Urocortin-dependent effects on adrenal morphology, growth, and expression of steroidogenic enzymes *in vivo*

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Abstract

Urocortin (UCN) 1, 2, and 3 are members of the corticotropin-releasing factor (CRF) family that display varying affinities to the CRF receptor 1 (CRFR1 (CRHR1)) and 2 (CRFR2 (CRHR2)). UCNs represent important modulators of stress responses and are involved in the control of anxiety and related disorders. In addition to the CNS, UCNs and CRFRs are highly expressed in several tissues including the adrenal gland, indicating the presence of UCN-dependent regulatory mechanisms in these peripheral organ systems. Using knockout (KO) mouse models lacking single or multiple *Ucn* genes, we examined the potential role of the three different *Ucns* on morphology and function of the adrenal gland. Adrenal morphology was investigated, organ size, cell size, and number were quantified, and growth kinetics were studied by proliferative cell nuclear antigen staining and *Ccnd1* expression analysis. Furthermore, mRNA expression of enzymes involved in steroidogenesis and catecholamine synthesis was quantified by real-time PCR. Following this approach, *Ucn2*, *Ucn1/Ucn2* dKO and *Ucn1/Ucn2/Ucn3* tKO animals showed a significant cellular hypotrophy of the adrenal cortex and an increase in *Ccnd1* expression, whereas in all other genotypes, no changes were observable in comparison to age-matched controls. For steroidogenesis, *Ucn2/Ucn3* dKO animals displayed the most pronounced changes, with significant increases in all investigated enzymes, providing indirect evidence for increased stress behavior. Taken together, these data suggest that mainly *Ucn2* and *Ucn3* could be involved in adrenal stress response regulation while *Ucn2* additionally appears to play a role in morphology and growth of the adrenal gland.

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Introduction

Corticotropin-releasing factor (CRF) was the first identified member of the CRF family and has been determined as the main mediator for stress response-dependent activation of the hypothalamus–pituitary–adrenal (HPA) axis (Vale *et al.* 1981, 1983). The neuropeptides urocortin 1 (UCN1), UCN2 (stresscopin-related peptide), and UCN3 (stresscopin) have been described more recently as additional members of the CRF family. *Ucn1* was first reported in 1995 in the rat midbrain region (Vaughan *et al.* 1995), while *Ucn2* and *Ucn3* were identified on the basis of sequence homology searching tools not until 2001 (Reyes *et al.* 2001). CRF, UCNs, and their receptors are involved in homeostatic mechanisms of the stress response and in the control of anxiety, cognitive functions, appetite, and related disorders (Fekete & Zorrilla 2007).

The effects of CRF and UCNs are mediated via two G-coupled receptors, CRFR1 (CRHR1) and CRFR2 (CRHR2), which share 71% amino acid sequence similarity (Grigoriadis *et al.* 1996). CRFR1 and CRFR2

signal largely by coupling to Gs, leading to the stimulation of adenylyl cyclase and activation of protein kinase A. However, stimulation of specific CRFRs in distinct tissues or cell types by receptor-selective CRF peptides has been demonstrated to activate a variety of further signaling pathways (Brar *et al.* 2002, Bale 2005). UCN1 has similar binding affinity to CRFR1 and CRFR2 while UCN2 and UCN3 are considered as specific ligands for CRFR2 (Fukuda *et al.* 2005). These specific binding properties translate into distinct phenotypic changes induced by targeted deletions of the CRF receptors. Animals deficient for CRFR1 have an impaired stress response (Smith *et al.* 1998, Timpl *et al.* 1998) and decreased anxiety-like behavior (Smith *et al.* 1998) and fail to display the characteristic HPA axis response to restraint stress. In contrast, *Crfr2*-mutant mice display increased anxiety-like behavior and are hypersensitive to stressors (Bale *et al.* 2000, Coste *et al.* 2000).

In addition to the well-characterized effects of *Ucns* in the CNS, over the last few years, a number of peripheral sites of action have been identified.

Ucn1 expression has been observed in adipocytes (Seres *et al.* 2004), heart (Nishikimi *et al.* 2000), skin, and immunological tissue including the thymus and spleen, among others (Baigent 2001). *Ucn2* is highly expressed in skin and skeletal muscle, and is differently regulated by circulating glucocorticoid levels, as manipulated by exogenous administration or adrenalectomy (Chen *et al.* 2004). *Ucn3* gene expression can be detected in pancreatic β cells, where it stimulates insulin and glucagon secretion (Li *et al.* 2003), and in the adipose tissue, heart, and skin. CRFR2 is broadly distributed in peripheral tissues including the heart and skeletal muscle (Dautzenberg & Hauger 2002, Suda *et al.* 2004). In addition to the expression of CRFR1 in different sites of the brain (Justice *et al.* 2008), *Crfr1* expression could be documented in many peripheral tissues such as the testis, endocrine pancreas, or colon (Tao *et al.* 2007, Huising *et al.* 2010, O'Malley *et al.* 2010). Similar to other peripheral organs, members of the CRF/UCN system are also present in the adrenal gland, with *Ucn2* and *Ucn3* as well as CRFR2 being predominantly expressed in the adrenal cortex, while *Ucn1* and CRFR1 are mainly detectable in the adrenal medulla, and CRF is distributed throughout the adrenal gland. A proposed intra-adrenal UCN-dependent regulatory system would thus be dependent on the balance between the local concentration of CRF and UCNs and the presence of their receptors. Following this notion, it has been suggested that a CRFR1 receptor/ligand system might be active in the medulla, while a parallel CRFR2 receptor/ligand system might modulate adrenocortical function (Tsatsanis *et al.* 2007). Some examples provide evidence for a functional significance of a local UCN system within the adrenal gland: human fetal adrenal cells are able to respond to CRF stimulation through activation of CRFR1 with an increase in steroidogenesis, which has been hypothesized to be required for the late gestational increase in fetal adrenal cortisol and DHEAS production (Sirianini *et al.* 2005). In addition, preliminary data suggest a stress-related upregulation of *Ucn2* in the adrenal gland (Liu *et al.* 2008).

However, the physiological role of the influence of *Ucns* on adrenal function and structure has not been investigated in depth so far. Thus, our approach was to investigate the effects of the three *Ucns* on the adrenal gland using six different *Ucn*-knockout (KO) mouse models: individual KO for each one of the three peptides genes (*Ucn1* KO, *Ucn2* KO, and *Ucn3* KO) as well as combined deletions of two (*Ucn1/Ucn2* KO and *Ucn2/Ucn3* dKO) or all the three *Ucns* (*Ucn1/Ucn2/Ucn3* tKO). To achieve this goal, we investigated the mRNA expression of enzymes involved in steroidogenesis and catecholamine production as well as the histological appearance of the adrenal gland of those mouse models.

Materials and methods

Animals and housing conditions

For the animal experiments, six different genotypes of male *Ucn* KO mice were used. Mouse breeding took place at the Weizmann Institute of Science, Rehovot, Israel. Mice lacking two or all the three *Ucn* genes (*Ucn1*, *Ucn2*, and *Ucn3*) were generated by cross-breeding of *Ucn1*, *Ucn2*, and *Ucn3* single KO mice, which has been reported earlier (Vetter *et al.* 2002, Chen *et al.* 2006, Neufeld-Cohen *et al.* 2010a,b). All mice were on a mixed C57BL/6 $\times$ 129 background. WT mice of the mixed C57BL/6 $\times$ 129 background were derived from the same breeding colony. Male mice that were used in this study were housed up to five mice per cage on a 12 h light:12 h darkness photoperiod (lights on at 1800 h) with food and water *ad libitum*. All experimental protocols were approved by the Institutional Animal Care and Use Committee of The Weizmann Institute of Science.

To exclude interferences due to the female estrous cycle, as documented earlier (Chen *et al.* 2006, Xu *et al.* 2006), only male mice were investigated. *Ucn1* KO ($n=10$), *Ucn2* KO ($n=9$), *Ucn3* KO ($n=9$), *Ucn1/Ucn2* dKO ($n=8$), *Ucn2/Ucn3* dKO ($n=11$), *Ucn1/Ucn2/Ucn3* tKO ($n=8$), and WT controls ($n=11$) were killed at the age of 3 months.

Morphometry and immunohistochemistry

Adrenal glands from all investigated genotypes were rapidly dissected and placed in 4% paraformaldehyde overnight. Tissues were dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) following standard protocols. H&E-stained adrenal sections were examined using a light microscope with a magnification of 40 $\times$ and 400 $\times$. Areas of medulla and cortex were quantified on four independent sections per mouse from the center of the adrenal gland using the SpotAdvanced Software (Spot, Burroughs, Sterling Heights, MI, USA). The medullary area was normalized for the cortical area and expressed as the medullary/cortical area ratio. Cell nuclei within the outer and inner adrenal cortex were counted on three independent sections per animal under standardized conditions. A decrease in cell size was defined as cellular hypotrophy, whereas a decrease in nuclear density was interpreted as evidence of cellular hypertrophy, as the amount of extracellular matrix was comparable between the lines. In the adrenal cortex, less cytoplasm can be regarded as a rough estimate of lower functional activity of the cells. Cell counts were expressed as cell number/high power field (ncl/hpf). For proliferative cell nuclear antigen (PCNA) immunohistochemistry (IHC), paraffin-embedded sections

were rehydrated, blocked with 0.3% H₂O₂ in methanol for 10 min, and incubated with blocking buffer for 15 min. PCNA was immunolocalized overnight at 4 °C by means of a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a dilution of 1:100 in blocking buffer containing 3% BSA (Roche Applied Science), 5% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and 0.5% Tween 20. After rinsing for 15 min in PBS, secondary antibody (goat-anti-rabbit-biotinylated IgG (Vector Laboratories, Burlingame, CA, USA)) in a dilution of 1:200 in blocking buffer was applied for 30 min at room temperature. For the visualization of the bound PCNA antibody, Vectastain Elite ABC system (Vector Laboratories) and Sigma Fast diaminobenzidine (Sigma) were used.

Quantitative real-time PCR

Adrenal glands from mice of all investigated genotypes were immediately cleansed of adjacent tissue under a stereo microscope, snap frozen in liquid nitrogen, and stored at −80 °C. Both adrenals from each individual animal were combined and homogenized in extraction buffer while still frozen. RNA was extracted using the SV Total RNA Isolation System according to the manufacturer's instructions (Promega). RNA quality was verified on a 1.5% agarose gel. For cDNA synthesis, 500 ng total RNA were reverse transcribed using the reverse transcription system (Promega).

Gene expression of selected genes involved in steroidogenesis, catecholamine production, and cell cycle regulation was investigated: steroidogenic acute regulatory protein (*Star*), cholesterol side-chain cleavage enzyme (*Cyp11a1*), 11- β -hydroxylase (*Cyp11b1*), aldosterone synthase (*Cyp11b2*), tyrosine hydroxylase (*Th*), phenylethanolamine *N*-methyltransferase (*Pnmt*),

and cyclin D1 (*Ccnd1*). β -Actin was used as housekeeping gene. For primer sequences, see Table 1.

Quantification of β -actin, *Star*, *Cyp11a1*, and *Cyp11b2* was accomplished for all genotypes using the FastStart DNA MasterPlus SYBR Green I reaction mix in the LightCycler 1.5 (Roche). Real-time PCR conditions in the LightCycler were preincubation at 95 °C for 10 min followed by amplification of 40 cycles at 95 °C for 10 s, the annealing temperature (primer dependent, see Table 1) for 5 s, and extension at 72 °C, at which the time is calculated by the product length in base pairs divided by 25. The melting curve analysis was performed between 65 and 95 °C (0.1 °C/s). For the quantification of *Cyp11b1*, *Th*, *Pnmt*, and *Ccnd1*, the Sso Fast EVA Green Supermix (Bio-Rad Laboratories) in the Mx3000P QPCR System (Stratagene, La Jolla, CA, USA) was used. Real-time PCR conditions were preincubation at 95 °C for 10 min followed by amplification of 40 cycles at 95 °C for 10 s, the annealing temperature for 5 s (primer dependent, see Table 1), and extension at 72 °C. The melting curve analysis was performed between 65 and 95 °C (0.1 °C/s).

Furthermore, all products were run on a 1% agarose gel to verify the correct size of the amplified product. Quantification was adjusted using the housekeeping gene β -actin. To facilitate overall comparison of individual real-time experiments, expression levels of the particular genes were set as 100% for WT animals.

Statistical analysis

All results are expressed as mean $\pm$ S.E.M. Statistical significance was determined using the Mann–Whitney *U*-test with the Prism 3.02 Software (GraphPad Software, La Jolla, CA, USA). Statistical significance was defined as $P < 0.05$.

Table 1 Primer sequences

Gene	Primer sequences	Annealing temperature (°C)	Nucleotides
β -actin	F: TCATGAAGTGACGTGGACATCC R: CCTAGAAGCATTTGCGGTGGACGATG	60	911–1195
<i>Star</i>	F: GACCTTGAAAGGCTCAGGAAGAAC R: TAGCTGAAGATGGACAGACTTGC	60	7–987
<i>Cyp11a1</i>	F: AGGACTTTCCCTGCGCT R: GCATCTCGTAATGTTGG	53	34–980
<i>Cyp11b2</i>	F: CAGGGCCAAGAAAACCTACA R: ACGAGCATTTTGAAGCACCT	63	268–636
<i>Cyp11b1</i>	F: GGTCTGCCACAGTCCTGGAGT R: ACCAACAGGATAGAGCCTCAAGGT	63	878–1171
<i>Th</i>	F: TGCAGGGCTGCTGTCTTCCTAT R: TTCACAGAGAATGGGCGCTGGA	60	1175–1381
<i>Pnmt</i>	F: GTGAAGCGAGTCCTGCCTATC R: AAGATGCCTTTGACATCATCTACC	60	484–851
<i>Ccnd1</i>	F: AGCAGACCATCCGCAAGCAT R: AAGTGCGTTGTGCGGTAGCA	60	756–927

Figure 1 Gene expression levels of enzymes involved in steroidogenesis: *Star* (A), *Cyp11a1* (B), *Cyp11b1* (C), and *Cyp11b2* (D) and in catecholamine synthesis: *Th* (E) and *Pnmt* (F) in the adrenal gland of *Ucn1* KO, *Ucn2* KO, *Ucn3* KO, *Ucn1/Ucn2* dKO, *Ucn2/Ucn3* dKO, and *Ucn1/Ucn2/Ucn3* tKO mice. Asterisks denote significant changes for each gene over WT expression levels that are set as 100% for each gene.

Results

Ucn1 KO

The area of the adrenal gland and the cortical/medullary area ratio showed no differences between *Ucn1* KO mice and WT controls. Morphometrical studies of the outer adrenal cortex displayed a significant increase in nuclei per hpf (ncl/hpf) in *Ucn1* KO mice in comparison with age-matched WT controls, indicating cellular hypertrophy. In the inner adrenal cortex, no structural differences in comparison with WT animals could be observed. On the molecular level, *Ucn1* KO mice displayed a significant decrease in *Cyp11b2* expression, but there was no significant differences in the expression of *Star*, *Cyp11a1*, or *Cyp11b1*. Investigation of the expression pattern of enzymes of the adrenal medulla, involved in catecholamine synthesis, showed a significantly lower *Th* and *Pnmt* expression in

Ucn1 KO mice (Fig. 1). Cell proliferation, assessed by IHC with PCNA, did not demonstrate any differences in the staining intensity between *Ucn1* KO and WT mice. Cell cycle activation was quantified with *Ccnd1* expression, but again no changes could be documented in the *Ucn1* KO animals (Fig. 2).

Ucn2 KO

Ucn2 KO mice had a significantly higher cell number per hpf in the adrenal cortex in comparison with WT animals. In contrast to the cellular hypertrophy, no changes in the area of the adrenal gland or the ratio between cortex and medulla could be found. On the molecular level, a pronounced upregulation of *Cyp11a1* expression in *Ucn2* KO animals was observed. In contrast, expression levels of *Star*, *Cyp11b1*, and *Cyp11b2* remained unchanged. As for the adrenal

Figure 2 Representative H&E staining out of the center of the adrenal glands of all genotypes investigated. M, adrenal medulla; C, adrenal cortex, scale bar represents 50 μ m; area of the adrenal gland, cortical/medullary area ratio, and morphometrical studies of the outer and inner adrenal cortex of *Ucn1* KO, *Ucn2* KO, *Ucn3* KO, *Ucn1/Ucn2* dKO, *Ucn2/Ucn3* dKO, and *Ucn1/Ucn2/Ucn3* tKO mice in comparison with age-matched WT animals; representative PCNA staining of all phenotypes investigated; and *Ccnd1* expression levels of *Ucn1* KO, *Ucn2* KO, *Ucn3* KO, *Ucn1/Ucn2* dKO, *Ucn2/Ucn3* dKO, and *Ucn1/Ucn2/Ucn3* tKO mice. Asterisks denote significant changes for each gene over WT expression levels that are set as 100% for each gene. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-11-0144>.

medulla, *Pnmt* expression was found to be significantly lower in *Ucn2* KO mice, whereas *Th* expression did not differ significantly between the two investigated groups showing, though, the same tendency (Fig. 1). Cell proliferation assessed by *Ccnd1* expression remained unchanged in this KO model (Fig. 2).

Ucn3 KO

Analysis of the structure of adrenal cortices of *Ucn3* KO mice displayed a higher number of cells per hpf in the outer adrenal cortex in comparison with WT animals. In contrast, the cell number of the inner adrenal cortex was comparable between the two groups as well as the

size of the adrenal and the cortical/medullary area ratio. Similarly, expression levels of *Star*, *Cyp11b1*, and *Cyp11b2* did not differ between *Ucn3* KO and WT mice. However, elevated levels of *Cyp11a1* expression in *Ucn3* KO animals could be observed. *Th* and *Pnmt* expression did not differ between *Ucn3* KO and WT mice (Fig. 1). *Ccnd1* levels showed a trend toward reduced expression, but PCNA staining did not reveal any changes in the proliferation rate in *Ucn3* KO animals (Fig. 2).

Ucn1/Ucn2 dKO

Ucn1/Ucn2 dKO mice displayed no differences in the area of the adrenal gland and in cortical/medullary

area ratio in comparison with WT controls. However, *Ucn1/Ucn2* dKO mice had a significantly higher cell number per hpf in the outer and inner adrenal cortex, indicating cellular hypertrophy. Significantly lower levels of expression for *Star* and *Cyp11b2* were found in adrenals from *Ucn1/Ucn2* dKO mice in comparison with WT controls. In contrast, *Cyp11a1* and *Cyp11b1* expressions were comparable between the two groups, with no significant difference between WT and *Ucn1/Ucn2* dKO animals. Both *Th* and *Pnmt* expression levels, involved in catecholamine production, showed no differences in the *Ucn1/Ucn2* dKO mice (Fig. 1). Cell proliferation proved to be significantly higher in *Ucn1/Ucn2* dKO mice. Likewise, IHC with PCNA presented an increased staining in both the medulla and cortex of those mice (Fig. 2).

***Ucn2/Ucn3* dKO**

Mice lacking both *Ucn2* and *Ucn3* displayed no significant differences in morphological studies of the adrenal cortex by counting the cell number per hpf in comparison with WT controls. However, *Ucn2/Ucn3* dKO mice displayed smaller adrenal glands with an unchanged ratio of the cortex to medulla area in comparison with WT animals. In contrast, significant upregulation of *Star*, *Cyp11a1*, and *Cyp11b1* could be shown. *Cyp11b2* levels did not differ between *Ucn2/Ucn3* dKO mice and WT controls. *Ucn2/Ucn3* dKO mice presented a significant upregulation of the expression levels of enzymes catalyzing catecholamine synthesis (Fig. 1). No changes in the *Ccnd1* levels could be observed in this investigated group, similarly as after PCNA IHC staining (Fig. 2).

***Ucn1/Ucn2/Ucn3* tKO**

Following morphometrical analyses, adrenals from tKO mice had a significantly higher cell number per hpf in the inner and outer adrenal cortex. Besides the cellular hypertrophy, a reduced surface of the adrenal gland could be found in comparison with WT controls. However, the medulla/cortex area ratio displayed no differences between triple KO and WT mice. Significantly lower expression levels of *Star* and *Cyp11b1* in the adrenal were evident in tKO animals when compared with WT controls while differences in *Cyp11a1* and *Cyp11b2* displayed the same trend but failed to reach statistical significance. Adrenal medulla enzymes showed no changes in their expression levels between tKO mice and WT controls (Fig. 1). Adrenal glands of tKO animals displayed a significant increase in the cell cycle protein CCND1. Accordingly, an increased IHC staining with PCNA could be documented in the adrenals of those animals (Fig. 2).

Discussion

To evaluate the impact of the three different UCNs on the adrenal gland in an *in vivo* situation, we made use of six UCN KO models and investigated the effect of these targeted deletions on adrenal structure and function. Following this morphological and molecular approach, it became evident that absence of specific UCNs results in a complex pattern of functional changes in the adrenal gland, which would not have been predicted in all instances.

Ucn1 and *Ucn2* have been implicated in fluid and sodium regulation and modulation of cardiac function during heart failure. Specifically, administration of *Ucn1* in a sheep model with acute heart failure has been reported to result in decreasing levels of plasma vasopressin, renin activity, as well as aldosterone and natriuretic peptide levels (Rademaker *et al.* 2002), which was associated with delayed development of overt heart failure (Rademaker *et al.* 2007). Similarly, *Ucn2* was shown to reverse furosemide-induced increases in plasma renin activity and induced greater decreases in plasma aldosterone and vasopressin (Rademaker *et al.* 2009). While these effects involve multiple systems including cardiac and vascular function, there is only limited information on UCN-dependent adrenocortical aldosterone secretion. Interestingly, *in vitro* treatment of the human adrenocortical cell line NCIh295 with *Ucn1* has been demonstrated to induce and increase in *Cyp11b2* expression and aldosterone secretion (Kageyama *et al.* 2010). In line with this finding in our KO model, lack of *Ucn1* and *Ucn2* alone or in combination was associated with cellular hypertrophy of the outer adrenal cortex and lower expression levels of *Cyp11b2*. Thus, while the interventional protocols using *Ucn1* and *Ucn2* in models of heart failure demonstrate an inhibition of several volume-retaining hormones including the renin–angiotensin aldosterone system, direct effects on aldosterone secretion from the adrenal cortex cannot be excluded and might impact on long-term effects of these peptides.

In previous studies, it has been described that *Ucn1* KO mice display normal hormonal responses to acute stress (Vetter *et al.* 2002, Wang *et al.* 2002). In accordance with these findings, we demonstrated that the structure of the inner adrenal cortex as well as the expression levels of the enzymes involved in corticosterone and catecholamine synthesis remained unchanged. As *Crf1* KO mice have been reported to have an atrophic adrenal medulla and lower epinephrine plasma levels (Yoshida-Hiroi *et al.* 2002), it is prudent to assume that functional redundancy through retained CRF-dependent stimulation of the CRFR1 could be the reason for the observed minor effects on zona fasciculata function but not for the significant effects in adrenomedullary function in *Ucn1* KO animals.

In previous experiments, we could demonstrate that female, but not male, *Ucn2* KO mice exhibit an increase in the basal daily rhythm of ACTH and corticosterone (Chen *et al.* 2006). These findings are endorsed by our present studies in which the absence of *Ucn2* caused cellular hypotrophy of the adrenal cortex in male animals with only minor differences in the expression of steroidogenic enzymes and enzymes involved in catecholamine synthesis. Almost identically, in *Ucn3* KO mice, no or only small differences regarding adrenal structure, steroidogenesis, and epinephrine synthesis were detectable, a finding that also correlates with earlier data demonstrating normal corticosterone values in *Ucn3* KO animals (Deussing *et al.* 2010). Similar to the situation in single *Ucn1* KO animals, it is well possible that the isolated lack of one of both UCNs acting via the CRFR2 might be compensated by the other, resulting in a rather mild phenotype in single *Ucn2* and *Ucn3* KO mice.

In line with this notion and in contrast to the isolated KO models, mice lacking both *Ucn2* and *Ucn3* showed a robust upregulation of all investigated enzymes of steroidogenesis and catecholamine synthesis and displayed smaller adrenal glands, whereas no structural differences on cellular level or on proliferation could be observed. Accordingly, the increase in *Star* expression in *Ucn2/Ucn3* dKO animals could be explained by the dominance of the stress phenotype of *Ucn1* peptide in this model leading to increased steroidogenesis. Similarly, in each of the *Ucn2* and *Ucn3* KO and more pronounced in the *Ucn2/Ucn3* dKO model, the dominance of the *Ucn1* peptide was associated with an increase in *Cyp11a1*. For *Cyp11b1*, although the expression data were not significantly different in the single KO animals as expected, its expression was significantly upregulated in *Ucn2/Ucn3* dKO mice and on the other hand significantly downregulated in *Ucn1/Ucn2/Ucn3* tKO animals. Consequently, *Ucn1* appears not to contribute to a rescue of the stress-coping phenotype, as described earlier, although it is known that it has a similar affinity to both CRF receptors. It has been previously shown that *Crfr2* KO mice display normal baseline concentrations of ACTH and corticosterone but increased sensitivity to stress (Bale *et al.* 2000, Coste *et al.* 2000, 2006), supporting the hypothesis that stimulation of CRFR2 results in a decrease in anxiety and to a stress-coping behavior (Hsu & Hsueh 2001).

In *Ucn1/Ucn2/Ucn3* tKO mice, a decrease in adrenal size, cellular hypotrophy of the adrenal cortex, reduced steroidogenic capacity, and increased proliferation rate were observable, indicating that absence of *Ucn1* outweighs the lack of *Ucn2* and *Ucn3*. However, *Ucn1* is mainly expressed in the adrenal medulla and functions via binding to its receptors CRFR1 and CRFR2, which are mainly located in the adrenal cortex.

A possible model of indirect or direct interaction between the adrenal cortex and the medulla has been proposed, for example, by Fukuda *et al.* (2005). Comparable to this phenotype is the *Crfr1/2* KO model described previously, where an atrophy of the zona fasciculata and a phenotype of glucocorticoid insufficiency could be documented (Preil *et al.* 2001). Apparently, absence of either all three UCNs or both their receptors from the adrenal gland leads to a similar phenotypic development of the organ structure. Concordant with the reduced levels of steroidogenic enzymes in triple KO animals is the observation that *Ucn1/Ucn2/Ucn3* tKO mice show a tendency toward reduced corticosterone levels under baseline conditions (Neufeld-Cohen *et al.* 2010b), although the adrenal insufficiency appears to be less pronounced in this model in comparison with *CRF1/2* KO animals.

Finally, in *Ucn1/Ucn2* dKO mice, significant downregulation of key enzymes of steroidogenesis was detectable, together with a significant cellular hypotrophy of the adrenal cortex and an increased proliferation rate. As this observation is not in absolute agreement with a recent study, in which older animals had been investigated (Neufeld-Cohen *et al.* 2010a), an explanation for this discrepancy could be that UCN-mediated effects display age dependency. While a number of parameters affecting adrenal formation and zonation have been found to be age dependent (Bielohuby *et al.* 2007, Hershkovitz *et al.* 2007), UCN-related effects on the adrenal glands will require further detailed experiments involving time-dependent functional studies. The fact that *Ucn1/Ucn2*-dependent phenotype is comparable with that of *Ucn1/Ucn2/Ucn3* tKO mice, together with the very slight changes observed in *Ucn3* single KO mice suggest that the role of *Ucn3* on adrenal function and growth is relatively limited. Additionally, as only in *Ucn2* KO, *Ucn1/Ucn2* dKO, and *Ucn1/Ucn2/Ucn3* tKO animals a cellular hypotrophy of the adrenal cortex could be documented, a potential role of *Ucn2* in the development and the structure of the adrenal gland can be hypothesized.

Taken together, the absence of UCNs influences both the adrenal cortex and the medulla. As UCN single KO mice showed relatively mild adrenal phenotypes, these findings suggest that regulation of adrenal structure and function is dependent on the balance of all UCNs and their receptors.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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