adrenals from both renal cancer patients (RC adrenal) and those from autopsy samples (autopsy adrenal). In all cases, the qPCR confirmed the increase of transcript seen using microarray analysis. Later, we provide detailed descriptions of the five GPCR transcripts that were expressed greater than fivefold in APA samples as shown with the qPCR methods.

Hundred percent of our 28 APA samples had a significantly increased CYP11B2 expression ($>$ mean $\pm$ 2 s.d.; Fig. 2). No studied CPA had an elevated CYP11B2 expression (Fig. 2). Microarray analysis indicated that GPR37 (putative endothe-lin receptor type B-like protein), GRM3 (metabotropic glutamate receptor 3), HTR4, LH–R, and MC2R rep-resented the five receptors with the most increased expression in APA tissue (Table 1). GPR37 exhibited the highest increase (14-fold) based on microarray analysis (Table 1) and a tenfold increase using qPCR (Table 3). Using qPCR data, we were able to compare GPR37 transcript levels in each normal and APA sample (Fig. 3). GPR37 expression levels were $>$2 s.d. above the mean of normal adrenals in 5 out of the 28 APA samples examined (Fig. 3). No CPA studied has an elevated GPR37 expression. GRM3 exhibited a 5·8-fold increase in transcript expression by microarray and a 5·3-fold increase by qPCR (Tables 2 and 3). Four APA samples increased GRM3 by $>$2 s.d. above the mean expression level seen in normal adrenal glands (Fig. 3). On the other hand, 1 out of 13 CPA samples had an elevated GRM3 expression (Fig. 3).

Expression of HTR4 in the adrenal has been well documented and a role for this receptor in the regulation of aldosterone production has been demonstrated (Lefebvre et al. 2000). Microarray analysis suggested that the level of HTR4 was increased by fivefold in APA, and this represented a

Table 2 G-protein-coupled receptor (GPCR) with an increase of $>$15-fold in one or more of the APA samples based on ten separate APA and five normal adrenal microarrays. The microarray results are presented as pooled data from the applicable microarray experiments. The mean values represent the fold-induction of APA over normal adrenal. These data are presented as the mean and s.e.m. The number of microarrays where the GPCR transcript was $>$15-fold above controls is indicated.

	Gene symbol	Affymetrix probe ID	Accession no.	Mean $\pm$ s.e.m. for all arrays	APA with $>$15-fold expression
Gene name					
GPCR transcripts with a mean increase of greater than or equal to 15-fold in one or more of the ten APA samples					
Putative endothelin receptor type B-like protein	GPR37	209631_s_at	U87460	14·43 $\pm$ 5·20	3
Glutamate receptor, metabotropic 3	GRM3	205814_at	NM_000840	5·76 $\pm$ 2·47	1
Luteinizing hormone/choriogonadotropin receptor	LH-R	207240_s_at	NM_000233	4·87 $\pm$ 3·49	1
Gonadotropin-releasing hormone receptor	GnRHR	211522_s_at	L03380	2·82 $\pm$ 1·68	1

Samples of total RNA from 10 aldosterone producing adenomas (APA) and 5 normal adrenal glands (AN) were used for 15 independent microarray assays and analyzed using GeneSpring software.
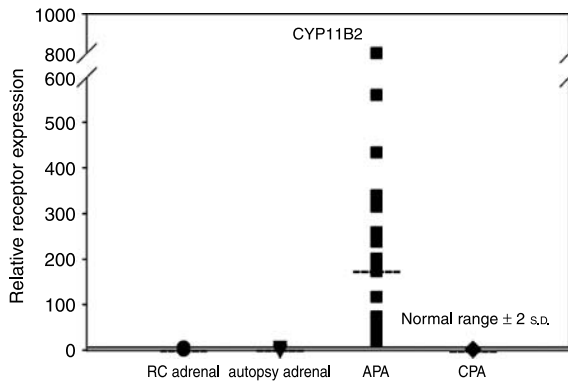
Figure 2 Individual adrenal samples from renal carcinoma surgery (RC adrenal), autopsy (autopsy adrenal), APA, and CPA tumor expression of CYP11B2. The vertical point scatterplots compare these G-protein-coupled receptor expression levels in four different groups using qPCR. Each dot on the plots represents one sample after normalization with 18S rRNA. The gray regions represent respective normal mean ± 2 s.d. One sample from autopsy adrenal, one from APA, and four from CPA are below our detection level.

significant increase when compared with that seen in normal adrenals (Table 1). Transcript examination with qPCR suggested a mean transcript increase of 17-fold, which was also significantly higher than the levels found in normal adrenals (Table 3). Examination of individual expression levels suggested that 23 APA samples had HTR4 levels that were >2 s.d. above normal adrenals (Fig. 3). Out of 13 CPA samples, 2 were found to have significantly elevated HTR4 expression.

MC2R had the highest signal level (an indicator of transcript abundance) of any of the genes found to be different between APA and normal adrenal (Fig. 1). Micro-array analysis showed a significant increase in the overall ACTH receptor expression (MC2R expression that was fourfold above normal adrenal (Table 1)). However, this result was not confirmed by qPCR which used more adrenals from renal carcinoma as normal controls (*n* = 9; Table 3). Only 1 out of the 28 individual APA samples had transcript levels that were >2 s.d. above normal adrenal glands (Fig. 3). Out of 13 CPA samples, 1 was found to have significantly increased MC2R expression (Fig. 3).

Two receptors normally associated with the reproductive axis (LH-R and GnRHR) were shown to be elevated in APA samples (Fig. 1 and Table 1). We previously demonstrated that LH-R transcripts are elevated in certain cases of APA (Saner-Amigh *et al.* 2006). Herein, we found in the pooled data from ten APA, only a 4·9-fold increase in LH-R transcript (Table 1). However, using qPCR one APA exhibited a 255-fold elevation, when compared with normal adrenals. It was the only sample in this cohort with >2 s.d.s beyond the mean of normal adrenals (Fig. 3). Out of 13 CPA samples, 2 were found to have significantly increased LH-R expression (Fig. 3). Microarray analysis suggested that the average increase in expression of GnRHR across the ten APA

Table 3 Confirmation of microarray analysis with real-time quantitative RT-PCR

Gene name	Gene symbol	ABI assay no.	RefSeq	APA mean ± s.e.m.	P value	CPA mean ± s.e.m.	P value
GPCR with the largest increase in transcript level based on microarray analysis (top 5 shown in Table 1)							
Putative endothelin receptor type B-like protein	GPR37	Hs00173744_m1	NM_005302.2	11·7 ± 5·09	0·27	1·53 ± 0·59	0·91
Glutamate receptor, metabotropic 3	GRM3	Hs00168260_m1	NM_000840.2	5·32 ± 2·26	0·78	1·98 ± 0·85	0·43
5-Hydroxytryptamine (serotonin) receptor 4	HTR4	Hs00410577_m1	NM_199453.2	16·76 ± 2·51	0·001	1·39 ± 0·51	0·98
Luteinizing hormone/choriogonadotropin receptor	LH-R	Hs00174885_m1	NM_000233.2	10·53 ± 8·15	0·62	4·83 ± 2·00	0·62
Melanocortin 2 receptor (adrenocorticotropic hormone)	MC2R	Hs00265039_s1	NM_000529.2	5·24 ± 0·71	0·11	9·23 ± 4·79	0·30
GPCR transcripts with a mean increase of greater than or equal to 15-fold in one or more of the ten APA samples based on microarray analysis							
Putative endothelin receptor type B-like protein	GPR37	Hs00173744_m1	NM_005302.2	11·7 ± 5·09	0·27	1·53 ± 0·59	0·91
Glutamate receptor, metabotropic 3	GRM3	Hs00168260_m1	NM_000840.2	5·32 ± 2·26	0·78	1·98 ± 0·85	0·43
Luteinizing hormone/choriogonadotropin receptor	LH-R	Hs00174885_m1	NM_000233.2	10·53 ± 8·15	0·62	4·83 ± 2·00	0·62
Gonadotropin-releasing hormone receptor	GnRHR	Hs00171248_m1	NM_001012763.1	20·67 ± 6·44	0·09	4·97 ± 2·05	0·44

Six GPCR transcripts were examined using quantitative real-time RT-PCR (qPCR) in 28 aldosterone-producing adenomas (APA; *n* = 28), 13 cortisol-producing adenomas (CPA; *n* = 13) and 9 normal adrenals (from renal carcinoma patients; *n* = 9) used for microarray assays. These data are presented as the mean and standard error. The *P* values are provided.
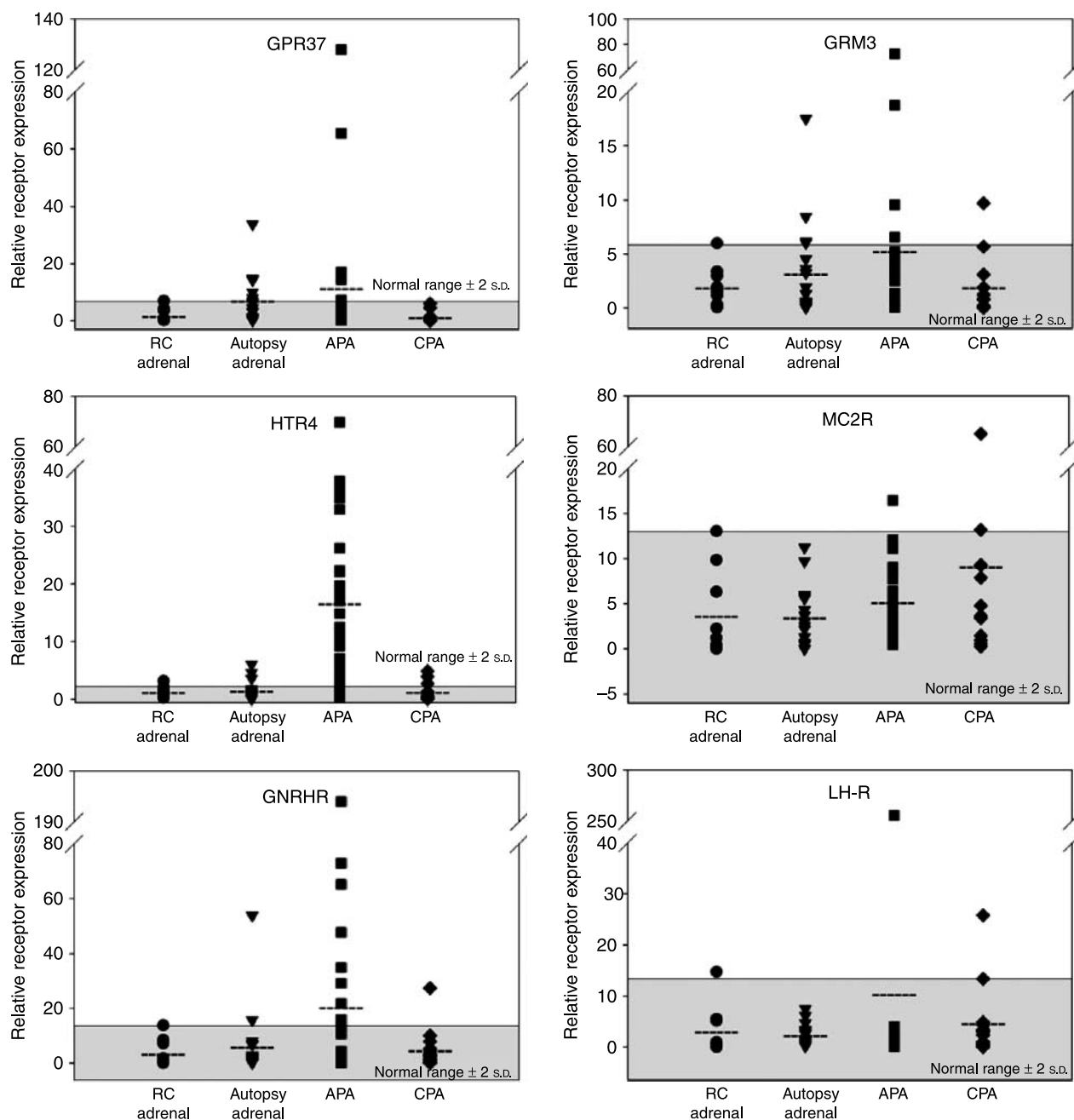
Figure 3 Individual adrenal samples from RC adrenal, autopsy adrenal, APA, and CPA tumor expression of GPR37 (five samples from APA and two from CPA are below our detection level), GRM32 (one sample from CPA is below our detection level), HTR4 (two samples from CPA are below our detection level), MC2R (one sample from autopsy adrenal is below our detection level), and GnRHR and LH-R (one sample from APA is below our detection level). The vertical point scatterplots compare these G-protein-coupled receptor expression levels in four different groups using qPCR. Each dot on the plots represents one sample after normalization with 18S rRNA. The gray regions represent respective normal mean ±2 S.D.

samples was 2·8-fold (Table 1). However, qPCR revealed an overall 21-fold increase in GnRHR with 11 out of 28 samples expressing GnRHR transcripts at levels that were 2 S.D. above those seen in normal adrenals (Table 3, Fig. 3). Only 1 out of 13 CPA samples had GnRHR levels higher than 2 S.D.

Discussion

The GPCRs are seven-helix transmembrane proteins that function in virtually every known physiological and pathological process. Numerous human diseases or disorders

have been associated with mutations or polymorphisms in GPCR (Spiegel 1996, Spiegel & Weinstein 2004). More recent studies have indicated that many GPCRs and their ligands are also involved in tumor progression (Li *et al.* 2005). Within the adrenal gland, ectopic expression of GPCR has been shown to cause excessive production of cortisol in certain cases of Cushing's syndrome (Schubert *et al.* 2001, Dall'Asta *et al.* 2004, Lacroix *et al.* 2004, Mazzuco *et al.* 2006*a,b*). Some GPCRs are also associated with the aldosterone production in Conn's syndrome (Rossi *et al.* 2000*a,b*, Mune *et al.* 2002, Perraudin *et al.* 2006, Rossi *et al.* 2006*a*). Thus, a single genetic event leading to inappropriate expression of a non-mutated *GPCR* gene appears to be sufficient to initiate the complete phenotypic alterations that ultimately lead to the formation of a benign adrenocortical tumor. However, the role of GPCR expression in APA has not been studied in detail. Herein, we showed that many APAs exhibit increased GPCR expression, and that the increase includes both receptors normally expressed in the adrenal and GPCR normally expressed at very low levels.

Aldosterone production can be regulated by a variety of GPCRs that are expressed in the adrenal gland. Two of these receptors, MC2R and HTR4, were found to be elevated in APA samples. While serotonin and ACTH do not appear to be primary physiological regulators of aldosterone production, both HTR4 and MC2R are expressed in the adrenal (Arnaldi *et al.* 1998, Schubert *et al.* 2001, Lefebvre *et al.* 2002). In addition, treatment with the HTR4 ligand, serotonin, or the MC2R ligand, ACTH, stimulates aldosterone production in isolated glomerulosa cells. These two receptors have also been studied previously as candidate regulators of APA aldosterone production (Arnaldi *et al.* 1998, Lefebvre *et al.* 2002).

Studies originally done by Tait *et al.* (1972) showed stimulation of rat glomerulosa cells with serotonin. Recent research has demonstrated the functional expression of HTR4 in adrenocortical APA using both *in vitro* and *in vivo* experiments (Lefebvre *et al.* 2002, Cartier *et al.* 2005). Their studies indicate a 4·7- to 47-fold increase of HTR4 mRNA in 11 APA samples. Our data confirm an elevation of HTR4 expression in the 28 APA used in our study. The effects of serotonin receptor antagonists have been studied in patients with APA. In the early eighties, Gross *et al.* (1981) showed that patients with idiopathic aldosteronism responded to cyproheptidine, a serotonin inhibitor. Later, the selective serotoninergic antagonist, ketanserin, was found to cause a transient decrease in blood pressure, plasma aldosterone, and cortisol levels in four patients with APA (Mantero *et al.* 1985). More recently, the specific HTR4 receptor antagonist, GR113808, has been shown to inhibit aldosterone production in patients with an APA as well as in adrenal cells isolated from these tumors (Lefebvre *et al.* 2002). GR113808 also inhibits cortisol production in patients with either bilateral macronodular adrenal hyperplasias (AIMAHs) or adenomas causing Cushing's syndrome (Louiset *et al.* 2004). In contrast, the HTR4 receptor agonist, cisapride, has a stimulatory effect to increase plasma aldosterone in PA patients (Lefebvre *et al.* 1997). Taken together, these data show that most

APAs, like normal glomerulosa cells, express a functional HTR4 receptor closely associated with steroidogenesis.

It is not surprising that the microarray found ACTH receptor transcripts (MCR2) to be elevated in APA samples. This finding is in agreement with a previous report that ACTH receptors are overexpressed in APA (Arnaldi *et al.* 1998, Mancini *et al.* 2003). However, because the magnitude of this elevation was not dramatic (mostly less than fivefold), the fold change of MCR2 in APA by qPCR was only slightly higher than the mean of variable expression seen in normal controls. Under physiological situations, ACTH can cause an acute increase in adrenal aldosterone production both *in vivo* and in isolated adrenal cells (Oelkers *et al.* 1988, Cozza *et al.* 1989, 1990*a,b*). However, chronic elevations in ACTH decrease plasma aldosterone levels in both human and animal models (Fuchs-Hammoser *et al.* 1980, Abayasekara *et al.* 1989, Aguilera *et al.* 1996, Mitani *et al.* 1996). The important issue is whether or not ACTH replaces the renin/Ang II system as a regulator of APA in the low-renin conditions seen in these patients. Adrenal vein sampling after ACTH stimulation was reported to amplify the differences between aldosterone secretion rates from normal and tumor-containing glands (Magill *et al.* 2001). Supporting a role for ACTH in the regulation of APA steroid production are studies showing that low-dose ACTH injection can induce a significant stimulation of aldosterone levels in patients with APA (Weinberger *et al.* 1979, Doppman & Gill 1996, Magill *et al.* 2001, Mancini *et al.* 2003). However, other studies suggest that ACTH infusion and subsequent venous sampling does not help differentiate between affected (APA containing) and unaffected adrenals (Rossi *et al.* 2006*b*). Meanwhile, the administration of dexamethasone to patients with APA lowers plasma aldosterone levels in some but not all patients (Ganguly *et al.* 1977, Wenting *et al.* 1978, Hoefnagels *et al.* 1980). These studies suggest that ACTH contributes but is not likely the sole agonist regulating APA steroid production.

Several APA also exhibited elevated expression of two receptors normally associated with the pituitary–gonadal axis, LH-R, and GnRHR. We have previously shown that LH-R can be highly expressed in certain APA samples, and that transgenic expression of LH-R in human adrenal cells can lead to LH-regulated aldosterone synthase expression (Saner-Amigh *et al.* 2006). Herein, we confirm that LH-R is overexpressed in some APA samples with only 1 out of the 28 APA samples used in this study having significant elevated LH-R when compared with the normal adrenal samples. In the current report, the number of APA with elevated LH-R was lower than previously reported. This is likely due to the variation observed in our larger collection of normal adrenal tissue – some of which appear to show higher LH-R transcript expression than in our previous study (Fig. 3). However, the known ability of this receptor to regulate steroid production in the gonads and the numerous reports of LH-R-mediated Cushing's syndrome (Hoefnagels *et al.* 1980, Christopoulos *et al.* 2004, Li *et al.* 2005) are highly supportive of a role for ectopic LH-R as the cause of adrenal disease and potentially APA.

Our results also detected elevated GnRHR expression in APA samples. One APA sample exhibited a 190-fold elevation in GnRHR expression over the normal controls. Out of 28 APA samples, 11 exhibited GnRHR levels that are >2 S.D. above normal adrenal levels. There have been no studies of GnRHR in normal or pathologic adrenal function. Normally, the key role of GnRHR is the control of the biosynthesis and secretion of LH and FSH from pituitary gonadotropes (Millar 2005). A number of studies regarding GnRH-mediated signal transductionhave been proposed, indicating that GnRHR activates both adenylate cyclase and phospholipase C via G-protein coupling (Arora *et al.* 1998, Liu *et al.* 2002). GnRHR have been shown to be expressed in numerous non-pituitary tissues and several different tumor types (Hapgood *et al.* 2005, Yeung *et al.* 2005). Its role in the regulation of tumor function is still not clear. However, its ability to regulate signaling pathways known to activate aldosterone production makes it a good candidate for regulation of APA steroidogenesis.

Glutamate is a neurotransmitter, which signals through glutamate receptors. However, glutamate has been reported to stimulate cultured lung carcinoma cells to proliferate. Moreover, glutamate-stimulated cell proliferation can be blocked by the glutamate receptor antagonists dizocilpine and GYKI52466 (Rzeski *et al.* 2001). Importantly, transgenic mice with an ectopically expressed glutamate receptor developed melanoma, further supporting a potential role for the metabotropic glutamate signaling pathway in tumorigenesis (Pollock *et al.* 2003). Herein, we found that GRM3 was increased in 15% APA samples with one APA having an increase of 72-fold. There have been no previous studies on the effects of glutamate on adrenal steroid production or the expression of this receptor in adrenocortical tissue.

GPR37 is ofspecial interest for pharmacology, since it was shown to contribute to Parkinson's disease (Imai *et al.* 2001, Marazziti *et al.* 2004). GPR37 is predominantly expressed in the mouse brain and testis (Zeng *et al.* 1997, Marazziti *et al.* 1998). A recent report found that its purified ligand is neuropeptide head activator (HA; Rezgaoui *et al.* 2006). After GPR37 binding to HA, it activates a pertussis toxin-sensitive inhibitory G-protein, which interacts with phosphoinositide 3-kinase and calmodulin-dependent protein kinase. The resulting Ca^{2+} influx activates a K^+ channel and leads to hyperpolarization, which is a prerequisite for cells to enter mitosis. GPR37 has not been studied with regard to adrenal physiology. Our microarray data indicated that GPR37 is expressed in the five normal adrenal arrays at a level that is about 100-fold lower than is seen in most of the GPCRs we studied (Fig. 1). This makes the fold change of GPR37 between APA and normal adrenal high (Fig. 1; Table 1).

In summary, the regulation of aldosterone is normally under tight control of Ang II, which acts through the type 1 Ang II receptor. However, in low-renin hyperaldosteronism normal feedback regulation is lost which leads to excessive renin-independent aldosterone production. The mechanisms regulating APA steroid production are currently not known.

However, ectopic GPCR expression has been shown to regulate some CPAs. Herein, we show that certain GPCRs are generally elevated in the majority of APA, while some appear to exhibit true ectopic expression. These receptors represent promising candidate regulators of APA aldosterone production and warrant further investigation.

Acknowledgements

References

Abayasekara DR, Vazir H, Whitehouse BJ, Price GM, Hinson JP & Vinson GP 1989 Studies on the mechanisms of ACTH-induced inhibition of aldosterone biosynthesis in the rat adrenal cortex. *Journal of Endocrinology* **122** 625–632.

Aguilera G, Kiss A, Lu A & Camacho C 1996 Regulation of adrenal steroidogenesis during chronic stress. *Endocrine Reviews* **22** 433–443.

Anand K, Mooss AN & Mohiuddin SM 2006 Aldosterone inhibition reduces the risk of sudden cardiac death in patients with heart failure. *Journal of the Renin-Angiotensin-Aldosterone System* **7** 15–19.

Arnaldi G, Mancini V, Costantini C, Giovagnetti M, Petrelli M, Masini A, Bertagna X & Mantero F 1998 ACTH receptor mRNA in human adrenocortical tumors: overexpression in aldosteronomas. *Endocrine Reviews* **24** 845–849.

Arora KK, Krsmanovic LZ, Mores N, O'Farrell H & Catt KJ 1998 Mediation of cyclic AMP signaling by the first intracellular loop of the gonadotropin-releasing hormone receptor. *Journal of Biological Chemistry* **273** 25581–25586.

Bassett MH, Mayhew B, Rehman K, White PC, Mantero F, Arnaldi G, Stewart PM, Bujalska I & Rainey WE 2005 Expression profiles for steroidogenic enzymes in adrenocortical disease. *Journal of Clinical Endocrinology and Metabolism* **90** 5446–5455.

Bugalho MJ, Li X, Rao CV, Soares J & Sobrinho LG 2000 Presence of a Gs alpha mutation in an adrenal tumor expressing LH/hCG receptors and clinically associated with Cushing's syndrome. *Gynecological Endocrinology* **14** 50–54.

Cartier D, Jegou S, Parmentier F, Lihrmann I, Louiset E, Kuhn JM, Bastard C, Plouin PF, Godin M, Vaudry H *et al.* 2005 Expression profile of serotonin4 (5-HT4) receptors in adrenocortical aldosterone-producing adenomas. *European Journal Endocrinology* **153** 939–947.

Christopoulos S, Bourdeau I & Lacroix A 2004 Aberrant expression of hormone receptors in adrenal Cushing's syndrome. *Pituitary* **7** 225–235.

Contesse V, Lenglet S, Grumolato L, Anouar Y, Lihrmann I, Lefebvre H, Delarue C & Vaudry H 1999 Pharmacological and molecular character-ization of 5-hydroxytryptamine(7) receptors in the rat adrenal gland. *Molecular Pharmacology* **56** 552–561.

Cozza EN, Ceballos NR & Lantos CP 1989 Effects of ACTH on the last step of aldosterone biosynthesis. *Journal of Steroid Biochemistry* **33** 1253–1255.

Cozza EN, Vila MC, Acevedo-Duncan M, Gomez-Sanchez CE & Farese RV 1990*a* ACTH increases *de novo* synthesis of diacylglycerol and translocates protein kinase C in primary cultures of calf adrenal glomerulosa cells. *Journal of Steroid Biochemistry* **35** 343–351.

Cozza EN, Vila MC, Acevedo-Duncan M, Farese RV & Gomez-Sanchez CE 1990*b* Treatment of primary cultures of calf adrenal glomerulosa cells with adrenocorticotropin (ACTH) and phorbol esters: a comparative study of the effects on aldosterone production and ACTH signaling system. *Endocrinology* **126** 2169–2176.

Dall'Asta C, Ballare E, Mantovani G, Ambrosi B, Spada A, Barbetta L, Colombo P, Travaglini P, Loli P & Beck-Peccoz P 2004 Assessing the presence of abnormal regulation of cortisol secretion by membrane hormone receptors: *in vivo* and *in vitro* studies in patients with functioning and non-functioning adrenal adenoma. *Hormone and Metabolic Research* **36** 578–583.

Doppman JL & Gill JR Jr 1996 Hyperaldosteronism: sampling the adrenal veins. *Radiology* **198** 309–312.

Fallo F, Pezzi V, Barzon L, Mulatero P, Veglio F, Sonino N & Mathis JM 2002 Quantitative assessment of CYP11B1 and CYP11B2 expression in aldosterone-producing adenomas. *European Journal Endocrinology* **147** 795–802.

Fuchs-Hammoser R, Schweiger M & Oelkers W 1980 The effect of chronic low-dose infusion of ACTH (1–24) on renin, renin-substrate, aldosterone and other corticosteroids in sodium replete and deplete man. *Acta Endocrinologica* **95** 198–206.

Funder J 2001 Mineralocorticoids and cardiac fibrosis: the decade in review. *Clinical and Experimental Pharmacology and Physiology* **28** 1002–1006.

Ganguly A, Chavarri M, Luetscher JA & Dowdy J 1977 Transient fall and subsequent return of high aldosterone secretion by adrenal adenoma during continued dexamethasone administration. *Journal of Clinical Endocrinology and Metabolism* **44** 775–779.

Gordon RD 2004 Primary aldosteronism–actual epidemics or false alarm? *Arquivos Brasileiros de Endocrinologia e Metabologia* **48** 666–673.

Gross MD, Grekin RJ, Gniadek TC & Villareal JZ 1981 Suppression of aldosterone by cyproheptadine in idiopathic aldosteronism. *New England Journal of Medicine* **305** 181–185.

Hapgood JP, Sadie H, van Biljon W & Ronacher K 2005 Regulation of expression of mammalian gonadotrophin-releasing hormone receptor genes. *Journal of Neuroendocrinology* **17** 619–638.

Hoefnagels WH, Drayer JI, Smals AG, Benraad TJ & Kloppenborg PW 1980 Nocturnal, daytime, and postural changes of plasma aldosterone before and during dexamethasone in adenomatous and idiopathic aldosteronism. *Journal of Clinical Endocrinology and Metabolism* **51** 1330–1334.

Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y & Takahashi R 2001 An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell* **105** 891–902.

Koshiyama H, Fujisawa T, Kuwamura N, Nakamura Y, Kanamori H, Oida E, Hara A, Suzuki T & Sasano H 2003 A case of normoreninemic aldosterone-producing adenoma associated with chronic renal failure: case report and literature review. *Endocrine* **21** 221–226.

Lacroix A, Baldacchino V, Bourdeau I, Hamet P & Tremblay J 2004 Cushing's syndrome variants secondary to aberrant hormone receptors. *Trends in Endocrinology and Metabolism* **15** 375–382.

Lampron A, Bourdeau I, Hamet P, Tremblay J & Lacroix A 2006 Whole genome expression profiling of glucose-dependent insulinotropic peptide (GIP)- and adrenocorticotropin-dependent adrenal hyperplasias reveals novel targets for the study of GIP-dependent Cushing's syndrome. *Journal of Clinical Endocrinology and Metabolism* **91** 3611–3618.

Lefebvre H, Dhib M, Godin M, Contesse V, Delarue C, Rieu M, Wolf LM, Vaudry H & Kuhn JM 1997 Effect of the serotonin 5-HT4 receptor agonist cisapride on aldosterone secretion in corticotropic insufficiency and primary hyperaldosteronism. *Neuroendocrinology* **66** 229–233.

Lefebvre H, Cartier D, Duparc C, Contesse V, Lihrmann I, Delarue C, Vaudry H, Fischmeister R & Kuhn JM 2000 Effect of serotonin4 (5-HT4) receptor agonists on aldosterone secretion in idiopathic hyperaldosteronism. *Endocrine Reviews* **26** 583–587.

Lefebvre H, Cartier D, Duparc C, Lihrmann I, Contesse V, Delarue C, Godin M, Fischmeister R, Vaudry H & Kuhn JM 2002 Characterization of serotonin(4) receptors in adrenocortical aldosterone-producing adenomas: *in vivo* and *in vitro* studies. *Journal of Clinical Endocrinology and Metabolism* **87** 1211–1216.

Li S, Huang S & Peng SB 2005 Overexpression of G protein-coupled receptors in cancer cells: involvement in tumor progression. *International Journal of Oncology* **27** 1329–1339.

Lim PO, Dow E, Brennan G, Jung RT & MacDonald TM 2000 High prevalence of primary aldosteronism in the Tayside hypertension clinic population. *Journal of Human Hypertension* **14** 311–315.

Liu F, Usui I, Evans LG, Austin DA, Mellon PL, Olefsky JM & Webster NJ 2002 Involvement of both G(q/11) and G(s) proteins in gonadotropin-releasing hormone receptor-mediated signaling in L beta T2 cells. *Journal of Biological Chemistry* **277** 32099–32108.

Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2($-\Delta\Delta$ C(T)) method. *Methods* **25** 402–408.

Louiset E, Cartier D, Contesse V, Duparc C, Lihrmann I, Young J, Bertherat J, Reznik Y, Kuhn JM, Laquerriere A et al. 2004 Paradoxical inhibitory effect of serotonin on cortisol production from adrenocortical lesions causing Cushing's syndrome. *Endocrine Reviews* **30** 951–954.

Magill SB, Raff H, Shaker JL, Brickner RC, Knechtges TE, Kehoe ME & Findling JW 2001 Comparison of adrenal vein sampling and computed tomography in the differentiation of primary aldosteronism. *Journal of Clinical Endocrinology and Metabolism* **86** 1066–1071.

Mancini T, Kola B, Mantero F & Arnaldi G 2003 Functional and nonfunctional adrenocortical tumors demonstrate a high responsiveness to low-dose adrenocorticotropin. *Journal of Clinical Endocrinology and Metabolism* **88** 1994–1998.

Mantero F, Rocco S, Opocher G, Armanini D, Boscaro M & D'Agostino D 1985 Effect of ketanserin in primary aldosteronism. *Journal of Cardiovascular Pharmacology* **7** S172–S175.

Marazziti D, Gallo A, Golini E, Matteoni R & Tocchini-Valentini GP 1998 Molecular cloning and chromosomal localization of the mouse *Gpr37* gene encoding an orphan G-protein-coupled peptide receptor expressed in brain and testis. *Genomics* **53** 315–324.

Marazziti D, Golini E, Mandillo S, Magrelli A, Witke W, Matteoni R & Tocchini-Valentini GP 2004 Altered dopamine signaling and MPTP resistance in mice lacking the Parkinson's disease-associated GPR37/parkin-associated endothelin-like receptor. *PNAS* **101** 10189–10194.

Mazzuco TL, Chabre O, Feige JJ & Thomas M 2006a Aberrant expression of human luteinizing hormone receptor by adrenocortical cells is sufficient to provoke both hyperplasia and Cushing's syndrome features. *Journal of Clinical Endocrinology and Metabolism* **91** 196–203.

Mazzuco TL, Chabre O, Sturm N, Feige JJ & Thomas M 2006b Ectopic expression of the gastric inhibitory polypeptide receptor gene is a sufficient genetic event to induce benign adrenocortical tumor in a xenotransplantation model. *Endocrinology* **147** 782–790.

Millar RP 2005 GnRHs and GnRH receptors. *Animal Reproduction Science* **88** 5–28.

Mitani F, Miyamoto H, Mukai K & Ishimura Y 1996 Effects of long term stimulation of ACTH and angiotensin II-secretions on the rat adrenal cortex. *Endocrine Reviews* **22** 421–431.

Mune T, Murase H, Yamakita N, Fukuda T, Murayama M, Miura A, Suwa T, Hanafusa J, Daido H, Morita H et al. 2002 Eutopic overexpression of vasopressin v1a receptor in adrenocorticotropin-independent macronodular adrenal hyperplasia. *Journal of Clinical Endocrinology and Metabolism* **87** 5706–5713.

Oelkers W, Boelke T & Bahr V 1988 Dose–response relationships between plasma adrenocorticotropin (ACTH), cortisol, aldosterone, and 18-hydroxycorticosterone after injection of ACTH-(1–39) or human corticotropin-releasing hormone in man. *Journal of Clinical Endocrinology and Metabolism* **66** 181–186.

Perraudin V, Delarue C, Lefebvre H, Do Rego JL, Vaudry H & Kuhn JM 2006 Evidence for a role of vasopressin in the control of aldosterone secretion in primary aldosteronism: *in vitro* and *in vivo* studies. *Journal of Clinical Endocrinology and Metabolism* **91** 1566–1572.

Plouin PF, Amar L & Chatellier G 2004 Trends in the prevalence of primary aldosteronism, aldosterone-producing adenomas, and surgically correctable aldosterone-dependent hypertension. *Nephrology, Dialysis, Transplantation* **19** 774–777.

Pollock PM, Cohen-Solal K, Sood R, Namkoong J, Martino JJ, Koganti A, Zhu H, Robbins C, Makalowska I, Shin SS et al. 2003 Melanoma mouse model implicates metabotropic glutamate signaling in melanocytic neoplasia. *Nature Genetics* **34** 108–112.

Rezgaoui M, Susens U, Ignatov A, Gelderblom M, Glassmeier G, Franke I, Urny J, Imai Y, Takahashi R & Schaller HC 2006 The neuropeptide head activator is a high-affinity ligand for the orphan G-protein-coupled receptor GPR37. *Journal of Cell Biology* **119** 542–549.

Rossi GP, Belloni AS, Nussdorfer GG & Pessina AC 2000a Endothelin-1 and the adrenal gland. *Journal of Cardiovascular Pharmacology* **35** S17–S20.

Rossi GP, Andreis PG, Neri G, Tortorella C, Pelizzo MR, Sacchetto A & Nussdorfer GG 2000b Endothelin-1 stimulates aldosterone synthesis in Conn's adenomas via both A and B receptors coupled with the protein kinase C- and cyclooxygenase-dependent signaling pathways. *Journal of Investigative Medicine* **48** 343–350.

Rossi GP, Sticchi D, Giuliani L, Bernante P, Zavattiero S, Pessina AC & Nussdorfer GG 2006a Adiponectin receptor expression in the human adrenal cortex and aldosterone-producing adenomas. *International Journal of Molecular Medicine* **17** 975–980.

Rossi GP, Ganzaroli C, Miotto D, De Toni R, Palumbo G, Feltrin GP, Mantero F & Pessina AC 2006b Dynamic testing with high-dose adrenocorticotrophic hormone does not improve lateralization of aldosterone oversecretion in primary aldosteronism patients. *Journal of Hypertension* **24** 371–379.

Rossi GP, Bernini G, Desideri G, Fabris B, Ferri C, Giacchetti G, Letizia C, Maccario M, Mannelli M, Matterello MJ *et al.* 2006c Renal damage in primary aldosteronism: results of the PAPY Study. *Hypertension* **48** 232–238.

Rossi GP, Bernini G, Caliumi C, Desideri G, Fabris B, Ferri C, Ganzaroli C, Giacchetti G, Letizia C, Maccario M *et al.* 2006d A prospective study of the prevalence of primary aldosteronism in 1,125 hypertensive patients. *Journal of the American College of Cardiology* **48** 2293–2300.

Rzeski W, Turski L & Ikonomidou C 2001 Glutamate antagonists limit tumor growth. *PNAS* **98** 6372–6377.

Saner-Amigh K, Mayhew BA, Mantero F, Schiavi F, White PC, Rao CV & Rainey WE 2006 Elevated expression of luteinizing hormone receptor in aldosterone-producing adenomas. *Journal of Clinical Endocrinology and Metabolism* **91** 1136–1142.

Schubert B, Fassnacht M, Beuschlein F, Zenkert S, Allolio B & Reincke M 2001 Angiotensin II type 1 receptor and ACTH receptor expression in human adrenocortical neoplasms. *Clinical Endocrinology* **54** 627–632.

Shigematsu K, Kawai K, Irie J, Sakai H, Nakashima O, Iguchi A, Shimamatsu J, Shimamatsu K, Kusaba Y & Takahara O 2006 Analysis of unilateral adrenal hyperplasia with primary aldosteronism from the aspect of messenger ribonucleic acid expression for steroidogenic enzymes: a comparative study with adrenal cortices adhering to aldosterone-producing adenoma. *Endocrinology* **147** 999–1006.

Spiegel AM 1996 Defects in G protein-coupled signal transduction in human disease. *Annual Review of Physiology* **58** 143–170.

Spiegel AM & Weinstein LS 2004 Inherited diseases involving g proteins and g protein-coupled receptors. *Annual Review of Medicine* **55** 27–39.

Tait SA, Tait JF & Bradley JE 1972 The effect of serotonin and potassium on corticosterone and aldosterone production by isolated zona glomerulosa cells of the rat adrenal cortex. *Australian Journal of Experimental Biology and Medical Science* **50** 833–846.

Weinberger MH, Grim CE, Hollifield JW, Kem DC, Ganguly A, Kramer NJ, Yune HY, Wellman H & Donohue JP 1979 Primary aldosteronism: diagnosis, localization, and treatment. *Annals of Internal Medicine* **90** 386–395.

Wenting GJ, Man in't Veld AJ, Derkx FH, Brummelen PV & Schalekamp MA 1978 ACTH-dependent aldosterone excess due to adrenocortical adenoma: a variant of primary aldosteronism. *Journal of Clinical Endocrinology and Metabolism* **46** 326–335.

Yeung CM, An BS, Cheng CK, Chow BK & Leung PC 2005 Expression and transcriptional regulation of the GnRH receptor gene in human neuronal cells. *Molecular Human Reproduction* **11** 837–842.

Zeng Z, Su K, Kyaw H & Li Y 1997 A novel endothelin receptor type-B-like gene enriched in the brain. *Biochemical and Biophysical Research Communication* **233** 559–567.