A triple *urocortin* knockout mouse model reveals an essential role for urocortins in stress recovery

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Responding to stressful events requires numerous adaptive actions involving integrated changes in the central nervous and neuroendocrine systems. Numerous studies have implicated dysregulation of stress-response mechanisms in the etiology of stress-induced psychopathophysiologies. The urocortin neuropeptides are members of the corticotropin-releasing factor family and are associated with the central stress response. In the current study, a triple-knockout (tKO) mouse model lacking all three *urocortin* genes was generated. Intriguingly, these urocortin tKO mice exhibit increased anxiety-like behaviors 24 h following stress exposure but not under unstressed conditions or immediately following exposure to acute stress. The inability of these mutants to recover properly from the exposure to an acute stress was associated with robust alterations in the expression profile of amygdalar genes and with dysregulated serotonergic function in stress-related neurocircuits. These findings position the urocortins as essential factors in the stress-recovery process and suggest the tKO mouse line as a useful stress-sensitive mouse model.

amygdala | anxiety-like behaviors | serotonergic system | corticotropin-releasing factor | corticotropin-releasing factor receptor type 2

Dysregulation of stress-response mechanisms is proposed to underlie a variety of stress-related psychopathologies (1, 2). Corticotropin-releasing factor (CRF) plays a pivotal and well-established role in regulating the hypothalamic-pituitary-adrenal (HPA) axis under basal and stress conditions (3, 4) and, via its type 1 receptor (CRFR1), integrates the autonomic, metabolic and behavioral stress responses (5).

The CRF peptide family includes also three urocortin (Ucn) peptides (Ucn1, Ucn2, and Ucn3) that bind and activate the CRF receptor type 2 (CRFR2) with high affinity (6–12). CRF has a relatively lower affinity for CRFR2 than for CRFR1; Ucn1 has equal affinities for both; and Ucns 2 and 3 appear to be selective for CRFR2 (6–9). These receptors are distributed differently throughout the brain: CRFR1 is widely expressed in various brain regions, whereas CRFR2 expression is more localized to selected stress-related brain nuclei, such as the amygdala, the bed nucleus of the stria terminalis (BNST), the lateral septum (LS), and the dorsal raphe nucleus (DRN) (13, 14).

Evidence from studies using competitive peptides or small-molecule CRF/urocortin receptor antagonists suggested that the brain CRF/urocortin systems play diverse roles in mediating behavioral responses to stress (15). Based on the complementary behavioral phenotypes of CRFR1- and CRFR2-deficient (KO) mice, opposing roles were suggested for the two CRF receptors systems in modulating anxiety-like behaviors. CRFR1KO mice display decreased anxiety-like behaviors coupled with an impaired HPA axis stress response (16, 17), whereas CRFR2KO mice show increased anxiety-like behaviors and an accelerated HPA-axis response to stress (18, 19). Thus, the CRF-CRFR1 system has been suggested as critical for initiating stress responses, whereas the urocortins-CRFR2 system was suggested to terminate stress responses or restore allostasis (1, 12). Nevertheless, the anxiety-related effects of CRFR2 agonists and administration of antagonists into the cerebral ventricles or into specific brain regions were less consistent, with some evidence for brain-site or ligand specificity (10).

Thus, to understand better the role of the endogenous CRFR2 ligands Ucn1, -2, and -3 in regulating the central stress response, a *urocortin* triple-knockout mouse model (tKO) was generated. Anxiety indices were compared between tKO and WT mice obtained from the same breeding colony under three conditions: unstressed, immediately following exposure to an acute stressor, and 24 h following stress. Under unstressed conditions and immediately following the acute stress, tKO mice exhibited anxiety-related behaviors comparable to those of WT mice but exhibited increased anxiety at 24 h poststress, suggesting a modified response to stress. This increased anxiety in tKO mice was associated with alterations in the regulation of amygdalar gene expression and with dysregulated serotonergic functions in stress-linked neurocircuits.

Results and Discussion

tKO Mice Exhibit a Persistent Behavioral Response to Stress. WT and tKO mice were tested in the open-field and light/dark transfer (LDT) tests under three conditions (different mice were used in each condition): (i) unstressed (no additional stressor other than the challenge of the test); (*ii*) immediately after 30 min of acute restraint stress; and (*iii*) 24 h poststress. Because these tests rely on the animals' exploratory behavior, general locomotion also was assessed to rule out motor dysfunctions. Further assessment of stress-induced anxiety used a nonexploratory index, the acoustic startle-response test (ASR).

In the open-field test, unstressed WT ($n = 12$) and tKO ($n = 15$) mice did not differ in the amount of time spent in the center (Fig. 1*A*) or in number of visits to it (Fig. 1*B*). However, tKO mice explored the arena significantly less ($P < 0.01$) than WT mice (Fig. 1*C*). In the stress conditions, two-way ANOVA and a follow-up contrast comparisons showed that immediately after stress WT ($n = 15$) and tKO ($n = 12$) mice did not differ in the amount of time spent in the center, in number of visits to the center, or in exploration of the center (Fig. 1 *D–F*, *Left*). However, at 24 h poststress tKO mice ($n = 12$) spent significantly ($P < 0.05$) less time in the center than WT mice ($n = 11$), visited the center significantly ($P < 0.05$) fewer times, and explored the arena significantly less ($P < 0.01$) (Fig. 1 *D–F*, *Right*). As depicted in Fig. 1*G*,

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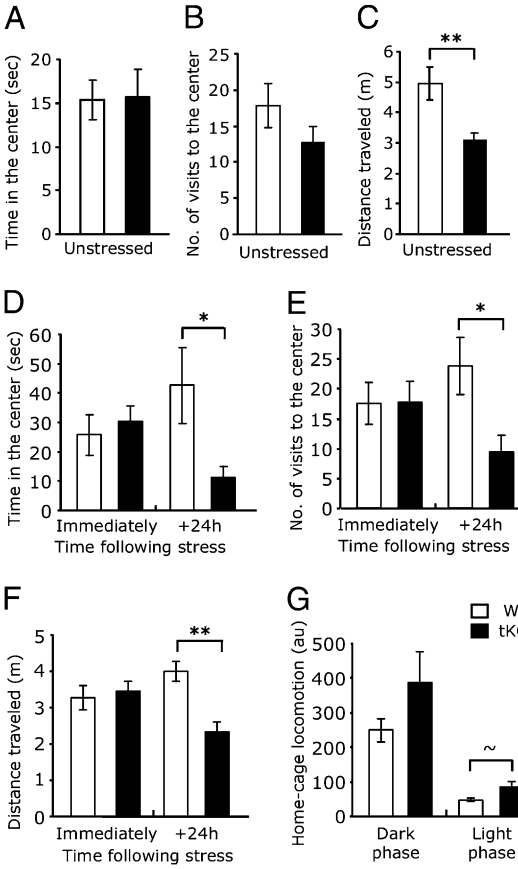

Fig. 1. tKO mice exhibit increased anxiety in the open-field test at 24 h poststress but not under unstressed conditions or immediately following stress. Under unstressed conditions (*A–C*), WT (n = 15) and tKO (n = 17) mice did not differ in time spent in the center (*A*) or in number of visits (*B*), but tKO mice traveled significantly less far than WT mice (*C*). Immediately following acute stress (*D–F*, *Left*), WT (n = 15) and tKO (n = 12) mice did not differ in time spent in the center (*D*), in number of visits to the center (*E*), or in total distance traveled (*F*). However, at 24 h poststress (*D—F*, *Right*), tKO mice (n = 12) spent significantly less time in the center (*D*), visited the center significantly fewer times (*E*), and traveled significantly shorter distances (*F*) than WT mice (n = 11). (*G*) tKO mice (n = 11) exhibited slightly higher levels of home-cage locomotion than WT mice (n = 11) in both the active dark phase and inactive light phase; however, these differences were not statistically significant. *P < 0.05; **P < 0.01; ~P = 0.067.

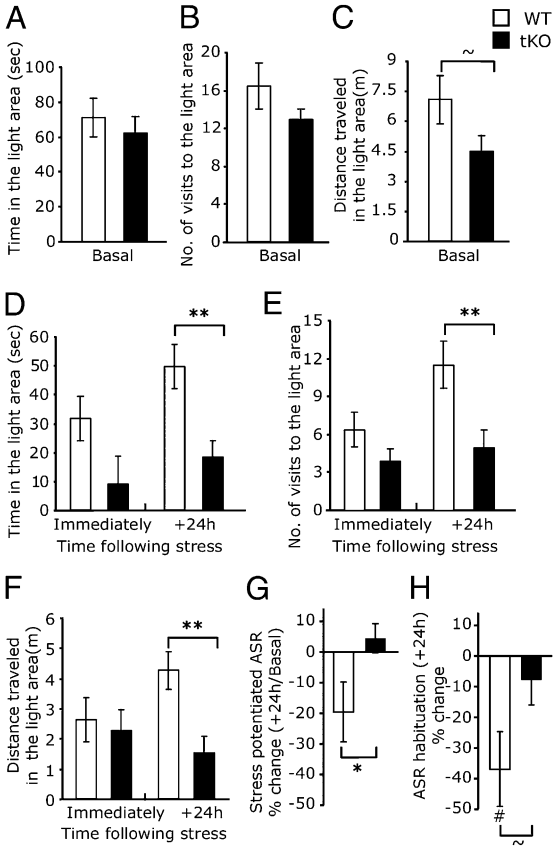

Fig. 2. tKO mice exhibit increased anxiety in the LDT test and in ASR at 24 h poststress but not under unstressed conditions or immediately following stress. Under unstressed conditions (*A–C*), WT (n = 8) and tKO (n = 10) mice did not differ in time spent in the light area (*A*), in number of visits to it (*B*), or in distance traveled in it (*C*). Immediately following stress (*D–F*, *Left*), WT (n = 12) and tKO (n = 12) mice did not differ in time spent in the light area (*D*), in number of visits to it (*E*), or in distance traveled in it (*F*). However, at 24 h poststress (*D*–F, *Right*), tKO mice (n = 13) spent significantly less time in the light area (*D*), visited it significantly fewer times (*E*), and traveled a significantly shorter distance in it (*F*) than WT mice (n = 11). (*G* and *H*) ASR. (*G*) At 24 h following stress, tKO mice (n = 11) exhibited a stress-induced increase in mean maximal ASR significantly higher than that in WT mice (n = 12). (*H*) Within-session ASR rate of habituation. tKO mice exhibited a marginally significant lower habituation rate than WT mice; however, although WT mice exhibited a significant rate of habituation, tKO mice did not. *Significant difference between groups, P < 0.05; **significant difference between groups, P < 0.01; #significant difference within group, P < 0.05; ~significant difference between group, P = 0.064.

tKO mice (n = 11) exhibited slightly higher levels of home-cage locomotion than WT mice (n = 11) in both the active dark phase and inactive light phase, but these differences were not statistically significant.

A similar pattern of effects was evident in the LDT test. Unstressed WT (n = 8) and tKO (n = 10) mice did not differ in the amount of time spent in light area (Fig. 2*A*), number of visits to it (Fig. 2*B*), or distance traveled in it (Fig. 2*C*). In the stress conditions, two-way ANOVA and a follow-up contrast comparisons showed that immediately after stress the genotypes (WT: n = 12; tKO: n = 12) did not differ in any of the indices (Fig. 2 *D–F*, *Left*). At 24 h poststress, however, tKO mice (n = 13) spent significantly less time (P < 0.01) than WT mice (n = 11) in the light area, visited it significantly fewer times (P < 0.01), and explored it significantly less (P < 0.01) (Fig. 2 *D–F*, *Right*).

Comparisons of mean maximal ASR in unstressed tKO (n = 11) and WT (n = 12) mice and again a week later at 24 h poststress (the same mice in both conditions) indicated that tKO mice exhibited a significantly higher stress-induced increase in ASR (P < 0.05) (Fig. 2*G*). Furthermore, comparing the within-session rate of habituation of WT and tKO mice to the startling stimuli indicated that, although unstressed WT and tKO mice exhibited a comparable lack of habituation (WT = 3.91 ± 7.69; tKO= −7.78 ± 6.20), (Fig. 2*H*) WT mice exhibited a significant habituation rate at 24 h poststress (P < 0.05), whereas tKO mice did not.

These data suggest that deleting all three *urocortin* genes neither induced a state of enhanced anxiety among unstressed mice nor seemed to alter the immediate behavioral stress responses. However, it appears that WT mice recover from the acute stress exposure 24 h following stress, whereas tKO mice fail to do so. It is of note that unstressed tKO mice, although appearing more active than unstressed WT mice in the home cage, exhibited reduced novel-setting exploration, implying an anxiogenic effect under nonchallenged (unstressed) conditions. A similar pattern of effects was indicated for the relative distance traveled in the center of the open field (Fig. S1). Collectively, the data suggest that lacking all urocortins has a limited effect on

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anxiety under nonchallenged conditions but renders the mice susceptible to the effects of stress, possibly by impairing recovery mechanisms.

In previous studies, pharmacologically manipulating Ucn2 or Ucn3 yielded inconsistent data, enhancing anxiety in some paradigms (20–22) but alleviating it in others (23–26). Similar inconsistencies exist in studies demonstrating differentially modulated hormonal stress responses (22, 26–28). Our data demonstrate a relationship between the integrative actions of all urocortins in modulating stress responses. Increases in the above-noted anxiety indices were reported following different paradigms of stress exposure and were related to altered structure and/or functions of stress-related neurocircuits, including the amygdalar-BNST complex, septal regions, raphe nuclei serotonergic circuits, and HPA-axis regulation (29–35).

To assess the involvement of the HPA axis in the observed behavioral phenotype, circulating corticosterone levels and paraventricular nucleus (PVN) CRF mRNA levels were measured in unstressed mice and at 24 h poststress. Corticosterone and PVN CRF mRNA levels did not differ in WT and tKO mice at either condition (Fig. S2). Thus it appears that lacking all urocortins does not affect tonic HPA-axis regulation. Other mouse models that lack CRFR2, to which all three urocortins bind, also have been reported to exhibit normal basal levels of adrenocorticotropic hormone (ACTH) and corticosterone (18, 19) but to exhibit enhanced ACTH and corticosterone stress responses (18).

Additional assessments evaluated the effect of urocortin depletion on learning and memory faculties, using the fear-conditioning paradigm and the Morris water maze (MWM). During fear-conditioning learning, tKO mice appeared more anxious than WT controls (Fig. S3*A*). In the retention tests, no differences were observed between the genotypes in the context test (Fig. S3*B*), which is dependent on both the amygdala and hippocampus. In the amygdala-dependent cue test, however, tKO mice exhibited enhanced freezing during and following the conditioned stimuli presentation (Fig. S3*C*).

Spatial learning in the MWM was compared between the genotypes in unstressed mice and mice that had undergone the stressful experience of fear conditioning 10 d earlier (stressed mice). Different mice were used in each assessment. Unstressed WT and tKO mice did not differ in spatial learning (Fig. S3*D*). Among stressed mice, however, tKO mice exhibited a significantly slower spatial learning process than WT mice (Fig. S3*E*). The fear-conditioning data indicate that depletion of all urocortins does not affect memories that rely on hippocampal functions (context) but enhances amygdala-dependent fear memories (cue). The hippocampal-dependent MWM data indicate that the mere depletion of all urocortins is insufficient to affect spatial learning, but the interaction with exposure to stress has enduring impairing effects. Collectively, these results correspond with findings that highlight the effects of stress exposure-induced amygdala modulation of learning and memory processes (36). More specifically, increased freezing responses have been associated with altered amygdalar activity (37, 38); thus the increased freezing exhibited by tKO mice corresponds to the increased stress-potentiated ASR and to the alterations in amygdalar functions described in detail below. The comparable freezing exhibited by both genotypes in the context test may result from an interaction between enhanced amygdalar functions and a stress-induced deficit in hippocampal functions.

CRFR2-expressing neurons in the LS and DRN are involved in modulating anxiety-like behaviors (31, 39). Thus LS and DRN CRFR2 mRNA levels were evaluated in unstressed WT and tKO mice and at 24 h poststress. Under both conditions, tKO mice exhibited higher CRFR2 mRNA levels in the LS and DRN than WT mice (Fig. S4). These increases may represent developmental compensatory changes caused by the absence of high-affinity innervating ligands and may contribute to the susceptibility of tKO mice to the effects of exposure to stress.

Restricted Stress-Induced Amygdalar Gene Modification in tKO Mice. To determine whether the observed differences between tKO and WT mice in anxiety-like behaviors 24 h following stress also are reflected in the gene-expression profile of the amygdala, an established modulator of fear- and anxiety-linked behaviors (40, 41), we evaluated the expression levels of selected amygdalar genes in unstressed tKO and WT mice and in tKO and WT mice 24 h poststress. The expression levels of 28 stress-related and housekeeping genes associated with amygdalar functions were assessed using a custom-made real-time PCR array (Tables S1 and S2).

Amygdalar stress-induced gene-expression profiles of the tKO and the WT mice were differentially regulated at 24 h poststress (Fig. 3*A*). As in the behavioral indices, the amygdalar gene-expression profile of unstressed WT and tKO mice did not differ (Fig. 3*B*); however, the genotypes differed significantly at 24 h poststress. A comparison of WT amygdalar cDNA samples obtained from unstressed mice and from mice 24 h poststress indicated that several genes were significantly ($P < 0.05$) up- or down- regulated, including CRFR1 (−1.59-fold); serotonin receptor 3A (Htr3a; +3.06-fold); dopamine receptor 2 (Drd2; −5.95-fold); dopamine receptor D1A (Drd1a; −4.73-fold); glutamic acid decarboxylase 1 (GAD1; −2.24-fold); opioid receptor κ1 (OPRK1; −2.45-fold), and opioid receptor μ1 (OPRM1; −1.47-fold) (Fig. 3*C*). Interestingly, the significant differences in amygdalar gene expression profile in WT mice were not significant among tKO mice (Fig. 3*D*). A full description of the expression profile is given in Table S1.

Overall, the stress-induced amygdalar gene-expression profile in WT mice, which coincided with an adequate behavioral recovery, was not evident in the tKO mice, which behaviorally appeared anxious at 24 h poststress. A detailed discussion of the putative role of the amygdalar genes that are differentially regulated in tKO mice can be found in *SI Results and Discussion*. Collectively, the results of the amygdalar gene-regulation profile suggest a key role for the urocortins/CRFR2 system in regulating the required changes in amygdalar gene expression that coincide with lower levels of anxiety 24 h following exposure to an acute stressor.

tKO Mice Exhibit a Modified Serotonergic Balance. Altered function of the serotonergic (5-HT) system was suggested to underlie the emergence of stress-related psychopathologies (42, 43). The raphe nuclei (RN) are the primary site of 5-HT neuronal projections to forebrain stress-related neurocircuits, including the septohippocampal and the amygdalar complexes (29, 32, 44–46). CRFR2, the high-affinity urocortin receptor, is highly expressed in the RN (14), and Ucn2 caudal-DRN infusion potentiated conditioned fear- and stress-induced escape deficits in a CRFR2-dependent manner (47). In addition, DRN CRFR2 activation increased 5-HT activity and 5-HT release in stress response-regulating nuclei, including the basolateral amygdala (BLA) (39, 48–50).

Therefore, serotonin metabolism was assessed by comparing 5-hydroxyindoleacetic acid (5-HIAA)/5-HT ratios (higher values indicate more serotonergic activity) within anxiety-related neurocircuits in unstressed WT ($n = 5$) and tKO ($n = 8$) mice and in WT ($n = 11$) and tKO ($n = 12$) mice 24 h poststress. The medial and lateral regions of the septum (MS and LS, respectively), BNST, BLA, central nucleus of the amygdala (CeA), CA1 region of the ventral and the dorsal hippocampus (CA1d and CA1v, respectively), the lateral entorhinal cortex, and the subiculum (S) were examined.

The serotonergic activity of tKO mice was dysregulated in limbic forebrain sites under unstressed conditions and at 24 h poststress; however, this dysregulation differed across the examined limbic sites (Fig. 4). Two-way ANOVA for Genotype (WT/tKO) and Stress (unstressed/24 h poststress) and the interaction of 5HIAA:5-HT ratios within each brain region indicated a main effect for Genotype in the LS and BLA and a main effect for Stress in the CeA ($P < 0.01$). The interaction Genotype × Stress was significant in the BLA, CeA, and S. There were no significant effects for Genotype, Stress, or interactions

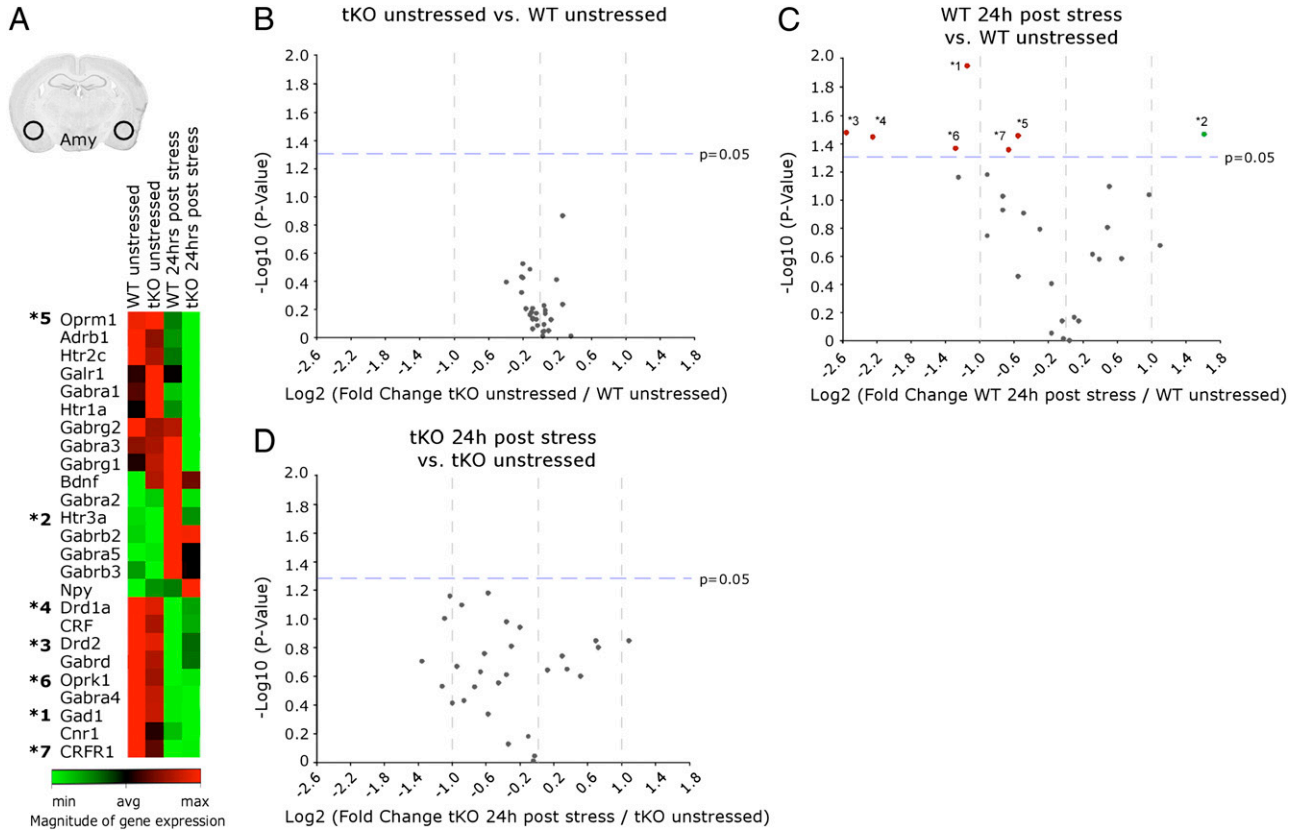

Fig. 3. Altered stress-induced amygdalar gene modification in tKO mice. Gene-expression profiles of amygdalar genes in WT and tKO mice under unstressed conditions and 24 h following stress. (*A*) Heat-map representation of the differential gene expression in WT and tKO mice in unstressed conditions and at 24 h poststress. Asterisks and numeration correspond to C: *1, GAD1, glutamic acid decarboxylase 1; *2, Htrt3a, serotonin receptor 3A; *3, Drd2, dopamine receptor 2; *4, Drd1a, dopamine receptor D1A; *5, OPRM opioid receptor μ1; *6, OPRK, opioid receptor κ1; *7, CRFR1, corticotropin-releasing factor receptor type 1. (*B–D*) Several genes are differentially regulated in tKO and WT mice, especially at 24 h poststress. Unstressed, WT: $n = 3$; tKO: $n = 3$; 24 h poststress, WT: $n = 6$; tKO: $n = 6$. (*B*) No significant differences in gene expression were observed between the WT and tKO genotypes under unstressed conditions. (*C*) WT mice exhibited a significant stress-induced change in amygdalar gene expression. In WT unstressed vs. WT 24 h poststress, GAD1 decreased 2.24-fold (1); Htrt3a increased 3.06-fold (2); Drd2 decreased 5.95-fold (3); Drd1a decreased 4.73-fold (4); OPRM decreased 1.47-fold (5); OPRK decreased 2.45-fold (6); and CRFR1 decreased 1.59-fold. (*D*) tKO mice exhibited a blunted stress-induced change in amygdalar gene expression. In tKO unstressed mice vs. tKO mice 24 h poststress, the changes observed in WT mice were not evident. Horizontal dashed lines indicate statistical significance at $P < 0.05$; Vertical dashed lines indicate no change and ± twofold change in mRNA levels as compared with the relevant control. *$P < 0.05$.

among these factors for 5-HT or 5-HIAA concentrations (pg/μg protein) independently (Table S3).

Post hoc pairwise comparisons of 5-HIAA:5-HT ratios within each region revealed that unstressed tKO mice exhibited lower 5-HIAA:5-HT ratios than unstressed WT mice in the BLA (Fig. 4*A*) and CeA (Fig. 4*B*) but not in the septohippocampal system. Conversely, at 24 h poststress, tKO mice exhibited lower 5-HIAA:5-HT ratios than WT mice in the MS (Fig. 4*D*), LS (Fig. 4*E*), and S (Fig. 4*G*) but not in the amygdala. Exposure to stress reduced 5-HIAA:5-HT ratios in the BLA and CeA in WT but not in tKO mice (Fig. 4 *A* and *B*) and in the S in tKO but not WT mice (Fig. 4*G*).

Because indices of anxiety under unstressed conditions were similar in tKO mice and WT controls, the differences in unstressed amygdalar serotonergic activity may reflect decreased activity in serotonergic systems innervating 5-HT_{2A} receptor signaling pathways in the amygdala in tKO mice. Activation of 5-HT_{2A} receptors in the BLA is thought to be excitatory to local GABAergic inhibitory neurons (51). Activation of these 5-HT_{2A} receptors may have little consequence for regulating unstressed anxiety-like behaviors but may have important consequences for the regulation of anxiety-like behaviors following stress-induced excitatory transmission. The failure of tKO mice to respond with stress-induced decreases of serotonergic activity in the BLA and CeA 24 h following stress suggests that in tKO mice amygdalar serotonergic activity is already at a minimum.

The genotype differences within brain regions of the septohippocampal system (MS, LS, and S), which differed from those in amygdalar nuclei, may reflect dysregulation of the mesolimbocortical serotonergic system innervating the septohippocampal system in tKO mice. This serotonergic system was implicated in neuromodulation of circuits involved in inhibitory control of the HPA axis and anxiety-like behaviors (45).

Collectively, the 5-HIAA:5-HT ratio data indicate differential dysregulation of serotonergic systems innervating the amygdala and septohippocampal system in tKO mice that is consistent with the complex functional anatomy of serotonergic regulation of anxiety (29, 32, 45, 52, 53). The functional relationship between CRF-CRFR1 and the 5-HT_{2A} receptor was described recently (54); our data demonstrate a relationship between the integrative action of all urocortins and their concomitant effects on anxiety-like behaviors and serotonergic functions.

Concluding Remarks

Several behavioral paradigms indicated that the tKO mouse model exhibited anxiety-like behaviors comparable to those in WT controls under unstressed conditions and immediately following stress but exhibited significantly more anxiety than WT

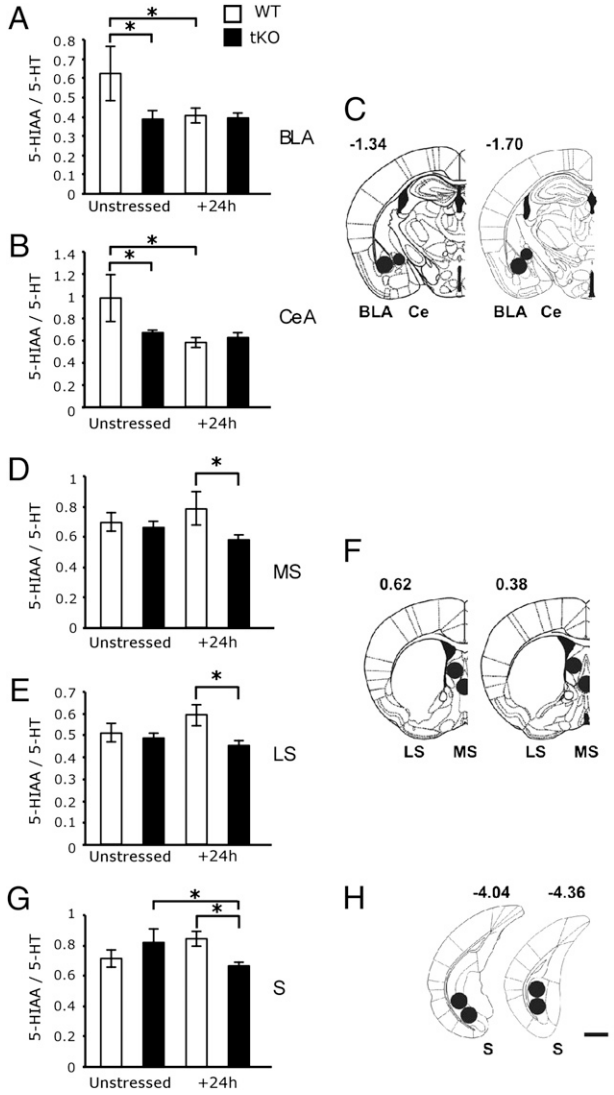

Fig. 4. tKO mice exhibit dysregulation of serotonergic function. In WT and tKO mice, serotonergic metabolism (5-HIAA:5-HT ratios) under unstressed conditions and at 24 h poststress differ in different brain regions. Unstressed, WT: $n = 5$; tKO: $n = 8$; 24 h poststress, WT: $n = 11$; tKO: $n = 12$. *C*, *F*, and *H* depict microdissection loci and median distance from bregma. In the BLA (*A*) and CeA (*B*), WT mice exhibited a stress-induced decrease in 5-HIAA:5-HT ratios at 24 h poststress relative to unstressed conditions; such a stress-induced decrease was not observed in tKO mice. In the MS (*D*) and LS (*E*), WT and tKO mice exhibited comparable 5-HIAA:5-HT ratios under unstressed conditions, but at 24 h poststress WT mice exhibited increased ratios compared with tKO mice. In the S (*G*), unstressed WT and tKO mice exhibited comparable 5-HIAA:5-HT ratios, but at 24 h poststress WT mice exhibited increased ratios compared with tKO mutants, which exhibited a significantly lower 5-HIAA:5-HT ratio at 24 h poststress as compared with unstressed conditions. $^*P < 0.05$.

mice at 24 h poststress. Furthermore, stress-induced amygdalar gene regulation in tKO mice differed significantly from that in WT mice at 24 h poststress. These differences included critical components of the GABAergic, opioid, CRF-CRFR1, dopaminergic, and serotonergic systems. Moreover, tKO mice differed from WT controls in serotonergic functions in amygdalar nuclei and within the septohippocampal complex both under unstressed conditions and at 24 h poststress.

Collectively these findings suggest that deleting all three *urocortin* genes induce a susceptibility to the effects of stress exposure by compromising amygdalar stress-induced gene regulation of inhibitory functions and several key neuromodulator receptors, while also differentially affecting serotonergic neuromodulation of amygdalar and septohippocampal subregions. Interestingly, a recent study (55) showed that Ucn1/Ucn2 double-KO mice exhibited an attenuated stress response in both sexes. It thus is suggested that Ucn3 central functions are pivotal to the observed phenotype of the tKO mouse model. Further examination of the contribution of each urocortin to the observed tKO phenotype using a longitudinal comparative study in both sexes should be a focus of future studies.

Ucn1 (56, 57), Ucn2 (58), and Ucn3 (59) individual KO mouse models have not indicated a clear anxious phenotype, perhaps because of differences in the time points of assessment following the stress exposure. Because the CRFR2 system was suggested to mediate restoration of allostasis (1, 12), further testing individual urocortin-KO models at time points that better reflect recovery processes, combined with the use of site-specific manipulations of those genes in adult mice (to avoid developmental compensatory changes), may promote further understanding of the role of each *urocortin* gene product in regulating the central stress response.

The urocortin tKO mouse model appears to be a useful, stress-sensitive line, highlights the roles of the urocortins-CRFR2 system in mediating recovery from stress, and further suggests potential mechanisms by which the urocortins-CRFR2 system interacts with other stress- and anxiety-regulating systems.

Materials and Methods

Animals. Mice lacking all three *urocortin* genes (Ucns1, -2, and -3) were generated by crossbreeding of Ucn1, Ucn2, and Ucn3 single-KO mice provided by the Vale laboratory (56, 58, 60). All mice were on a mixed C57BL/6 × 129 background. Ucn1 and Ucn2 KO mice and Ucn2 and Ucn3 KO mice were crossbred to produce double-KO mice that then were crossbred to produce tKO offspring homozygous for all genes. WT mice of the mixed C57BL/6 × 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/dark photoperiod (lights on at 18:00 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.

Behavioral Manipulation and Assessments of Anxiety, Learning, and Memory. Acute stress consisted of 30-min restraint stress. Anxiety was assessed using the open-field, LDT, and ASR tests. (A detailed description of these tests is provided in *SI Materials and Methods*). Learning and memory faculties were evaluated using the fear-conditioning paradigm, and spatial learning was evaluated in the MWM. (A detailed description of the apparatus and protocols is given in ref. 61 and *SI Materials and Methods*).

General Locomotion. Home-cage locomotion was assessed individually over a 72-h period using the InfraMot system (TSE Systems). (A detailed description is given in ref. 62 and *SI Materials and Methods*).

Blood Collection and Assessment of Corticosterone Levels. Corticosterone was quantified using a corticosterone enzyme immunoassay as described in *SI Materials and Methods*.

Quantifications of mRNA Levels. CRF mRNA levels in the PVN and CRFR2 mRNA levels in the LS and DRN were determined using real-time PCR as previously described (55). Detailed protocols of the brain tissue collection, RNA preparation, quantitative PCR, and the amygdalar gene-expression profile are described in *SI Materials and Methods*.

HPLC Analyses of 5-HT and 5-HIAA Tissue Concentrations. HPLC analyses of 5-HT and 5-HIAA tissue concentrations were performed as previously described (52, 55).

Statistical Analyses. Results are expressed as mean ± SEM. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 15.0, unless specified otherwise.

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