Chapter 2

CCKergic System, Hypothalamus-Pituitary-Adrenal (HPA) Axis, and Early-Life Stress (ELS)

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1. Introduction

Early-life exposure to adverse experience or stress, simply termed early-life stress (ELS), is a worldwide problem that has a significantly negative impact in human health [1, 2]. In the United States, about 50% of adults had experienced some kind of stress before age 18 [3], and up to 15-25% of adults had traumatic ELS such as sexual abuse [4]. Most ELS is parents-originated, such as neglect, maltreatment, and abuse [5, 6]. In addition to the immediate, dreadful, and destructive effects on a child's life, ELS may produce a series of mental [7, 8], cardiovascular [9, 10], metabolic [11, 12], and many other types of disease [13, 14], at a later life stage. For example, adults who were sexually abused during childhood have a 5.7-fold increase in risk for drug abuse over those without ELS [7], and the prevalence of posttraumatic stress disorder (PTSD), a predominant form of anxiety disorders (ADs), is highly associated with ELS, with a 4-5 fold difference between adults with ELS and those without ELS [15]. Moreover, cognitive dysfunctions [16-18] such as learning and memory impairment [19-21] are also highly associated with ELS. Given that children, especially early adolescents, have a higher possibility to expose to a traumatic insult [22], adolescent trauma (AT) is an important risk factor for these post-ELS disorders.

Over the past decades, considerable insights have been gained into the molecular/neuronal mechanisms regarding how ELS impacts brain function and behavior [23-26]. Generally, it is now accepted that ELS can produce changes, most permanently, at multiple levels [25, 27]. Following ELS, for example, the overall volume of the hippocampus [28-30], corpus callosum [31-33], and cortex [34-36] all becomes smaller, compared to that of those brain regions in age-matched subjects. Besides these neuroanatomical changes, the neuronal activity and the synaptic function in the brain in ELS-victims are impaired [37-39], and most neurotransmitter systems are significantly affected too. By using positron emission tomography or fMRI, it has been found that a significantly increased release of dopamine in the ventral striatum is associated to ELS [40, 41]. The turnover rate of the serotonin (5-HT)

metabolism or the 5-HT receptor density [42, 43] is altered following ELS. Similarly, the activity of the glutamatergic system [44, 45] and the cholinergic system [46, 47] are also altered in the brain of individuals following ELS. However, it should be emphasized that the changes in the hypothalamic-pituitary-adrenal (HPA) axis activity is of the most interest [48-52].

As the most important stress-related neuroendocrine system in the body, the HPA axis is anatomically and functionally composed of three major structures: the paraventricular nucleus of the hypothalamus (PVN), the anterior lobe of the pituitary gland, and the adrenal gland [53, 54]. The PVN contains magnocellular neurosecretory neurons that synthesize and release a corticotropin-releasing factor (CRF). CRF is a 41 amino acid peptide [55, 56], and can bind to three types of G-protein-coupled receptors: CRFR1, CRFR2, and CRFR3 [57-59]. In the mammalian brain, both CRF and CRFR1 are mainly distributed in the limbic system, while CRFR-2 is in the hypothalamus [60-62]. The essential role for the CRF system is to maintain the basal HPA axis activity as well as to trigger the HPA axis in response to stresses. After released from the PVN, the CRF binds to CRFR1 at the anterior pituitary and increase the release of adrenocorticotropic hormone (ACTH). The ACTH consequently stimulates the release of glucocorticoids from the adrenal gland [63]. Once released, glucocorticoids bind both high-affinity mineralocorticoid receptors and lower-affinity glucocorticoid receptors. The glucocorticoids, or cortisol in humans and corticosterone in rodents, play an essential role in energy metabolism, growth processes, immune function, and brain functions [63, 64].

In response to stress, CRF system plays an essential role in modifying peripheral physiological response to support "fight or flight" reactions, such as mobilizing energy stores, increasing blood sugar and heart rate, inhibiting digestive functions etc [65,66]. In addition, CRF itself may act on CRFR2 in the brain to directly regulate adaptive behavioral changes encountering stress [67-69]. Taken together, the CRF/HPA system plays a primary role in coordinating the endocrine, autonomic, immune, and behavioral response to stress. As stress, either real or imaged, is a necessary inducer for ADs, the CRF/HPA system must play a unique role in anxiety-related behaviors. Indeed, a huge body of evidence has documented this notion. For example, administration of CRF [70-72] or CRFR1 agonists [69,73,74] or overexpression of the CRF gene [75-77] produces Anxiety-like behaviors (ALBs) in the animals. On the other hand, CRFR1 antagonists exert significantly anxiolytic effects [78-80]. Knockout of CRF or CRFR1 in mice significantly reduces ALBs to stress and dramatically blunts stress-induced HPA axis activity [61,81,82]. Remarkably, previous chronic stress is able to enhance HPA axis activity in response to a novel acute stress, despite the negative feedback effects of increased glucocorticoids produced by the chronic stress [83-85]. For example, CCK-4-induced panic status in healthy volunteers significantly increases HPA axis activities [86]. Even the effects of early-life stress on HPA axis function are found to be associated with CCK sensitivity [130]. Most interestingly, interactions between the CCKergic system and the CRF/HPA system exist [88-90]. For example, the CCKergic system was found to be involved in this chronic stress-enhanced responsiveness, since chronic stress can specifically facilitate the release of CCK into the PVN, which directly projects to the pituitary, in response to acute stress [125]. All these findings have not only established the role of the CRF/HPA system in initiating behavioral responses to stresses,

but also indicate that a significant interaction may exist between the CRF/HPA system and CCKergic system to regulate stress-related behaviors.

However, the vulnerability among different individuals to AT is different. This variability may at least partially attribute to a genetic variability [91]. A twin study of Vietnam veterans revealed that about 37.9% of vulnerability to PTSD was genetically related [92]. Further genetic evidence comes from clinical association studies, by which several candidate genes for ADs including PTSD have been associated, although a causative gene has not been yet established [91]. Among those candidate genes, cholecystokinin (CCK) receptor-2 (CCKR-2) has been linked to panic disorder, another major form of ADs [93,94].

As the most abundant neuropeptides, CCK distributes broadly in the brain and mainly in the limbic system [95,96]. CCK binds to CCK receptor-1 (CCKR-1) and CCKR-2, of which the CCKR-2 is predominantly found in the brain with the highest level in cortical area and the limbic system [97], a brain region that is critically involved in emotion response and behavior. Virtually, the CCKergic system has long been recognized as an anxiogenic factor for the animals [98], and this effect has been well validated in human populations as well [89,99,100]. Our recent study also showed that overexpression of CCKR2 in neurons of the forebrain of mice significantly enhanced ALBs [101]. At the same time, some candidate genes that are linked to ADs are also associated with HPA axis activity. For example, a common polymorphism at the serotonin transporter (5-HTT) gene, namely 5HTTLPR, is a strong candidate genetic variation for ADs and depression [102-103], and also is significantly implicated in HPA axis activity [104]. Similar to the CCKergic system, the HPA axis system has long been recognized as a stress hormone [105,106], and plays a critical role in the pathogenesis of ADs [107,108]. Indeed, following ELS, the activity of the HPA axis system is dysfunctional [109-111]. Moreover, given the overall role of both the HPA axis system [112-114] and the CCKergic system [115-117] in regulating neuronal, cardiovascular, and metabolic functions in the body, these two systems may play an integrative role in the pathogenesis of post-ELS disorders.

In this study, by using our previously engineered inducible forebrain-specific CCKR-2 transgenic (IF-CCKR-2 tg) mice [101], we demonstrated that the elevated CCKergic tone in the brain significantly facilitated the effect of AT on the impairment of the glucocorticoid negative feedback inhibition in response to a novel acute stressor during the adult stage in the mouse, providing direct evidence that reveals a molecular basis for this co-effect.

2. Materials and methods

2.1. Experimental animals

The procedures for the generation of IF-CCKR-2 tg (simply dtg) mice were described in our previous publication [101]. Briefly, we used the tTA/tetO-inducible gene expression system to produce these dtg mice. This system requires two independent transgenic mouse strains, tTA transgenic and tetO/CCKR-2 transgenic mice. Accordingly, two constructs were made. The first was for tTA transgenic mice, in which the expression of the tTA was under the control of an alpha-Ca^{2+} calmodulin kinase II (CaMKII) promoter. The tTA transgene cassette consists of

0.6 kb of exon-intron splicing signal (pNN265), 1.0 kb of tTA encoding sequence (pTet-Off, Clontech), and 0.5 kb of SV-40 poly-A signals (pTet-Off, CLONTECH). The other construct is for CCKR-2 transgenic mice, in which the expression of the CCKR-2 transgene was under the control of the tetO promoter. The CCKR-2 transgene cassette consisted of 1.3 kb of mouse CCKR-2 cDNA, an upstream 0.6 kb of splicing signal (pNN265), and a downstream 1.1 kb of b-globin poly-A signals. All these components were subcloned into the pTRE2 vector (CLONTECH). CCKR-2 cDNA was cloned by RT-PCR from the total RNA extracted from the brain of a male B6/CBA F_1 mouse (The Jackson Laboratory) with the primers of 5'-CGG GAT CCA TGG ATC TGC TCA AGC TG-3' and 5'-GCT CTA GAT CAG CCA GGT CCC AGC GT-3'. A commercial RNA extraction kit (Invitrogen) and a reverse transcription kit (Stratagene) were used. The cloned cDNA was confirmed by sequencing. The plasmid constructs were then linearized with suitable enzymes and separately injected into the pronucleoli of B6/CBA F_1 zygotes, as described [118]. Transgenic founders and the transgene copy numbers were determined by Southern blot analyses of the tail DNA. Founder mice with suitable gene copy numbers were backcrossed into B6/CBA F_1 mice first to produce hemizygous single transgenic mice and then to produce double hemizygous transgenic mice. We have totally generated nine CaMKII-tTA transgenic founders and seven tetO-CCKR-2 transgenic founders. Southern blot analyses indicated that the gene copy numbers were from 2 to 70 for tTA transgenic founders and 2-150 for CCKR-2 transgenic founders (data not shown). To map the tTA expression pattern in the brain, we crossed a tetO-Lac-Z reporter mouse line (SJL-TgN-tetoplacZ, the Jackson Laboratory) into different independent CaMKII-tTA mouse lines to produce different tTA-LacZ double transgenic mouse lines. For Lac-Z staining, a commercial X-Gal staining kit (Invitrogen) and the recommended staining protocol were used with sagittal brain sections (30 μm), by which we identified a tTA transgenic line that was of the capacity to drive tetO/gene expression in almost all the neurons in the forebrain region (data not shown). Genotyping was determined by PCR analyses of both tTA (5'-AGG CTT GAG ATC TGG CCA TAC-3' and 5'-AGG AAA AGT GAG TAT GGT G-3') and the CCKR-2 (5'-ACG GTG GGA GGC CTA TAT AA-3' and 5'-GAG TGT GAA GGG CATG CAA-3') transgenes. Dtg mice used here were around 12-16 generations since they were generated, during which duration dtg mice were backcrossed into B6/CBA F_1 mice in every 5-6 generations, in order to avoid an inbreed effect. Single transgenic (tTA or tetO-CCKR-2 only) and wild-type (wt) littermates of dtg mice were used as controls, and are collectively and simply called wt mice hereafter. Mice used here were kept in standard laboratory mouse cages under the standard condition (12 hours light/dark cycle, temperature at 22 ± 1 ^{o}C, humility at 75%) with food and water *ad libitum*. All experimental procedures for the use of animals were previously reviewed and approved by the institutional animal care and use committee at the Louisiana State University Heath Sciences Center at New Orleans, and all of the experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.2. *In situ* hybridization

The hybridization was used to detect the expression level and pattern of the CCKR-2 transgene in the brain. Brains from both wt and dtg mice were collected by decapitation,

and were frozen with powered dry ice immediately. Sagittal sections (20 μm) were made with a Cryostat (Leica, CM 1900, Richmond, IL). An oligo probe for tTA and a cRNA probe for the total CCKR-2 mRNAs were labeled with ^{35}S UTP (>1,000 Ci/mmol; NEN, Boston, MA) by a random labeling kit and *in vitro* transcription kit (Invitrogen, Carlsbad, CA), respectively. The hybridization was performed overnight at 55°C, and after washing, slides were exposed to Kodak BioMax film (NEN) for the same time.

2.3. Adolescent trauma (AT)

Both wt and dtg mice at the age of P25 were individually put into a small shock-box (4 X 4 X 10 inch in high) that was modified from the shock box from a fear-conditioning system (Coulbourn Instruments, Whitehall, PA), in order to ensure that the mice did not have much space for escaping during shocking. The current of the footshock was higher (1.0 mA) than it was commonly used in the fear-conditioning test (0.6-0.8 mA). The footshock was conducted for 5 times (trials), in total, during a period of 1 minute, and each trial lasted for 2 seconds, with an interval of 10 seconds between trials.

2.4. Acute stressor (AS)

Additional acute stressor (AS; 0.8 mA for 2 seconds for one trial) with a standard fear-conditioning paradigm as described previously [119], was used to trigger HPA axis reaction at the age of P60 (2 months).

2.5. ELISA

Commercially available kits for both the adrenocorticotropic hormone (ACTH) (MD Bioproducts, St. Paul, MN) and corticosteroid hormone (CORT) (R&D systems, Minneapolis, MN) were used to determine the serum level of these hormones. Experimental procedures followed the recommended steps. In order to have samples enough for triplicate measurements, blood was collected with a retroorbital eye bleeding method. In order to minimize non-specific effects, blood collection was conducted at 9:00 Am, and the procedure was completed within 30 seconds, by which time any possible change that might be produced by the sampling procedure was not yet measurable.

2.6. Statistical analysis

Both female and male mice were almost equally distributed in each group. Data were analyzed with one-way ANOVA, followed by post-hoc tests. The p value less than 0.05 is considered significant.

3. Results

3.1. Expression of the CCKR-2 transgene in the brain of dtg mice

As shown in Fig 1, *in situ* hybridization revealed that the expression of the tTA was forebrain-specific in dtg mice (Fig. 1B), but was not detectable in wt mice (Fig. 1A). The

expression pattern of the CCKR-2 transgene (data not shown) was the same as both the pattern of the tTA expression and the CCKR-2 transgene expression reported in our previous study [101].

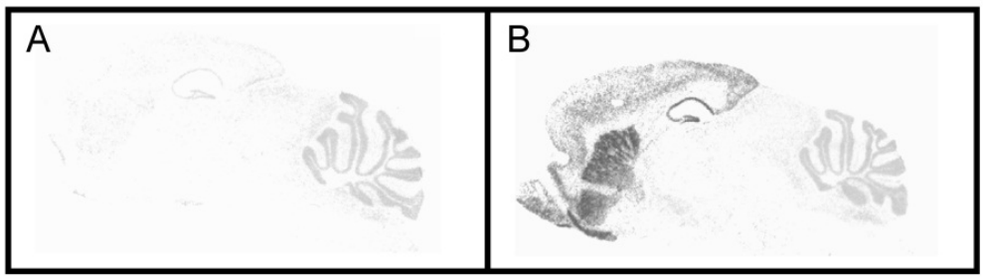

Figure 1. Expression pattern of the tTA mRNA detected by *in situ* hybridization with saggital brain sections in wt (A) and dtg (B) mice.

3.2. Dtg mice with AT exhibit an increased HPA axis activity in response to AS

Either wt (n = 60) or dtg mice (n = 60) were subjected to AT, and then were divided into 5 groups (n = 12) for a time-course study, in which both ACTH and CORT were examined before the AS for the basal level, and 1, 2, 4, and 8 hours following the AS. As shown in Fig. 2, although the difference in the basal level of ACTH (Fig. 2A) or CORT (Fig. 2C) between these mice was not significant, a tendency of a lower level ACTH ($p = 0.0741$) and CORT ($p = 0.0648$) was observed in dtg groups, compared to wt groups. Following the AS, an one-way ANOVA revealed a significant effect of the AT and CCKR-2 transgene on ACTH [$F(1,8) = 6.781$, $p < 0.01$] and CORT [$F(1,8) = 9.201$, $p < 0.01$]. Detailed post-hoc tests revealed that both ACTH (Fig. 2B) and CORT (Fig. 2D) in either wt or dtg mice reached the peak level at 1 hr after the AS, while a significant difference was observed at 1 and 2 hr in ACTH between wt and dtg groups ($p > 0.05$), and at 1 and 2 hr in CORT between wt and dtg groups ($p > 0.05$). In both wt and dtg mice, ACTH returned to the basal level at 4 hr (Fig. 2B), while CORT returned to the basal level at 4 hr (Fig. 2D). All these results indicate that the interaction between the AT and CCKR-2 transgene does not only increase the activity of the HPA axis following a novel stressor, but also impairs the CORT negative feedback in response this stressor.

3.3. Disassociation of the CCKR2 transgene expression and AT largely diminishes the effect of AT on HPA axis activity in response to AS

In this study, both wt and dtg mice were treated with doxycycline (doxy, 2 mg/100 ml in drinking water) for 5 days prior to AT, so that the transgene expression in dtg mice was inhibited during the episode of AT, and this inhibition lasted for about 3-5 days after the doxy treatment. At 2 months old, these mice were subjected to AS, and 1 hr later, which is the peak time of HPA axis response, as described in Fig. 2, the HPA axis activity was measured. Surprisedly, the levels of both ACTH and CORT were indistinguishable between wt and dtg mice, indicating that the coupling of AT and the transgene expression is critical for the AT to produce impaired glucocorticoid negative feedback inhibition in the animals.

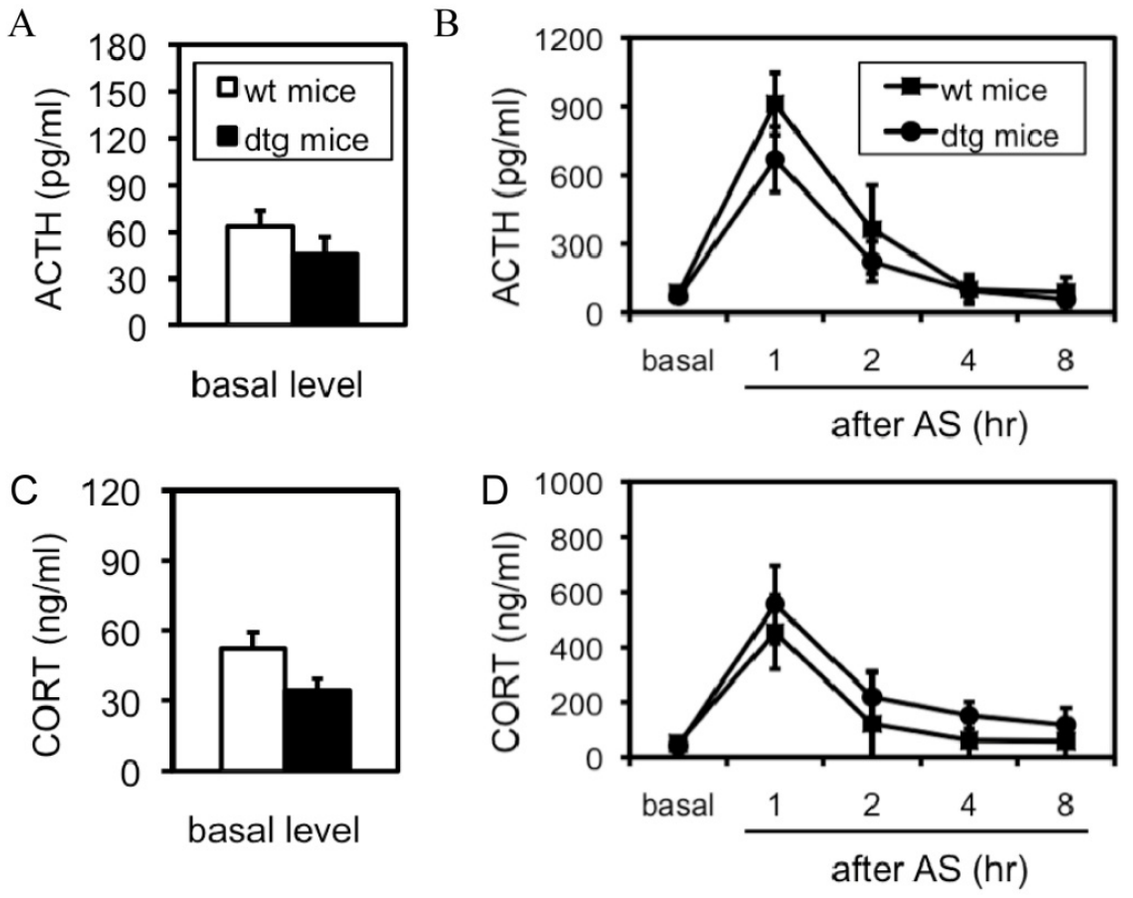

Figure 2. Increased HPA axis activity in dtg mice with AT/AS. A. Basal serum level of ACTH in naïve wt mice and naïve dtg mice. A tendency of a difference is shown, but it is not significant. Data are expressed as mean ± SEM. **B.** Time-course of ACTH response following the AS. **C.** Basal serum level of CORT in naïve wt mice and naïve dtg mice. A tendency of a difference is shown, but it is not significant. Data are expressed as mean ± SEM. **D.** Time-course of CORT response following the AS. The same groups of mice above were examined.

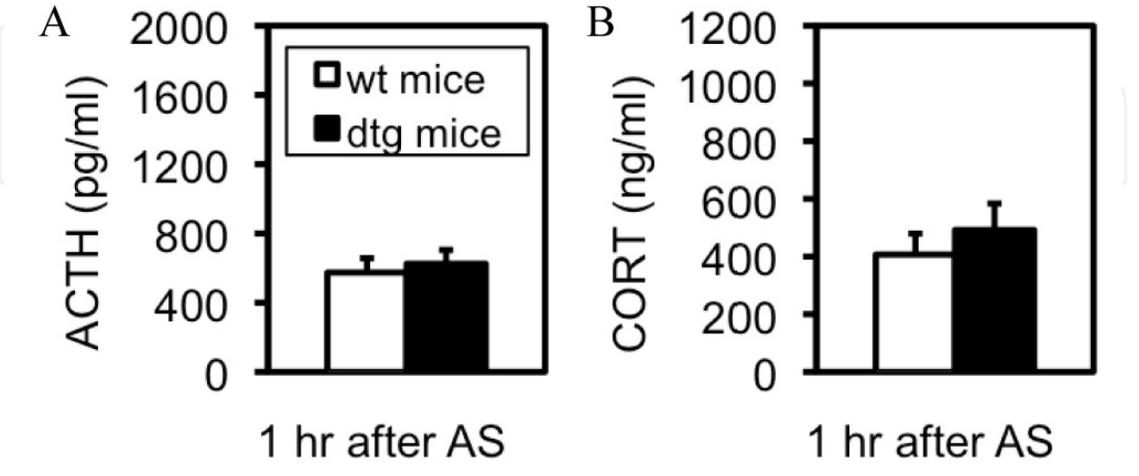

Figure 3. Level of ACTH (A) and CORT (B) in the mice after AT/AS. No significant difference was found between wt and dtg mice when the expression of the CCKR-2 transgene was suppressed during AT.

4. Discussion

We have for the first time demonstrated that a coupling of a higher CCKergic tone with an ELS event is a causative factor for the development of an impairment of glucocorticoid negative feedback inhibition in the animals in response to additional acute stressor at a later life stage.

This demonstration is achieved based on the technical merit in our transgenic mice, in which the transgene expression is inducible/reversible. The time resolution for this inducible/reversible feature is within 1 week, which is high enough for this time-coupling analysis. However, it is still not clear how this real-time coupling occurs, partially due to the fact that the functional significance of the CCKergic system is still not fully understood. As G protein-coupled receptors, CCKR are associated with Ca^{2+} release, PKC activation, PLA2 activity, and cAMP production [120]. In addition, there are robust interactions between the CCKergic system and other neurotransmitter systems including dopaminergic, serotonergic, and GABAergic systems at both the structural and functional levels [121,122], and therefore, the mechanism underlying this associative effect should be complicated, and need to be further studied.

An important finding in this study is the discovery of the change in the HPA axis activity, and these changes include (1) a slightly lower basal level of the HPA axis activity in dtg mice, compared to wt mice, (2) a synergistic effect of AT and the CCKR-2 transgene on the peak level of the HPA axis activity in response to the AS; (3) a prolonged decay time of the HPA axis activity following the AS in dtg mice with AT, and (4) a requirement of real-time coupling of the transgene expression and TA. It should be mentioned that it has been well established that a previous chronic stress in the animals down-regulates the HPA axis activity, but enhances their response to a novel acute stress, despite the negative feedback effects [83,123,124]. Because chronic stress can specifically facilitate the release of CCK into the PVN, which directly projects to the pituitary, in response to acute stress [88], the elevated CCKergic tone in our dtg mice may mimic the effect of a chronic stress by working as an "intrinsic stressor" for the animals. Therefore, this intrinsic stressor constitutes a basis for the higher vulnerability of dtg mice to AT. At the same time, the impaired AS-induced CORT negative feedback response may, in tern, significantly alter many other physiological functions, and eventually lead to a pathological condition.

As described above, following ELS, neuroanatomical changes were found in different brain regions. In addition, neuronal activity is altered too {125}. Consistent to the current study, the activity of the HPA axis system in the subject who experienced ELS was dysregulated [48-52]. Moreover, many other neurotransmitter systems were also affected by ELS [40, 126-128]. Therefore, the finding from the current study has provided additional evidence regarding how the CCKergic system and the HPA axis system are involved in the pathogenesis of post-ELS disorders.

The most important finding in this study is the demonstration of that if the transgene was temporally suppressed during the time of AT exposure, this impaired HPA axis inhibition

in response to another acute stressor was largely diminished, indicating that the temporal association of the elevated CCKergic tone with AT is critically pathogenic. This finding has a potential translational significance. It is well know that the endogenous CCKergic activity, or the CCKR-2 level in the brain, plays a dominant role in the expression of anxiety. For example, the expression of anxiety was correlated with the increased CCKergic tone, which was evidenced by a higher CCK receptor-binding capacity in the brain of anxious animals, in comparison with non-anxious animals [129-131]. Different fear responses among different strains of the same animal species were attributed to different expression levels of CCKR-2 [132-134]. On the other hand, evidence also indicates that the CCKergic tone in the brain is dynamically regulated by stress. Following stress, for example, both CCK peptide immunoreactivity and CCK receptor density in the brain were significantly increased [135-139]. Social isolation, an anxiogenic stress, increased the CCK mRNA expression in the brain [140]. Especially, the effect of ELS on the HPA axis activity was associated with CCK activity [87]. Chronic stress could specifically facilitate the release of CCK into the PNV in response to acute stress [84,141]. Consistently, CCKR-2 agonists could only produce, or produce more pronounced, anxiogenic effect in stressed animals, but not in un-stressed animals [88, 142-144]. Patients with ADs were more sensitive to CCKR-2 agonists than normal controls [145-148]. Together with all these findings, it seems conclusive that the CCKergic system is dynamically involved in ELS-triggered mental disorders, and thus, an inhibition of the CCKergic tone timely associated with an ELS event might be useful to prevent the development of post-ELS disorder, especially ADs.

In summary, our study has revealed a Novel molecular underpinning for the development of post-ESL disorders, especially for mental disorders, and provide insightful information regarding how can we develop a preventive strategy for these post-ESL disorders in the humans.

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