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ORIGINAL ARTICLE

Urocortin-1 and -2 double-deficient mice show robust anxiolytic phenotype and modified serotonergic activity in anxiety circuits

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The urocortin (Ucn) family of neuropeptides is suggested to be involved in homeostatic coping mechanisms of the central stress response through the activation of corticotropin-releasing factor receptor type 2 (CRFR2). The neuropeptides, Ucn1 and Ucn2, serve as endogenous ligands for the CRFR2, which is highly expressed by the dorsal raphe serotonergic neurons and is suggested to be involved in regulating major component of the central stress response. Here, we describe genetically modified mice in which both Ucn1 and Ucn2 are developmentally deleted. The double knockout mice showed a robust anxiolytic phenotype and altered hypothalamic–pituitary–adrenal axis activity compared with wild-type mice. The significant reduction in anxiety-like behavior observed in these mice was further enhanced after exposure to acute stress, and was correlated with the levels of serotonin and 5-hydroxyindoleacetic acid measured in brain regions associated with anxiety circuits. Thus, we propose that the Ucn/CRFR2 serotonergic system has an important role in regulating homeostatic equilibrium under challenge conditions.
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Introduction

The neuropeptide corticotropin-releasing factor (CRF), and the more recently identified family members, the urocortins (Ucns), are proposed to integrate the neuroendocrine, autonomic, metabolic and behavioral responses to stressors.[1–4] CRF, Ucns and their receptors are implicated in the control of arousal, anxiety, cognitive functions and appetite. Chronic hyperactivation of the CRF system has been linked to stress-related emotional disorders such as anxiety, anorexia nervosa and depression.[5–8] However, the brain circuits and downstream signals responsible for their stress-related responses are not well understood.

The dorsal raphe nucleus (DRN) is a primary site of the serotonergic neurons projecting to the forebrain and brainstem targets, among them to the forebrain stress circuits.[9] Serotonin (5-HT) was found to regulate the hypothalamic–pituitary–adrenal (HPA) axis activity[9–11] and a variety of behavioral responses[12] during stressful challenges. Disturbed 5-HT functioning has a key role in the etiology of depression, as evidenced by altered expression of 5-HT receptors, decreased cerebrospinal fluid 5-hydroxyindoleacetic acid (5-HIAA) levels and the therapeutic efficacy of selective 5-HT reuptake inhibitors.[13]

All three Ucns were identified as high-affinity ligands for the CRF receptor type 2 (CRFR2[14–17]). CRFR2 is expressed in discrete regions of the brain, including the lateral septum (LS) and ventromedial hypothalamus in the forebrain, and the nucleus of the solitary tract and DRN in the hindbrain.[18,19] The high expression levels of CRFR2 in the DRN might suggest a possible involvement of Ucns in the regulation of 5-HT DRN neurons during different stages of the central stress response.

Several studies suggested that Ucns, through the activation of CRFR2, can modulate serotonergic neuronal activity. Ucn2 injection into the caudal DRN potentiated conditioned fear and escape deficits after stress. This behavioral effect could be reversed by a CRFR2-, but not by a CRF receptor type 1 (CRFR1)-specific antagonist.[20] Activation of CRFR2 in the DRN was further shown to increase firing rates and c-Fos expression of 5-HT neurons,[21,22] as well as increase 5-HT release in stress-related brain nuclei such as the basolateral amygdaloid (BLA) nucleus.[23–26]

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Ucn1 is synthesized mainly by the midbrain Edinger–Westphal nucleus, and is involved in the regulation of behavioral responses to stress.[27–29] Descending projections of Ucn1-like immunoreactivity, presumed to originate from the Edinger–Westphal nucleus, were observed in several caudal brain structures, including the DRN.[30]

Ucn2 exhibits a restricted subcortical expression pattern in the rat and mouse brain, mainly in the locus ceruleus, supraoptic nucleus, magnocellular subdivision of the paraventricular nucleus (PVN) of the hypothalamus and the arcuate nucleus.[15,16] Afferent projections from Ucn2-expressing nuclei to the DRN were shown in studies using both anterograde and retrograde tracers.[31–34] Thus, the expression of Ucn2 in known anatomical projections to the DRN suggests a possible role for Ucn2 in the functioning of 5-HT neurons during stress. Interestingly, detailed anatomical studies showed that Ucn3-expressing neurons do not innervate CRFR2–DRN neurons,[35] suggesting Ucn1 and Ucn2 as the endogenous ligands for CRFR2 activation in DRN neurons.

To further explore the physiological role of Ucn1 and Ucn2 in mediating the central stress response, and in accord with the neuroanatomical studies that position these two peptides as the endogenous ligands for DRN–CRFR2-expressing neurons, we generated double Ucn1 and Ucn2-deficient knockout (Ucn1/Ucn2 dKO) mice. The Ucn1/Ucn2 dKO mice showed a robust anxiolytic phenotype in both male and female mice, a modified HPA axis activity in males, changes in the expression levels of CRF family members and altered levels of 5-HT and 5-HIAA measured in brain regions associated with anxiety circuits.

Materials and methods

Animals

Ucn1/Ucn2 dKO mice were generated by crossbreeding Ucn1[36] and Ucn2[37] single knockout mice, on mixed C57BL/6 × 129 background, to produce offspring heterozygous for both genes. Male and female heterozygous mice were then bred to produce control, Ucn1, Ucn2 and double-mutant mice. Mice were housed under controlled conditions of 12 h light/dark photoperiod (lights on at 1800 hours) with access to food and water *ad libitum*.

RNA preparation and real-time PCR

Immediately after decapitation, the brain was removed and placed into a 1 mm metal matrix (Stoelting Co., Wood Dale, IL, USA, catalog no. 51386). The brain was sliced using standard razor blades (GEM, Personna American Safety Razor Co., Cedar Knolls, NJ, USA, 62–0165) into 1 mm (for DRN punch) or 2 mm slices (for the LS, amygdala and the bed nucleus of the stria terminalis (BNST) punches) that were quickly frozen on dry ice. The area of interest was punched using a microdissecting needle of an appropriate size and stored in −80 °C. RNA was extracted using a 5 PRIME Manual PerfectPure RNA Cell & Tissue kit (5 Prime GmbH, Hamburg, Germany). The extracted RNA was treated with RQ1 DNAse (Promega, Madison, WI, USA) to avoid false-positive results caused by DNA contamination. The RNA samples were reverse transcribed to generate cDNA pools that were later used as templates for quantitative real-time PCR analysis using specific primers. The expression of hypoxanthine–guanine phosphoribosyltransferase (HPRT) mRNA served as an internal control. The real-time reaction was performed in a Rotor-Gene 6000 thermocycler (Corbett Life Science, Mortlake, NSW, Australia) using fluorescent SYBR Green technology (ABgene, Epsom, Surrey, UK). The following specific primers were designed using Primer Express software (PE Applied Biosystems, Perkin Elmer, Foster City, CA, USA): mCRFR1 primers: 5′-TGCCAGGAGATTCTCAACGAA-3′ and 5′-AAAGCCGAGATGAGGTTCCAG-3′ corresponding to nucleotides 495–515 and 656–676, respectively. CRFR2 primers: 5′-TACCGAATCGCCCTCATTGT-3′ and 5′-CCACGCGATGTTTCTCAGAAT-3′ corresponding to nucleotides 479–498 and 640–620, respectively. CRF primers: 5′-GCAGTTAGCTCAGCAAGCTCAC-3′ and 5′-CAAATGATATCGGAGCTGCG-3′ corresponding to nucleotides 683–705 and 824–844, respectively. HPRT primers: 5′-GCAGTACAGCCCCAAAATGG-3′ and 5′-GGTCCTTTTCACCAGCAAGCT-3′ corresponding to nucleotides 540–559 and 571–591, respectively. The PCR conditions were as follows: cDNA equivalent to 1 μg of total RNA was amplified by PCR for 45 cycles at an annealing temperature of 61 °C. Each quantitative PCR reaction contained 10 μl 2 × SYBR Green Mastermix, a final primer concentration of 250 nM and 10 ng of template cDNA. The specificity of the amplification products was checked by melting curve analysis.

Adrenals obtained from both wild-type (WT) ($n = 6$) and Ucn1/Ucn2 dKO ($n = 5$) mice were immediately cleaned from adjacent tissue using 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 extraction was performed using the SV Total RNA Isolation System according to the manufacturer's instructions (Promega, Mannheim, Germany). For cDNA synthesis, 1 μg of total RNA was reverse transcribed using the reverse transcription system (Promega). Quantification of investigated genes was accomplished using the FastStart DNA MasterPlus SYBR Green I reaction mix in the LightCycler 1.5 (Roche, Indianapolis, IN, USA). Primers used were 5′-TCATGAAGTGTACGTGGACATCC-3′ and 5′-CCTAGAAGCATTTGCGGTGGACGATG-3′ for β-actin; 5′-CAGGGCCAAGAAAACCTACA-3′ and 5′-ACGAGCATTTTGAAGCACCT-3′ for aldosterone synthase (Cyp11β2); 5′-AGGACTTTCCCTGCGCT-3′ and 5′-GCATCTCGGTAATGTTGG-3′ for P450-mediated cholesterol side-chain cleavage; and 5′-GACCTTGAAAGGCTCAGGAAGAAC-3′ and 5′-TAGCTGAAGATGGACAGACTTGC-3′ for steroidogenic acute regulatory protein gene. Real-time PCR conditions were pre-incubation at 95 °C for 10 min followed by amplification

of 40 cycles at 95 °C for 10 s, annealing at 56 °C for β-actin, 63 °C for aldosterone synthase, 53 °C for P450-mediated cholesterol side-chain cleavage and 60 °C for steroidogenic acute regulatory gene (for 5 s), and extension at 72 °C, at which the time is calculated by the product length in base pairs divided by 25 (Roche). The melting curve analysis was performed between 65 and 95 °C (0.1 °C sec^{-1}) to determine the melting temperature of the amplified product and to exclude undesired primer dimers. Furthermore, the products were run on a 1% agarose gel to verify 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.

In situ hybridization and cell counts

Adult C57BL/6 × 129 WT and Ucn1/Ucn2 dKO mice were used in the present study. Animals were anesthetized with chloral hydrate (1.4 μg g^{-1} body weight, intraperitoneally) and perfused transcardially with 10 ml of phosphate-buffered saline followed by 100 ml of 4% paraformaldehyde in borate buffer, pH 9.5. The brains were removed and post-fixed in 30% sucrose in the same fixative at 4 °C. The brains were frozen and sectioned coronally at 25 μM using a sliding microtome and stored in phosphate-buffered saline at 4 °C.

Antisense and sense (control) RNA probes were generated using rat CRF cDNA and labeled with DIG-11-UTP using a labeling kit from Roche Molecular Biochemicals. *In situ* hybridization of CRF mRNA was carried out with the free-floating section method, as reported by Korosi *et al.*[38]

Adrenal glands from both WT and Ucn1/Ucn2 dKO mice were rapidly dissected and placed in 4% paraformaldehyde overnight. Tissues (WT, $n=4$; Ucn1/Ucn2 dKO, $n=5$) were dehydrated, embedded in paraffin, sectioned and stained with hematoxylin and eosin according to the standard protocols. Hematoxylin and eosin-stained adrenal sections from WT and Ucn1/Ucn2 dKO mice were examined with a standard light microscope using ×400 magnification. Cell nuclei within the zona fasciculata and glomerulosa were counted on two independent sections per animal under standardized conditions. Cell counts were expressed as cell number per high power field.

Blood collection and hormone analysis

Basal dark phase blood samples were obtained at 1400 hours, 8 h after the beginning of the dark phase, from individually housed WT and Ucn1/Ucn2 dKO male (KOM) and female (KOF) mice. For evaluation of the endocrine response to stress, male tail blood samples were collected before (basal), immediately after 15 min of restraint stress, and 30, 60 and 90 min from stress initiation. Female measurements were collected under basal (non-stress), and 30 and 90 min from stress initiation. The restraint stress was induced using a 50 ml ventilated conical tube. Plasma samples were immediately centrifuged and stored at −20 °C until assays for hormone measurement were conducted. Corticosterone, in male plasma samples, was quantified using specific corticosterone antibody and enzyme-linked immunosorbent assay protocol,[39] kindly provided by Dr Kenyon and Dr Aldujaili (Endocrinology Unit, Centre for Cardiovascular Science, The Queen's Medical Research Institute, Edinburgh, UK). Female samples were quantified using Corticosterone EIA kit (Cayman Chemical Company, Ann Harbor, MI, USA).

Behavioral analysis

Elevated plus maze. Male and female mutant and WT mice were used. The elevated plus maze (EPM) apparatus and experimental conditions were as previously described.[40] The time spent on the open arms was expressed as a percentage of the total test duration. The number of entries into the open arms, and the number of total entries to both open and closed arms were scored. All four paws were required to be on the arm for it to be counted. The EPM is a validated animal model for anxiety that is based on the natural aversion of rodents to height and open spaces.[41,42]

Open-field test. Male and female mutant and WT mice were used. The open-field apparatus consists of a white Plexiglas box (50 × 50 × 40 cm^3), with a lamp directed to the center of the field providing a dim 120 lux illumination on the floor. Testing was conducted during the dark phase of the circadian cycle. Each mouse was placed in the corner of the apparatus to initiate a 10-min test session. Behavior was recorded using a camera mounted above the apparatus and analyzed by TSE software VideoMot2 (Bad Homburg, Germany). The time spent in the center of the arena, the latency to visit the center, number of rearing events and the total distance traveled were measured. For the evaluation of high-stress conditions on WT and Ucn1/Ucn2 dKO mice behavior in the open-field test, the mice were immobilized for 30 min in a ventilated 50 ml tube before the test.

Light/dark transfer test. Male and female mutant and WT mice were used. The light/dark transfer test takes advantage of the natural conflict of a rodent between the exploration of a novel environment and the aversive properties of a large, brightly lit open-field. A greater amount of time spent in the light compartment and a greater number of transitions are indicative of decreased anxiety-like behavior.[43] The test apparatus consists of a Plexiglas box divided by a partition into two environments. One compartment is dark (14 × 27 × 26 cm^3) and the other compartment (30 × 27 × 26 cm^3) is highly illuminated (1000–1100 lux) by a light source located above it. The compartments are connected by an opening located at the floor level in the center of the partition. The mice were placed in the dark compartment to initiate the test session. Behavior was recorded using a camera mounted above the apparatus and analyzed by TSE software

VideoMot2. The time spent in each compartment, the number of transitions between compartments, the distance traveled in the light compartment and the latency entering the light compartment were measured. All four paws were required to be in a compartment for it to be counted. Each mouse was tested in a single 5-min session during the dark phase of the circadian cycle. For the evaluation of high-stress conditions on WT and Ucn1/Ucn2 dKO mice behavior in the dark/light transfer test, the mice were immobilized for 30 min in a ventilated 50 ml tube immediately before the test.

Brain microdissection and high-pressure liquid chromatography analysis of tissue concentrations of 5-HT and 5-HIAA

Methods for brain microdissection combined with high-pressure liquid chromatography with electrochemical detection of 5-HT and 5-HIAA have been described previously.[44]

To investigate the effects of genotype and sex on 5-HT metabolism within these anxiety-related circuits, the midline DRN, dorsal part (DRD) and DRN, caudal part (DRC) and bilateral BNST, central amygdaloid (CeA), BLA, CA1 region of the ventral hippocampus (CA1v), lateral entorhinal cortex (LEnt) and subiculum (S) were selected for microdissection (see Figure 5, Supplementary Figure S3 and Table 1) and identified by comparisons with a standard mouse brain stereotaxic atlas.[45]

Microdissected tissue punches in acetate buffer were thawed at 4 °C and centrifuged (13 000 r.p.m.; ~12 000 *g*) for 3 min. The supernatant was drawn off and the pellet was reconstituted with 0.2 M NaOH for assay of protein content (Pierce Protein Microassay Protocol, Perbio Science UK, Cramlington, UK). A total of 35 µl of the supernatant from each sample was then placed in an ESA 542 autosampler (ESA Analytical, Huntington, UK) maintained at 4 °C with the oven temperature set to 37 °C. A total of 10 µl of each sample were then injected onto the chromatographic system. Chromatographic separation and electrochemical detection were accomplished using a previously described method.[44]

Statistical analyses

Differences between groups for behavioral tests under basal conditions were determined by two-way analysis of variance (ANOVA) for stress and genotype, followed by Bonferroni corrected student-*t* test. Differences between the responses of each group to basal or stress conditions were analyzed using ANOVA with repeated measures (JMP IN Statistical Discovery Software, version 7.0, SAS Institute, Cary, NC, USA). High-pressure liquid chromatography results were statistically analyzed using Statistical Package for the Social Sciences version 11.5.0 (SPSS, Woking, UK). When appropriate, Fisher's Protected LSD tests were used for *post-hoc* comparisons. Adjustments for multiple comparisons were made, when indicated, using Bonferroni's correction. All estimates of central tendency are mean values with standard errors of the mean (s.e.m.).

Results

Altered HPA axis activity of Ucn1/Ucn2 dKO mice

Given that both Ucn1 and Ucn2 are expressed by neuroendocrine and stress-related brain nuclei in the mouse hypothalamus, and that serotonergic projections are known to regulate the HPA axis, we were able to determine the HPA axis activity both under basal (pre-stress) and stress conditions. The basal a.m. corticosterone levels of the KOM mice revealed an augmented level, but no significant difference was observed when compared with WT controls (Figure 1a, $P = 0.064$). To examine the response of the HPA axis to stress, animals were subjected to 15 min of restraint stress and the corticosterone levels were measured 15, 30, 60 and

Table 1 HPLC analysis of tissue concentrations of serotonin and 5-HIAA

Brain region	*Rostocaudal levels (mm bregma)*	*Microdissections (no. (diameter (mm)))*
Bed nucleus of the stria terminalis, lateral division, dorsal, posterior and juxtacapsular parts (BST)	0.29 to −0.01	1 (690)
Central amygdaloid nucleus, medial, lateral and capsular parts (Ce)	−1.21 to −1.51	2 (500)
Basolateral amygdaloid nucleus, anterior part (BLA)	−1.51 to −1.81	2 (690)
CA1 region of the ventral hippocampus (CA1v)	−2.71 to −3.01	2 (1000)
Lateral entorhinal cortex (LEnt)	−3.01 to −3.31	2 (690)
	−3.31 to −3.61	2 (690)
Subiculum (S)	−3.61 to −3.91	2 (690)
	−3.91 to −4.21	4 (690)
	−4.21 to −4.51	4 (690)
Dorsal raphe nucleus, dorsal part (DRD)	−4.21 to −4.51	1 (410)
	−4.51 to −4.81	1 (410)
Dorsal raphe nucleus, caudal part (DRC)	−4.81 to −5.11	1 (690)

Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; HPLC, high-pressure liquid chromatography.

90 min after stress initiation (Figure 1b). A higher corticosterone profile was found in the plasma of KOM mice compared with WT control mice ($P = 0.04$, repeated measures). No significant changes in basal or stress-induced levels of corticosterone were observed between KOF and female WT (WTF) mice (Supplementary Figure S1a and b). To further explore the possible mechanism mediating these differences, CRF mRNA level in the PVN of male (Figures 1c and d) and female (Supplementary Figures S1c) mice was determined using real-time PCR. The amplified CRF mRNA levels, isolated from the mouse PVN and quantified relative to HPRT mRNA levels, were found to be significantly elevated in both KOM and KOF mice compared with control mice (males: $P = 0.005$, Figure 1c; females: $P = 0.01$, Supplementary Figure S1c). *In situ* hybridization for CRF further supported the observed real-time PCR differences (Figures 1e–j). Brain sections obtained from KOM mouse revealed a stronger PVN CRF mRNA expression compared with WT mice (Figures 1e–h). Interestingly, no differences in CRF mRNA expression levels were observed, using *in situ* hybridization, in the CeA nucleus at the same brain sections (Figures 1e–f and i–j). Ucn1 and Ucn2 transcripts were previously found to be expressed by the adrenal gland.[16,46,47] To assess the contribution of

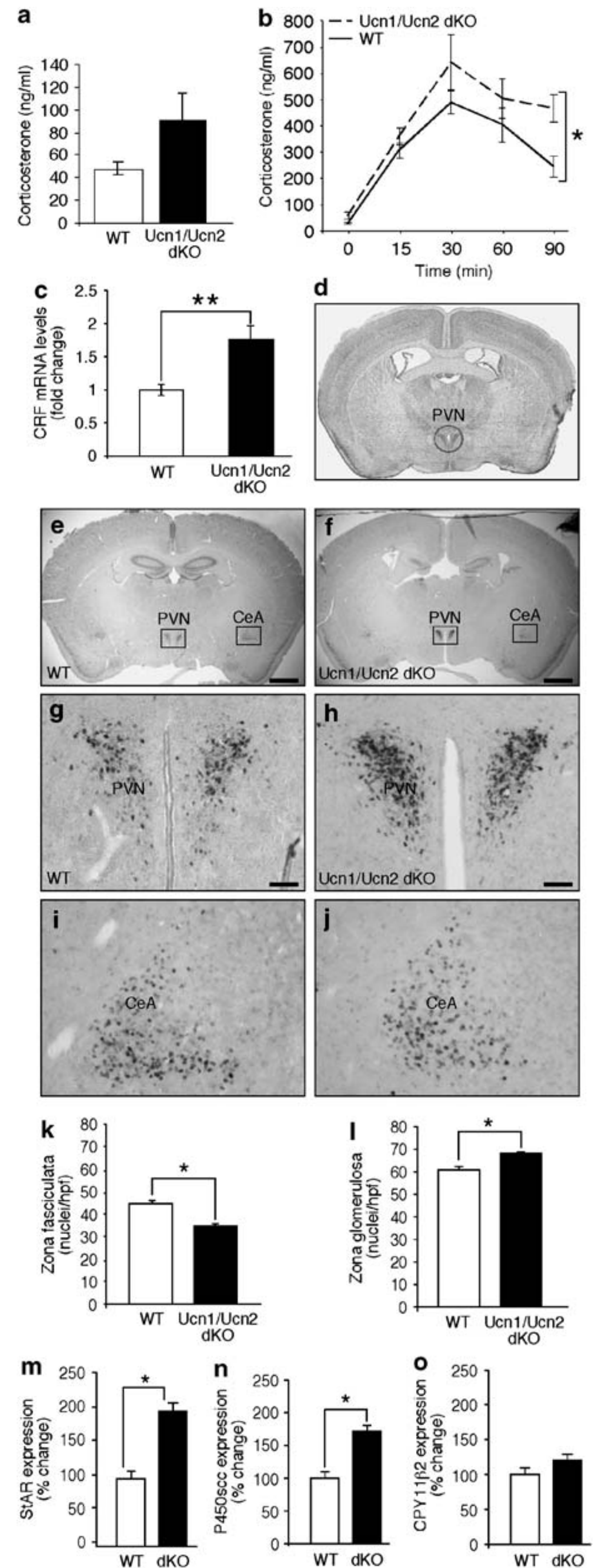


Figure 1 Altered hypothalamic–pituitary–adrenal (HPA) axis response and corticotropin-releasing factor (CRF) expression levels for urocortin-1 and urocortin-2 double knockout (Ucn1/Ucn2 dKO) male mice. (**a**) Basal corticosterone plasma levels of Ucn1/Ucn2 dKO mice are augmented compared with wild-type (WT) mice. The values represent mean ± s.e.m (WT, $n = 11$; Ucn1/Ucn2 dKO, $n = 12$; $P = 0.0649$). (**b**) Plasma corticosterone levels of WT and Ucn1/Ucn2 dKO mice obtained before and after 15 min of acute restraint stress. Ucn1/Ucn2 dKO show elevated levels at all time points and altered kinetics of corticosterone levels after stress (WT, $n = 11$; Ucn1/Ucn2 dKO, $n = 12$; $P = 0.04$). (**c** and **d**) Elevated levels of CRF mRNA in the paraventricular nucleus (PVN) of the hypothalamus of Ucn1/Ucn2 dKO mice, relative to hypoxanthine–guanine phosphoribosyltransferase (HPRT) mRNA levels, as shown by real-time PCR (WT, $n = 5$; Ucn1/Ucn2 dKO, $n = 7$; $P = 0.005$). Samples of the PVN were punched as depicted (**d**, modified from Paxinos and Franklin[45]). (**e–j**) CRF mRNA expression levels in the PVN, but not in the amygdala, are increased in the Ucn1/Ucn2 dKO. Representative photomicrographs of WT (**e**, **g** and **i**) and Ucn1/Ucn2 dKO (**f**, **h** and **j**) brain sections processed for *in situ* hybridization for CRF are represented. The PVN and central amygdala (CeA) areas are indicated in boxes and enlarged below as **g** and **h** and **i** and **j**, respectively. Scale bars: **e** and **f** = 1 mm; **g–j** = 100 μm. (**k** and **l**) Morphometrical analysis of adrenal cortices from age-matched male WT and Ucn1/Ucn2 dKO mice (WT, $n = 4$; Ucn1/Ucn2 dKO, $n = 5$) indicates a significant decrease of the cell count/high power field (hpf) within the zona fasciculata in Ucn1/Ucn2 dKO animals (**k**, $P < 0.001$), whereas a significant increase in the zona glomerulosa was evident in the same group (**l**, $P < 0.001$). (**m–o**) Although increased expression of StAR (**m**, $P < 0.05$) and P450scc (**n**, $P < 0.05$) in Ucn1/Ucn2 dKO mice in comparison with age-matched WT controls was detectable (WT, $n = 6$; Ucn1/Ucn2 dKO, $n = 5$), no significant differences in expression of aldosterone synthase could be observed between both groups (**o**, $P = 0.52$). *$P < 0.05$ and **$P < 0.01$.

the adrenal gland to the altered HPA response, we performed morphometrical analyses within the zona fasciculata and the zona glomerulosa (Figures 1k and l, respectively). After this analysis, Ucn1/Ucn2 dKO mice had a significantly lower cell number/high power field in the zona fasciculata, indicating cellular hypertrophy in comparison with WT animals (Figure 1k, $P<0.001$). The opposite could be observed for the zona glomerulosa, wherein Ucn1/Ucn2 dKO animals had significantly higher cell numbers/high power field (Figure 1l, $P<0.001$). We further analyzed adrenal steroidogenic enzymes gene expression using real-time PCR. Significantly higher levels of expression for steroidogenic acute regulatory protein and P450-mediated cholesterol side-chain cleavage protein were found in adrenal glands from Ucn1/Ucn2 dKO mice in comparison with WT controls (Figure 1m, $P<0.05$ and Figure 1n, $P<0.05$). In contrast, aldosterone synthase expression was comparable between the two groups, with no significant difference between WT and Ucn1/Ucn2 dKO animals (Figure 1o, $P=0.52$). Taken together, these findings indicate effects mediated by loss of Ucn1 and Ucn2 that mainly affects zona fasciculata, including hypertrophy and steroidogenic enzyme expression, whereas zona glomerulosa-specific genes, such as aldosterone synthase, seem to remain unchanged.

Reduced anxiety-like behavior of Ucn1/Ucn2 dKO under basal conditions

The mutant and control mice were tested using three different anxiety-related behavioral paradigms to determine whether Ucn1/Ucn2 dKO mice exhibit different anxiety-like behavior. The EPM test revealed that both KOM and KOF mice show a significant reduction in anxiety-like behavior (Figures 2a–d). Two-way ANOVA of the percent time spent in the open arms for genotype, sex and their interaction indicated a significant main effect only for the genotype ($F_{(1,43)}=19.757$; $P<0.001$). Further *post-hoc* pairwise comparisons (Bonferroni corrected) indicated that the percent time spent in the open arms was significantly higher for the Ucn1/Ucn2 dKO mice (Figures 2a and b; males: $P=0.014$; females: $P=0.001$). Similarly, a significant main effect for genotype was found for the number of entries to the open arms ($F_{(1,43)}=24.962$; $P<0.001$), and further *post-hoc* pairwise comparisons (Bonferroni corrected) indicated significantly more entries for the Ucn1/Ucn2 dKO mice (Figures 2c and d; males: $P<0.001$; females: $P=0.004$). This decrease in anxiety-like behavior was not due to altered locomotor activity, as the total entries (to both open and closed arms) did not differ between the Ucn1/Ucn2 dKO and WT controls for both males and females (Data not shown; males: Ucn1/Ucn2 dKO, 13.72 ± 1.15 versus WT, 15.75 ± 1.39, $P=0.323$; females: Ucn1/Ucn2 dKO, 19.08 ± 1.66 versus WT, 17.63 ± 1.98, $P=0.58$). In the open-field test, two-way ANOVA of the percent time spent in the center of the arena for genotype, sex and their interaction indicated a significant main effect for the genotype ($F_{(1,39)}=8.6$; $P=0.006$). Further *post-hoc*

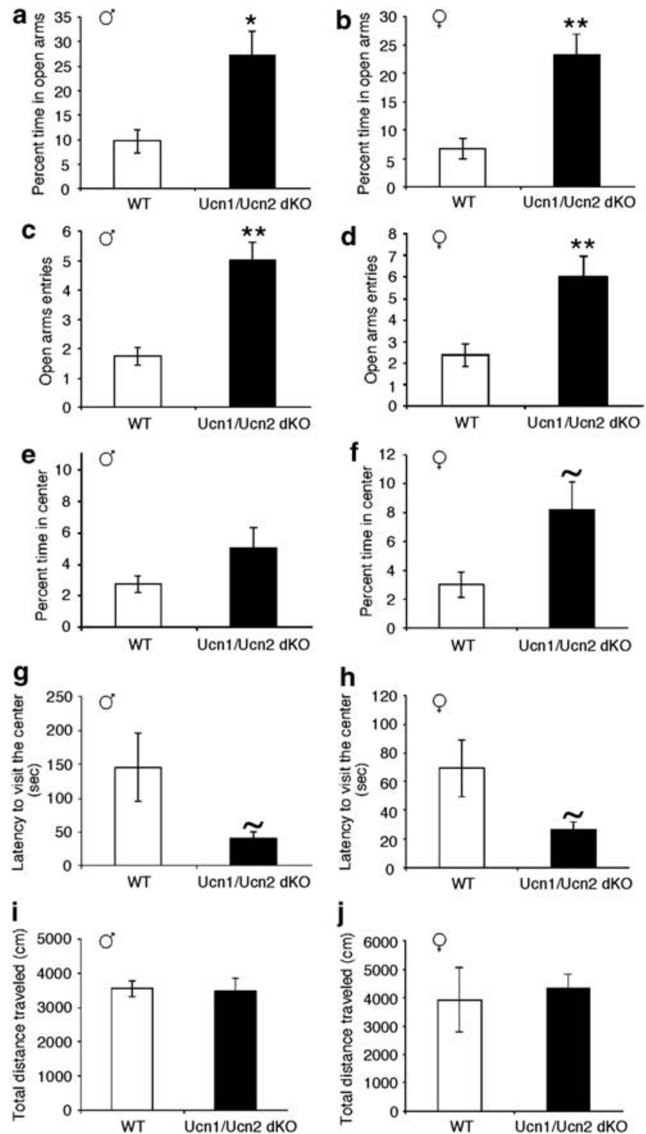


Figure 2 Decreased anxiety-like behavior of urocortin-1 and urocortin-2 double knockout (Ucn1/Ucn2 dKO) mice as measured by the elevated plus maze and open-field tests. (**a** and **b**) The percentage of time spent in the open arms is significantly higher for both male (**a**) and female (**b**) Ucn1/Ucn2 dKO mice (male, wild-type (WT), $n=11$; Ucn1/Ucn2 dKO, $n=12$; $P=0.014$ and female, WT, $n=10$; Ucn1/Ucn2 dKO, $n=12$; $P=0.001$). (**c** and **d**) The number of entries to the open arms was significantly higher for both male (**c**) and female (**d**) Ucn1/Ucn2 dKO mice in comparison with WT controls ($P=0.00014$ and $P=0.004$, respectively). In the open-field test, we observed decreased anxiety-like behavior of Ucn1/Ucn2 dKO mice. The percentage of time spent in the center was higher for Ucn1/Ucn2 dKO males (**e**, WT, $n=12$; Ucn1/Ucn2 dKO, $n=12$; $P=0.12$) and significantly higher for Ucn1/Ucn2 dKO female mice (**f**, WT, $n=11$; Ucn1/Ucn2 dKO, $n=12$; $P=0.028$). The latency to first enter the center of the arena was longer for both the male and the female WT controls (**g**, $P=0.032$ and **h**, $P=0.028$). Ucn1/Ucn2 dKO and WT mice show similar locomotor activity, as measured by total distance traveled in the open-field apparatus (**i**, males, $P=0.89$; **j**, females, $P=0.5$). All values represent mean ± s.e.m and are Bonferroni corrected: $\sim P<0.032$, $*P<0.025$ and $**P<0.005$.

pairwise comparisons (Bonferroni corrected) indicated that KOF mice spent more time in the center of the arena (Figures 2f), ($P = 0.028$). In addition, two-way ANOVA of the latency to enter the center revealed again a main effect for the genotype ($F_{(1,39)} = 7.1$; $P = 0.011$). Further *post-hoc* pairwise comparisons (Bonferroni corrected) indicated that Ucn1/Ucn2 dKO mice show shorter latency entering the center (Figures 2g and h, male: $P = 0.032$; female: $P = 0.028$). Locomotor activity between Ucn1/Ucn2 dKO mice and WT controls, male and female, did not differ, as shown by the total distance traveled in the arena (Figures 2i and j). To further examine the reduced anxiety-like behavior in the Ucn1/Ucn2 dKO mice, dark/light transfer test was used. Although KOF mice did not differ from WT controls (Supplementary Figures S2b, d, f and h), KOM mice spent significantly more time in the light compartment (Supplementary Figure S2a, $P = 0.0017$). The traveled distance in light was significantly higher for the KOM mice (Supplementary Figure S2e, $P = 0.02$), in accordance with the time spent in the light. KOM mice also displayed a trend toward anxiolytic phenotype in the number of transitions to the light compartment (Supplementary Figure S2c, $P = 0.073$) and the latency to first enter the light (Supplementary Figure S2g, $P = 0.09$).

Reduced behavioral response of Ucn1/Ucn2 dKO mice to acute stress

To further study the anxiety-like behavior response of Ucn1/Ucn2 dKO mice to acute stress, mice were subjected to 30 min of restraint stress, immediately before the behavioral tests. Figure 3 exhibits the results of KOF and WT controls in the open-field and the dark/light transfer tests after exposure to acute

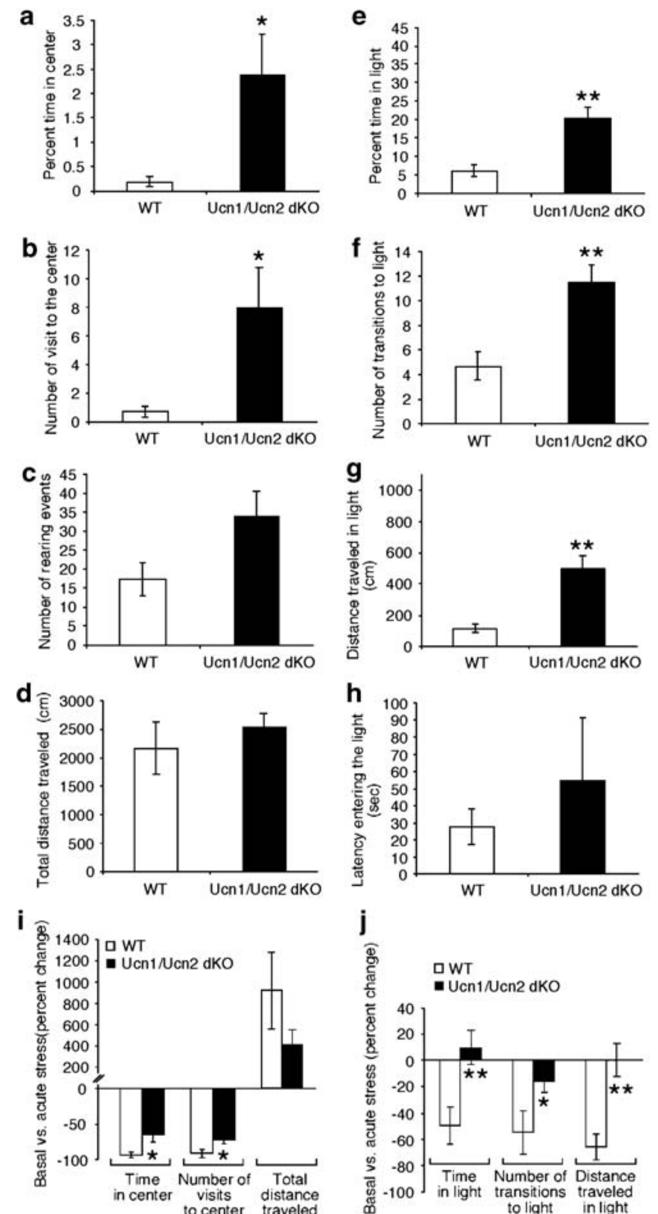


Figure 3 Urocortin-1 and urocortin-2 double knockout (Ucn1/Ucn2 dKO) mice show reduced stress-induced anxiety-like behavior. Female Ucn1/Ucn2 dKO and wild-type (WT) mice were subjected to 30 min of restraint stress followed by either open-field (**a**–**d**, **i**) or dark/light transfer tests (**e**–**h**, **j**). WT mice show a stronger significant increase to acute stress-induced anxiety-like behavior in comparison with Ucn1/Ucn2 dKO mice. The percentage of time spent in the center of the arena was significantly higher for the Ucn1/Ucn2 dKO mice (**a**, WT, $n = 10$; Ucn1/Ucn2 dKO, $n = 11$; $P = 0.0148$). The number of visits to the center was also less affected by stress in the Ucn1/Ucn2 dKO mice (**b**, WT, $n = 10$; Ucn1/Ucn2 dKO, $n = 11$; $P = 0.026$). The number of rearing events (**c**, WT, $n = 9$; Ucn1/Ucn2 dKO, $n = 11$; $P = 0.09$) and the total distance traveled (**d**, $n = 10$ for both groups, $P = 0.491$) did not differ significantly between WT and Ucn1/Ucn2 dKO. In the dark/light transfer test, the percentage of time spent in the light after stress was significantly higher for the Ucn1/Ucn2 dKO (**e**, WT, $n = 12$; Ucn1/Ucn2 dKO, $n = 11$; $P = 0.00022$). The Ucn1/Ucn2 dKO also exhibited a significantly higher number of transitions to the light compartment (**f**) and significantly longer distance traveled (**g**, $P = 0.0012$ and $P = 0.00016$, respectively; $n = 12$ in each group). The latency to enter the light compartment did not differ between the Ucn1/Ucn2 dKO and WT controls (**h**, $P = 0.76$). To better evaluate the effect of acute stress on both groups, the percent change between results measured in the open-field and dark/light transfer under basal conditions (no restraint) and after 30 min of restraint stress were calculated. (**i**) In the open-field test, Ucn1/Ucn2 dKO mice were significantly less influenced by the stress for both the time spent in the center ($P = 0.02$) and the number of visits to the center ($P = 0.05$). A smaller change was also seen in the latency to visit the center for the Ucn1/Ucn2 dKO ($P = 0.21$). (**j**) Percent change between dark/light transfer results under basal conditions and after restraint stress. The percent change of time spent in light differs significantly between the Ucn1/Ucn2 dKO and WT controls ($P = 0.0057$). A similar significantly reduced change of the Ucn1/Ucn2 dKO results was also shown by the number of transitions to the light compartment and the distance traveled in the light ($P = 0.043$ and $P = 0.0005$, respectively). * $P < 0.05$ and ** $P < 0.01$.

stress. The open-field test reveals a significant difference between the groups: the Ucn1/Ucn2 dKO mice spent significantly more time in the center, in addition to visiting the center more times than WT controls (Figures 3a and b, $P=0.014$ and $P=0.026$, respectively). The number of rearing events was higher for the Ucn1/Ucn2 dKO mice, although not significantly (Figure 3c). Despite the marked differences in the time spent in the center and visits to the center, no difference was found in the total distance traveled in the arena (Figure 3d, $P=0.49$), indicating a similar influence of stress on the locomotor activity. Calculated percent changes in the open-field between results obtained under basal conditions and after acute stress are illustrated in Figure 3i. KOF mice show a significant percent change compared with WT controls in both the time spent in the center ($P=0.02$) and in the number of visits to the center ($P=0.05$). KOM mice, on the other hand, did not differ significantly from WT controls.

Dark/light transfer test performed immediately after 30 min of restraint stress further shows the decrease in the behavioral response of the KOF mice to acute stress. Although WT controls showed marked reduction in the percentage of time spent in the light compartment (Figure 3e and j) and the number of transitions to the light (Figure 3f and j), Ucn1/Ucn2 dKO performances were similar to those under basal stress ($P=0.0002$ and $P=0.0012$, respectively). No significant difference between the groups was found for the latency to first enter the light zone (Figure 3h, $P=0.76$). Calculated percent changes in the dark/light transfer between results obtained under basal conditions and after acute stress are illustrated in Figure 3j. Percent changes were significantly higher for the WT mice, as measured by the time in light ($P=0.0057$), the number of transitions ($P=0.043$) and the distance traveled ($P=0.0005$). Thus, although WT controls display typical stress-induced anxiety-like behavior, Ucn1/Ucn2 dKO show little or no difference from the basal conditions. Similar results were obtained using Ucn1/Ucn2 dKO and WT male (WTM) mice, showing a significant increase in time spent in the light compartment ($P=0.0018$) and in the distance traveled in the light ($P=0.0012$, data not shown).

Compensatory expression profile of CRF and CRFRs mRNA levels in Ucn1/Ucn2 dKO mice

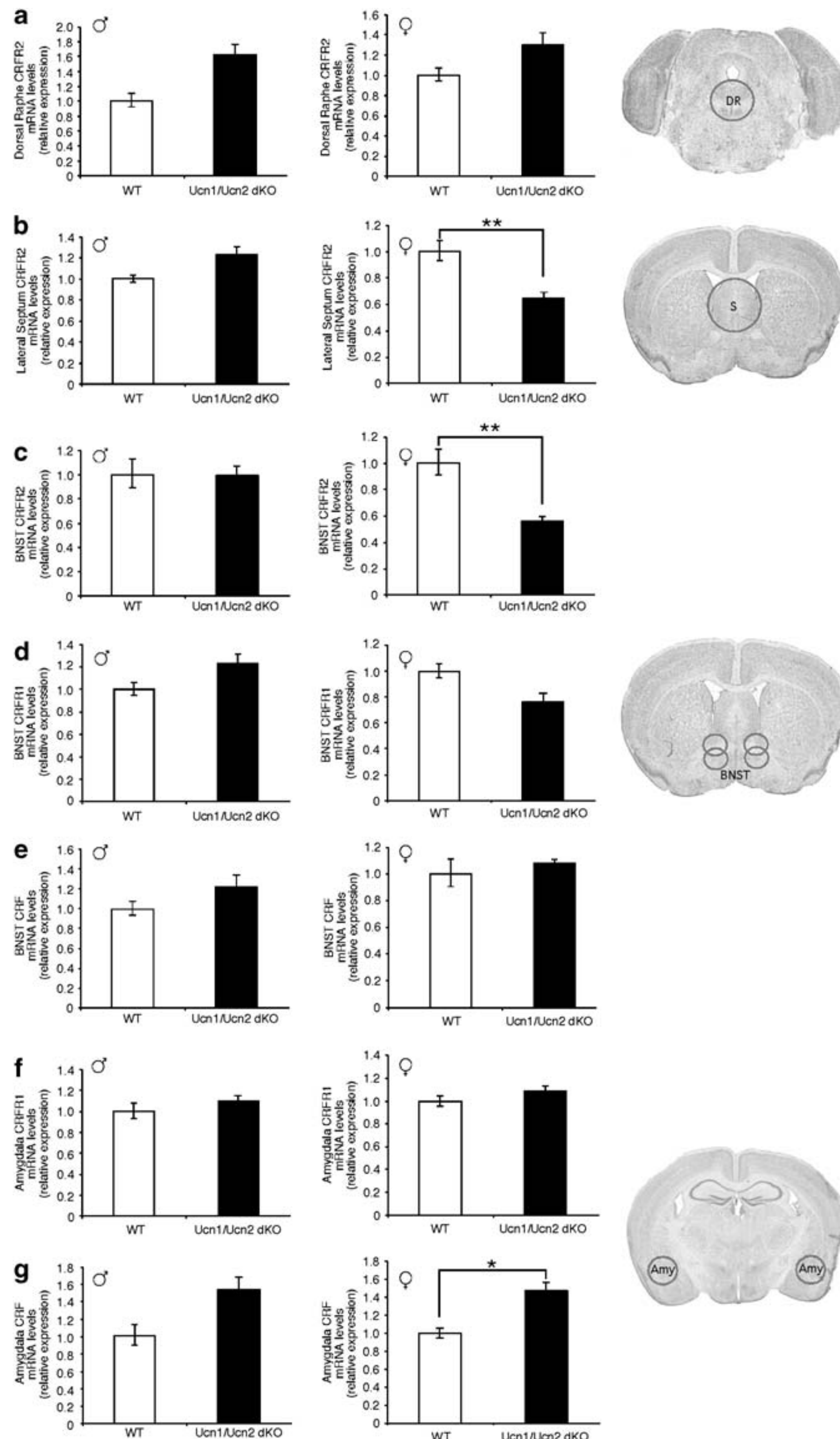
To evaluate the possible developmental compensatory changes in the expression levels of other components of CRF/Ucn signaling, we used real-time PCR to determine the mRNA levels of CRF and CRFR1 and -2 in relevant brain areas of Ucn1/Ucn2 dKO mice compared with WT controls. The CRFR2 mRNA levels in the male Ucn1/Ucn2 dKO DRN (area depicted in Figure 4a) were higher, although not reaching significance (Figure 4a, left graph, $P=0.09$). CRFR2 mRNA levels in female Ucn1/Ucn2 dKO DRN did not differ from those of WTF mice (Figure 4a, right). Interestingly, CRFR2 mRNA expression in the male LS (depicted in Figure 4b) did not differ between the groups, whereas KOF mice showed significantly lower level of CRFR2 mRNA expression in the LS ($P=0.006$, Figure 4b, right graph). In addition, KOF, but not KOM, mice showed significantly lower level of CRFR2 mRNA expression in the BNST ($P=0.003$, Figure 4c). CRFR1 is known to bind CRF and Ucn1 with high affinity. No change in CRFR1 mRNA levels was detected in KOM or KOF and WT mice in both the BNST (Figure 4d, male: $P=0.1$; female $P=0.07$) and the amygdala (Figure 4f, male: $P=0.35$; female: $P=0.35$) nuclei. Furthermore, CRF mRNA expression levels did not differ between Ucn1/Ucn2 dKO and WT mice in the BNST (Figure 4e, male: $P=0.32$; female: $P=0.68$). However, CRF mRNA expression level in the amygdala of KOF is significantly higher than the expression level of WTF mice ($P=0.04$, Figure 4g). No such change was detected between KOM and WTM mice CRF levels in the amygdala.

Altered serotonergic transmission in anxiety-related neural circuits of the Ucn1/Ucn2 dKO mice

Administration of Ucn2 has been shown to increase c-Fos expression in the mid-rostrocaudal DRD and the DRC.[22,48] Therefore, we have microdissected the DRD, DRC and the regions of the brain that are innervated by these subdivisions of the DRN, and that were previously identified as part of an anxiety-related brain circuit.[49] These areas include the nodal structure of the BLA, structures that provide input to the BLA (CA1v, LEnt and S) and structures involved in the regulation of anxiety states that are innervated by the BLA (the BNST and the CeA nucleus). Figure 5; Supplementary Figure S3 depicts tissue concentrations of 5-HT and 5-HIAA from WTM, WTF, KOM and KOF. Depicted on the right are diagrams indicating location of microdissections as well as median distance from bregma. Tissue concentrations of 5-HT within an anxiety-related neural circuit were influenced by Ucn1/Ucn2 dKO genotype as well as sex. Two-way univariate analyses of variance for tissue concentrations of 5-HT revealed a main effect of genotype in the BLA ($F_{(1,30)}=-20.105$; $P<0.001$, Figure 5a), CA1v ($F_{(1,32)}=12.155$; $P=0.001$, Figure 5b) and LEnt ($F_{(1,32)}=14.797$; $P=0.001$, Figure 5c), with an effect only approaching significance in the S ($F_{(1,31)}=3.847$; $P=0.059$, Figure 5d). Similarly, there were main effects of genotype in structures innervated by the BLA, the BNST ($F_{(1,36)}=13.251$; $P<0.001$, Supplementary Figure S3) and CeA ($F_{(1,35)}=21.270$; $P<0.001$, Supplementary Figure S3). There was an effect of sex on tissue concentrations of 5-HT in the DRD ($F_{(1,29)}=13.130$; $P=0.001$, Figure 5e). Further *post-hoc* pairwise comparisons of effects of genotype within sex groups (WTM–KOM and WTF–KOF) in the BLA, CA1v, LEnt, BNST and CeA, or of sex within genotype groups (WTM–WTF and KOM–KOF) in the DRD revealed greater tissue concentrations of 5-HT in KOF versus WTF in the BLA ($P<0.001$), LEnt ($P=0.001$), BNST ($P=0.003$) and CeA ($P=0.001$); greater tissue concentrations of 5-HT in KOM versus WTM in the CA1v ($P=0.002$)

and CeA ($P=0.007$); and greater concentrations of 5-HT in WTF versus WTM in the DRD ($P=0.003$).

Tissue concentrations of 5-HIAA within an anxiety-related neural circuit were influenced by Ucn1/Ucn2 dKO genotype as well as sex. Two-way univariate ANOVA for tissue concentrations of 5-HIAA revealed main effects of both genotype and sex on tissue concentrations of 5-HIAA in the BLA (genotype, $F_{(1,30)}=54.558$, $P<0.001$; sex, $F_{(1,30)}=11.495$, $P=0.002$, Figure 5a), CA1v (genotype, $F_{(1,32)}=30.710$, $P<0.001$; sex, $F_{(1,32)}=13.993$, $P=0.001$, Figure 5b) and LEnt (genotype, $F_{(1,31)}=48.468$, $P<0.001$; sex, $F_{(1,31)}=10.949$, $P=0.002$, Figure 5c). There was an effect of genotype, but not sex, in the S ($F_{(1,32)}=9.108$; $P=0.005$, Figure 5d), DRC ($F_{(1,29)}=12.656$; $P<0.001$, Figure 5f), BNST ($F_{(1,35)}=30.315$; $P<0.001$, Supplementary Figure S3) and CeA ($F_{(1,35)}=24.875$; $P<0.001$, Supplementary Figure S3). Finally, there was an effect of sex,



but not genotype, in the DRD ($F_{(1,29)}=16.715$; $P=0.001$, Figure 5e). Further *post-hoc* pairwise comparisons of genotype within sex groups (WTM–KOM and WTF–KOF) and sex within genotype groups (WTM–WTF and KOM–KOF) revealed greater tissue concentrations of 5-HIAA in both KOM and KOF with respect to their WT counterparts in the BLA (KOM–WTM, $P=0.001$; KOF–WTF, $P<0.001$), CA1v (KOM–WTM, $P=0.001$; KOF–WTF, $P<0.001$), LEnt (KOM–WTM, $P=0.010$; KOF–WTF, $P<0.001$), BNST (KOM–WTM, $P=0.002$; KOF–WTF, $P<0.001$) and CeA (KOM–WTM, $P=0.006$; KOF–WTF, $P<0.001$). In addition, KOF had higher tissue concentrations of 5-HIAA than KOM in the BLA ($P<0.001$), CA1v ($P=0.002$) and LEnt ($P<0.001$). In the DRD, both KOF and WTF had greater tissue concentrations of 5-HIAA than their respective male counterparts (KOF–KOM, $P=0.003$; WTF–WTM, $P=0.017$). In the DRC and S, an effect of genotype was observed in females with greater tissue concentrations of 5-HIAA in KOF as compared with WTF (DRC, $P=0.004$; S, $P=0.025$).

Discussion

Mice deficient for Ucn1 and Ucn2 display modified HPA response

In the current study, we established a mouse model, which is deficient for both Ucn1 and Ucn2 peptides, and determined their neuroendocrine and behavioral responses to stress. Ucn1/Ucn2 dKO and WTM mice show similar basal plasma corticosterone levels, but elevated levels after exposure to stress, which is partially in correlation with the accelerated and sensitive HPA axis responses observed for the CRFR2 knockout mice models.[50,51] KOF mice did not differ from WTF mice in their plasma corticosterone levels under basal or stress-induced conditions. Mice lacking Ucn2 exhibited a sex-specific phenotype, in which female (but not male) Ucn2-null mice showed an increase in basal daily rhythms of adrenocorticotrophic hormone and corticosterone.[37] Ucn1-deficient mice, generated independently by two groups, exhibited normal basal and stress-induced HPA hormone levels.[36,52] It is therefore possible to assume that Ucn1 and Ucn2, through their activation of CRFR2, have a role in modifying the recovery phase of the HPA axis. The difference between the HPA responses of each studied model also suggests a possible sex-dependent compensatory relationship between the two ligands.

Neuroanatomical studies of CRF revealed a differential expression of this neuropeptide in anxiety and HPA-related brain nuclei.[53,54] In the Ucn1/Ucn2 dKO mice, the CRF mRNA expression in the PVN of male mice was significantly higher than that in WT mice, whereas the levels in the CeA were unchanged. The higher levels of CRF in the PVN of Ucn1/Ucn2 dKO mice may explain their overactivated HPA axis. In a study performed on CRFR2-deficient mice, Bale *et al*.[51] found the CeA to contain higher CRF mRNA levels than did control mice, whereas no such changes were observed for the PVN. The differences in amygdalar CRF expression, between the CRFR2 knockout and the Ucn1/Ucn2 dKO mice models may contribute to the opposite anxiety-like behavior phenotype observed for these two models. The role of the CeA in anxiety-like behavior is under debate, and it is more often ascribed to fear responses, orchestrating the rapid and primary behavioral, autonomic and endocrine responses (reviewed in Rosen[55]), suggesting the possible involvement of changes in other brain regions in mediating the observed behavioral changes. Here, we described that KOF, but not KOM, mice show an increase in CRF mRNA expression in the amygdala (Figure 4). Sex differences in HPA axis activation and the prevalence of mood disorders are well documented,[56,57] and evident in both basal circadian rhythm and stress-induced activation of the HPA axis.[56] Our data suggests that Ucn1 and Ucn2 may be involved in mediating some of the sex differences observed in both the corticosterone levels after stress and the compensatory profile of CRF-related mRNA expression in various brain loci.

The response to stress involves initiation, maintenance and recovery processes. CRF and CRFR1 are known to be critical for initiation of the HPA hormone cascade.[58,59] As CRF levels in the PVN and plasma corticosterone levels are higher, it is reasonable to assume that the blunted behavioral

Figure 4 Compensatory profile of corticotropin-releasing factor (CRF) and CRF receptors (CRFRs) mRNA levels in anxiety-related brain regions. CRF and CRF receptors mRNA expression levels in male and female urocortin-1 and urocortin-2 double knockout (Ucn1/Ucn2 dKO) and wild-type (WT) controls were measured using the real-time PCR. Right panels depict the punch location (modified from Paxinos and Franklin[45]). All mRNA levels were quantified relative to hypoxanthine–guanine phosphoribosyltransferase (HPRT) mRNA. (**a**) CRFR2 mRNA levels in the dorsal raphe nucleus (DRN) of Ucn1/Ucn2 dKO and WT mice (male: $P=0.09$; female: $P=0.41$). (**b**) The lateral septum (LS) CRFR2 mRNA levels show difference between Ucn1/Ucn2 dKO and WT female (middle panel, $P=0.006$), but not between male mice (left panel, $P=0.37$). Similarly, CRFR2 mRNA levels in the bed nucleus of the stria terminalis (BNST) differ between female, but not male mice (**c**, male: $P=0.97$; female: $P=0.003$). mRNA expression levels of CRFR1 did not differ between Ucn1/Ucn2 dKO and WT male and female mice in both the BNST (**d**, male: $P=0.1$; female: $P=0.07$) and the amygdala (Amy) (**f**, male: $P=0.35$; female: $P=0.35$). (**e**) CRF mRNA levels in the BNST of Ucn1/Ucn2 dKO mice did not differ from WT controls (male: $P=0.32$; female: $P=0.68$). (**g**) CRF mRNA expression level was significantly higher in the amygdala of female Ucn1/Ucn2 dKO compared with WT mice, but not between male groups (**g**, male: $P=0.24$; female: $P=0.04$). Male group size: WT, $n=5$; Ucn1/Ucn2 dKO, $n=7$ and female group sizes: $n=7$–9 in each group. * $P<0.05$, ** $P<0.01$. S, subiculum.

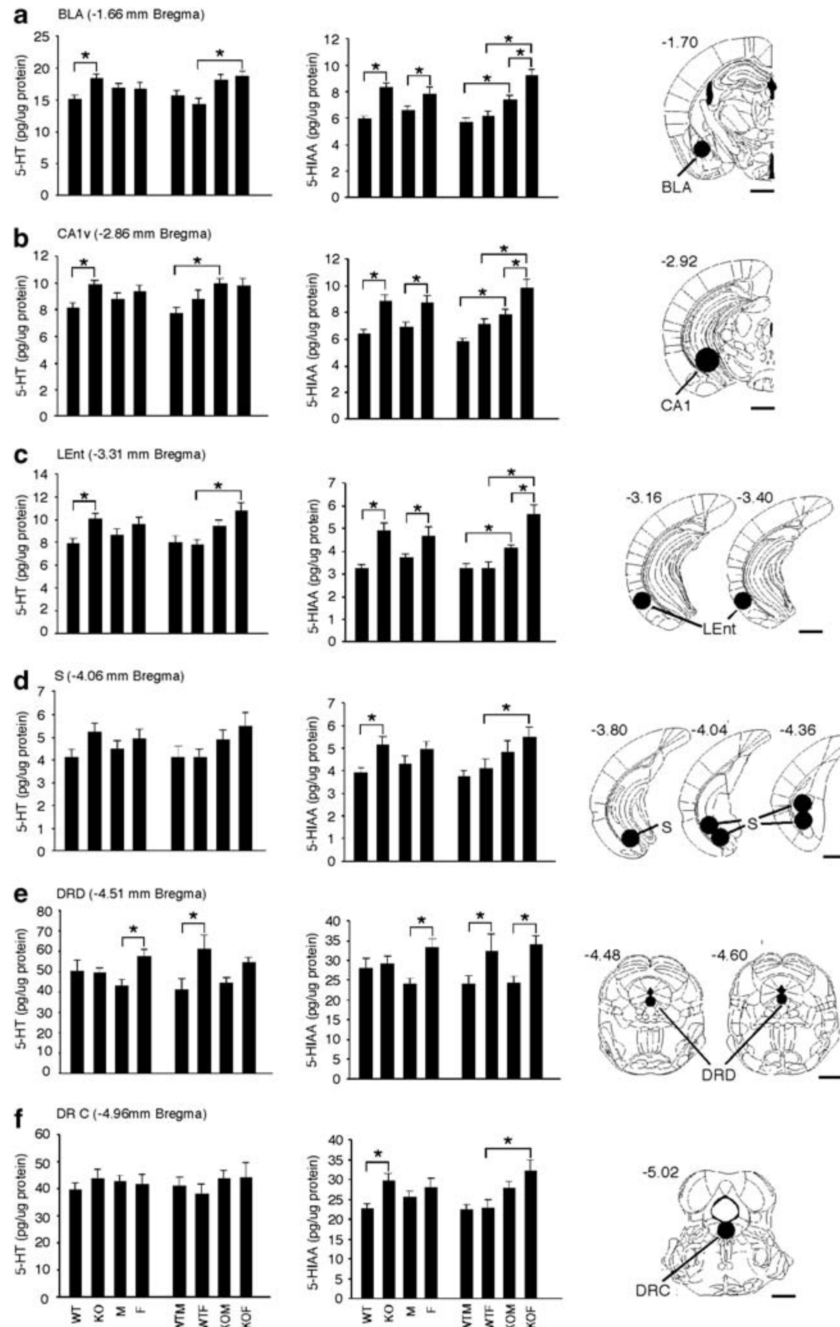


Figure 5 Male and female urocortin-1 and urocortin-2 double knockout (Ucn1/Ucn2 dKO) mice show elevated tissue concentration of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) levels in anxiety-related brain regions. The graphs depict tissue concentrations of 5-HT (left) and 5-HIAA (right) from Ucn1/Ucn2 dKO and wild-type (WT) mice, as measured by high-pressure liquid chromatography (HPLC) analysis. Four bars to the left of each graph indicate main effects of genotype and/or sex and the four bars to the right of each graph indicate tissue concentrations in male (WTM, KOM) and female (WTF, KOF) mice. Depicted on the right are diagrams indicating location of microdissections as well as median distance from bregma. (**a**) 5-HT and its metabolite 5-HIAA were found in higher concentrations in the basolateral amygdaloid (BLA) of KOM and KOF mice. For 5-HIAA in this site, a main effect was found for both the genotype and the sex. In the CA1 region of the ventral hippocampus (**b**) and in the lateral entorhinal cortex (LEnt) (**c**) a significantly higher 5-HT level was found in the Ucn1/Ucn2 dKO male and female mice, and significant differences between both genotype and sex for 5-HIAA. (**d**) A main effect of genotype was found in measurements of 5-HIAA in the Subiculum (S). Although in the dorsal raphe nucleus, dorsal part (DRD) we found significantly higher concentrations of 5-HT in WTF than in WTM, and higher 5-HIAA concentrations in females of both genotypes (**e**), the dorsal raphe nucleus, caudal part (DRC) concentrations of 5-HIAA did not differ between males and females. The DRC 5-HT concentration also did not differ between groups (**f**); however, a marked change of 5-HIAA concentrations between WT and Ucn1/Ucn2 dKO was evident, especially between the female groups (**f**). Concentrations represent means ± s.e.m.

responses to basal and acute stress challenges are not due to blunted initiation processes of the neuroendocrine stress response. Thus, it is possible that the anxiolytic phenotype shown by the behavioral tests in both males and females may reflect central, anxiety-related circuits influenced by Ucn1 and Ucn2.

Ucn1/Ucn2 dKO mice show robust anxiolytic behavior and sex-specific compensatory profiles

Behaviorally, the two models for Ucn1 deficiency displayed different phenotypes. Although Vetter *et al.*[36] observed increased anxiogenic-like behavior in both the open-field and the EPM tests, Wang *et al.*[52] showed normal anxiety-like behavior. Ucn2-null mice showed a significant decrease in depressive-like behavior, in addition to altered HPA axis activity. These effects were evident only in the female null mice, suggesting a role for Ucn2 in mediating the sex differences observed in the stress response.[37] Both KOM and KOF mice exhibited a stable anxiolytic phenotype along different paradigms (Figure 2 and Supplementary Figure S2). When subjected to restraint stress before the tests, WTM and WTF mice exhibited an expected increase in anxiety-like behavior (decreasing locomotion and exploration; Figure 3). However, KOM and KOF mutant mice showed a significantly smaller stress-induced change in behavior.

The changes in neuroendocrine and behavioral responses to stress observed in the Ucn1/Ucn2 dKO mice may be due to altered gene expression of other components of the CRF signaling pathway. CRFR1 mRNA expression levels were unchanged at important anxiety-related brain nuclei such as the amygdala and the BNST. In addition, CRFR2 mRNA expression levels of KOM in the LS and the BNST did not differ from that of WTM. Interestingly, KOF mice show a significant reduction in CRFR2 mRNA levels in the LS and the BNST. Previous studies have shown a robust Ucn1 projection from the Edinger–Westphal nucleus to the LS in mouse.[60] A lack of such innervation in the dKO mice could lead to the observed compensation, and may contribute to the anxiolytic phenotype, as described by Henry *et al.*[61] It is important to note that, although Ucn3 is not innervating the DRN–CRFR2-positive neurons, it may influence anxiety-related behavior in the Ucn1/Ucn2 dKO, by activating the CRFR2 expressed by other brain regions such as the LS and amygdala. i.c.v. administration of mouse Ucn3 was shown to cause reduced anxiety-like behaviors in both the open-field and dark/light transfer tests in mice[62] and increased the time spent on the open arms of the EPM in rats.[63]

Elevated 5-HT and 5-HIAA levels in anxiety-related brain circuit of the Ucn1/Ucn2 dKO mice

The DRN contains CRFR2 mRNA expression in abundance.[18,64] In addition, collateral serotonergic projections arise from the DRN to target functionally related regions, such as those involved in stress response, anxiety and conditioned fear.[9,49] Introduction of rodents to stressful stimuli associated with increased anxiety and fear[65–68] or i.c.v. infusion of CRF, Ucn1 or Ucn2[14,22,48,69] is effective at increasing immediate-early gene expression within caudal regions of the DRN. In addition, several stress paradigms, CRF and its related peptides and the use of CRFR1 antagonists cause different changes in levels of 5-HT and 5-HIAA in the terminal regions,[70–76] underscoring the role of CRF and CRF-related peptides in the regulation of extracellular 5-HT levels under basal and stress conditions.

The physiological and behavioral arousal associated with anxiety states and anxiety-related behavior seems to be regulated by a distributed and interconnected system of forebrain and hindbrain structures[49] including the septo-hippocampal system and entorhinal cortex,[77] medial prefrontal cortex,[78] BLA complex[79,80] and midbrain raphe complex.[81–83] The Ucn1/Ucn2 dKO mice had marked increases in 5-HT and 5-HIAA in different loci of the anxiety-related circuit (Figure 5; Supplementary Figure S3), effects that may be due to the altered input to the DRN, caused by the absence of Ucn1 and Ucn2 innervations. It is noteworthy that female mice show a significantly greater difference in 5-HT and 5-HIAA concentrations in DRN terminal fields. These data suggest that the role of Ucn2 in sex differences is (at least in part) to modulate DRN activity on anxiety- and depression-related brain nuclei, and thus may explain female-specific sensitivity to mood disorders.

We have seen in Ucn1/Ucn2 dKO mice a consistent increase in 5-HT and 5-HIAA in different brain areas associated with the anxiety-related circuit described by Hale *et al.* The S is an important region in the regulation of anxiety-related behavior and in HPA axis response to stress-related anxiogenic stimuli.[84,85] The CA1v is also a part of anxiety-related brain circuit, projecting to the BLA and exhibiting increased c-Fos expression after exposure to mild stress.[49,86,87] The CA1 role in modulating anxiety-related behavior was also revealed by lesions specific to the ventral hippocampus, causing reduction in anxiety-like behavior.[88,89] 5-HT and 5-HIAA were elevated in all the above anxiety-related areas; however, the DRN subpopulations of DRD and DRC exhibited a discrete pattern of expression. Although the DRD has shown no difference of 5-HT or 5-HIAA between Ucn1/Ucn2 dKO and WT mice (but did show sex differences), the DRC samples show significant elevation of 5-HIAA in the Ucn1/Ucn2 dKO mice. The DRD is selectively innervated by the BNST,[34] and gives rise to projections to anxiety-related forebrain regions (for review, see Lowry *et al.*[90]). The DRC gives rise to serotonergic projections to the periventricular thalamic and hypothalamic structures, and also projects to the limbic forebrain structures including the dorsal and ventral hippocampus,[91,92] S,[91] BLA[83] and entorhinal cortex.[91]

A growing body of evidence suggests that Ucn2 may influence stress-related physiology and behavior by

modulating the DRN serotonergic system. Ucn2 i.c.v. administration caused an increase in c-Fos immunostaining in topographically organized subpopulations of serotonergic neurons in the DRN, specifically within the DRC and DRD.[22,48] Moreover, although injection of mouse Ucn2 to the DRC leads to increased 5-HT release in the BLA[23] and potentiation of conditioned fear, as well as escape deficits in a model of learned helplessness,[20] the injection of the CRFR2 antagonist, antisauvagine-30, and not of CRFR1 antagonists, showed anxiolytic effects, including reversal of the potentiation of conditioned fear and the escape deficits after exposure to inescapable stress.[20,93,94] Thus, these behaviors indicating heightened anxiety in response to uncontrollable stress seem to be mediated by CRFR2 receptors on serotonergic neurons in the DRC.[24] Some overall conclusions that can be drawn from the present neurochemistry data set are that tissue concentrations of 5-HT and 5-HIAA are generally elevated in Ucn1/Ucn2 dKO mice within an anxiety-related neural circuit that receives projections from the caudal and dorsal parts of the DRN, regions of the DRN that are targeted by anxiety-related stimuli, anxiogenic drugs and anxiety-related neuropeptides, such as Ucn1 and Ucn2.[20,22,48,67,82,90,95,96] These effects are largely driven by effects of genotype on both 5-HT and 5-HIAA tissue concentrations within females, with a smaller effect of genotype in males. Analyses of additional 5-HT projection regions will reveal how widespread the effect of genotype is on increased tissue concentrations of 5-HT and 5-HIAA. It will be useful to examine projection regions of other populations of serotonergic neurons.

It is important to note that, although our results strongly indicate a DRN serotonergic modulation on anxiety-related nuclei, some of the observed effects may be due to the absence of Ucn1 and Ucn2 innervations to other brain nuclei and possible compensatory mechanisms. Therefore, further studies involving nuclei-specific modulations of Ucn1 or Ucn2 may further elucidate the importance of these neuropeptides as modulators of behavioral and neuroendocrine responses to stress.

Conflict of interest

Wylie Vale is a co-founder, equity holder, member of the Board of Directors and Scientific Advisory Board of Neurocrine Biosciences and Acceleron Pharma, Inc. The remaining authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)