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Limbic corticotropin-releasing hormone receptor 1 mediates anxiety-related behavior and hormonal adaptation to stress

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Corticotropin-releasing hormone (CRH) is centrally involved in coordinating responses to a variety of stress-associated stimuli. Recent clinical data implicate CRH in the pathophysiology of human affective disorders. To differentiate the CNS pathways involving CRH and CRH receptor 1 (Crhr1) that modulate behavior from those that regulate neuroendocrine function, we generated a conditional knockout mouse line (*Crhr1*$^{loxP/loxP}$*Camk2a-cre*) in which *Crhr1* function is inactivated postnatally in anterior forebrain and limbic brain structures, but not in the pituitary. This leaves the hypothalamic-pituitary-adrenocortical (HPA) system intact. *Crhr1*$^{loxP/loxP}$*Camk2a-cre* mutants showed reduced anxiety, and the basal activity of their HPA system was normal. In contrast to *Crhr1* null mutants, conditional mutants were hypersensitive to stress corticotropin and corticosterone levels remained significantly elevated after stress. Our data clearly show that limbic *Crhr1* modulates anxiety-related behavior and that this effect is independent of HPA system function. Furthermore, we provide evidence for a new role of limbic *Crhr1* in neuroendocrine adaptation to stress.

CRH is the key mediator of neuroendocrine, autonomic and behavioral responses to stress (for review, see ref. 1). Essential to the stress response are hypothalamic paraventricular neurons expressing CRH and other jointly secreted hormones, such as arginine vasopressin (AVP). Neurochemical (*e.g.*, autonomic) signals conveying potential threats reach the hypothalamus, releasing CRH from neurons of the paraventricular nucleus (PVN) to influence rapid secretion of the pituitary corticotropin (ACTH). ACTH, in turn, stimulates adrenal glucocorticoid release. Stress, and stress-associated dysfunction of CRH neuronal circuitries in particular, has been implicated in the onset and maintenance of specific psychiatric disorders such as major depression and anxiety disorders[2], as well as the consequences of early-life stress[3,4]. An increased abundance of both CRH-positive neurons and CRH receptor target cells in the developing hippocampus indicates that limbic CRH/CRH-receptor pathways have age-specific roles in mediating the influences of stress on hippocampal function[3,4]. This, in turn, contributes substantially to individual differences in HPA responsivity to stressful stimuli[5,6], which may predispose some people to the development of affective disorders later in life. The results of first clinical trials in which selective antagonists against CRHR1 were used to treat depression are encouraging, thus underlining the importance of the CRH/CRHR1 neuronal pathways for the development and maintenance of affective disorders[7]. Previous studies on conventional knockout mice deficient for either *Crhr1* or both CRH receptors (*Crhr1* and *Crhr2)* firmly establish the requirement of pituitary Crhr1 for endocrine responses to stress[8,9]. In *Crhr1* null mutants, both basal and stress-induced HPA system activity are markedly impaired[8–10].

In addition to its key role in modulating neuroendocrine function, the CRH/Crhr1 system is critically involved in regulating a variety of behavioral[11] traits such as locomotor activity, sleep, addictive behavior[12] and, in particular, anxiety-related behavior[11,13]. Accordingly, conventional knockout of *Crhr1* results in reduced anxiety-related behavior[8,14]. However, the behavioral analyses of *Crhr1* null mutants are hampered by the fact that *Crhr1* knockout mice show severe glucocorticoid deficiency[8,14]. Glucocorticoids are known to influence a variety of emotional and cognitive processes, such as learning and memory[15]. In addition, glucocorticoids are importantly involved in modulating fear and anxiety-related behavior[16,17]. Disruption of glucocorticoid receptor signaling in the central nervous system reduces anxiety-related behavior in mice[18]. The anxiolytic effect observed in conventional *Crhr1* knockout mice may, therefore, result either from *Crhr1* deficiency itself or from a marked reduction in circulating glucocorticoid hormone levels in these animals.

To address this question and to selectively dissect CRH/*Crhr1* central nervous system pathways modulating behavior from those regulating neuroendocrine function, we generated a conditional *Crhr1* knockout using the *Cre/lox*P system[19] driving Cre recombinase

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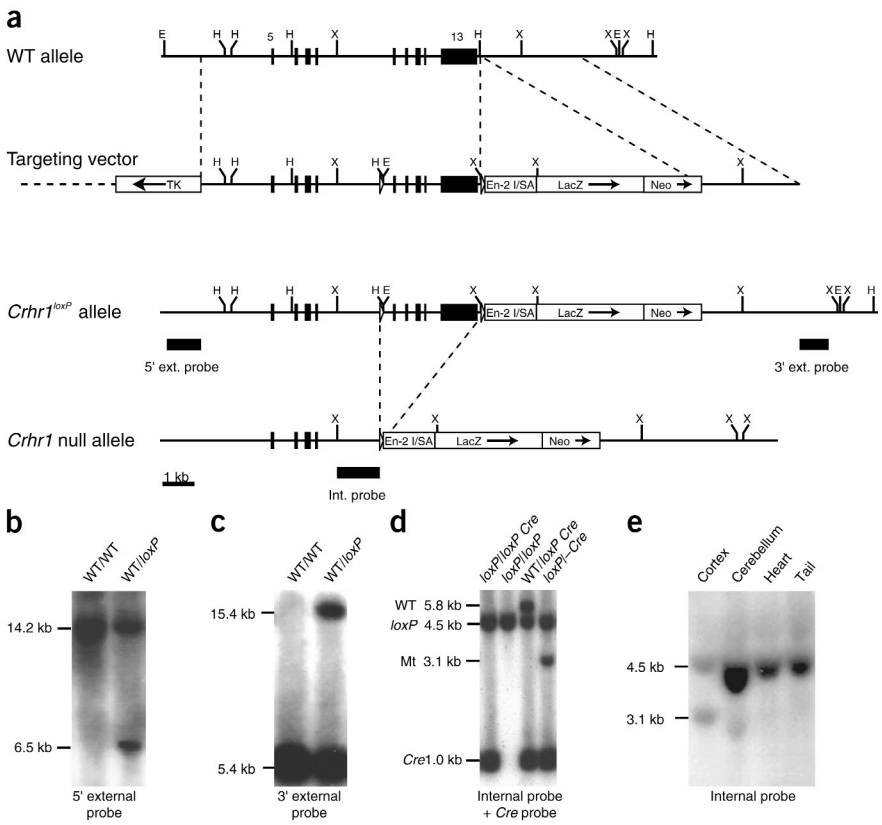

Figure 1 Generation of mice deficient for *Crhr1* in the limbic system. (**a**) Strategy for conditional inactivation of the *Crhr1* gene. Partial restriction maps of the wild-type *Crhr1* locus, targeting vector, *Crhr1*^{loxP} allele and mutant *Crhr1* locus are depicted. In the targeting vector, exons 9–13 encoding transmembrane domains 4–7, a G-protein coupling domain and the cytoplasmic tail were flanked with *lox*P sites. An engrailed 2 splice-acceptor site fused to a β-galactosidase and a PGK-neomycin cassette were inserted 3′ of exon 13. External and internal probes are indicated (E, *Eco*RV; H, *Hind*III; X, *Xba*I). (**b**) Southern blot analysis of wild-type and targeted ES cell clones. The 5′-external probe was hybridized to *Eco*RV-digested genomic DNA. The targeted allele is indicated by the presence of an additional 6.5-kb fragment. (**c**) The 3′-external probe was hybridized to *Hind*III-digested DNA from the same ES cell clones, confirming homologous recombination by the presence of an additional mutant 15.4-kb *Hind*III fragment. (**d**) Southern blot analysis of *Xba*I-digested tail DNA from conditional *Crhr1* mutant mice hybridized simultaneously with an internal probe and a Cre recombinase–specific probe. The restriction fragments obtained correspond to the indicated genotypes (Mt, mutant). (**e**) Southern blot analysis of *Xba*I-digested DNA from various tissues of a *Crhr1*$^{loxP/loxP}$*Camk2a-cre* animal hybridized with the internal probe, showing the deletion pattern and efficiency of *Camk2a-cre*.

expression by the calcium/calmodulin-dependent kinase IIα (CaMKIIα) promoter[20]. The *Camk2a* gene is expressed with tissue-specificity predominantly in the mouse anterior forebrain during postnatal development with high expression levels in hippocampal neurons (pyramidal and granule cell layer), cortical layers and the amygdala[21]. This pattern of *Camk2a*-driven *cre* expression closely matches the murine expression of functional *Crhr1* receptors in neuronal circuitries involved in anxiety-related behavior[22,23]. However, hypothalamic and pituitary expression sites of *Crhr1* are spared by *Camk2a*-driven, *cre*-mediated conditional inactivation, leaving basal and stress-induced activation of the HPA system intact. Therefore, the *Camk2a-cre* mouse line is a powerful genetic tool to study the particular role of *Crhr1* in anxiety-related limbic neuronal pathways without directly manipulating neuroendocrine function. Our results allow us to genetically differentiate CRH/*Crhr1* neuronal pathways modulating behavior from those regulating neuroendocrine (HPA system) function.

RESULTS

Generation of the conditional *Crhr1* knockout

We used homologous recombination in embryonic stem (ES) cells to generate a modified *Crhr1* allele (*Crhr1*^{loxP}) in which exons 9–13, which code for transmembrane regions 4–7 including the G-protein coupling domain and the intracellular cytoplasmatic tail, are flanked by two *lox*P sites (**Fig. 1**). *Crhr1*^{loxP} is sensitive to Cre recombinase, which catalyzes site-specific recombination between the *lox*P sites, inactivating the *Crhr1* gene in any cell expressing the recombinase (for review, see ref. 19). Mice homozygous for *lox*P (*Crhr1*$^{loxP/loxP}$) behave like wild-type littermates and do not show any behavioral or neuroendocrine phenotype (data not shown).

In a second step, the *Crhr1*$^{loxP/loxP}$ mouse line was crossed to an effector mouse line that expresses Cre recombinase in a region- and cell type–specific manner under the control of the *Camk2a* promoter[20].

Verification of the region-specific *Crhr1* knockout

We used three different approaches to verify the regional pattern and specificity of Cre recombinase activity *in vivo*. First, the transgenic line *Camk2a-cre* was crossed to a double transgenic reporter mouse, the *lacZ*/human placental alkaline phosphatase (Z/AP) reporter mouse, in which AP becomes active following *cre*-mediated excision of the *lacZ* gene[24] (**Fig. 2**). Sagittal and coronal brain sections after AP-staining showed a specific, intense labeling of the mouse anterior forebrain, including the hippocampal formation, the cortex and the amygdaloid nuclei, indicating *Camk2a*-driven *cre* expression in these selected brain regions. Second, we analyzed the pattern of *cre* expression by means of immunohistochemistry. The pattern of Cre-like immunoreactivity was similar to the pattern of Cre activity as indicated by AP staining in the Z/AP mouse line with high and specific expression in the cortex, the hippocampus and the amygdaloid nuclei (**Fig. 2**). Cre-like immunoreactivity was absent in the hypothalamus. Third, conditional inactivation of *Crhr1* was verified by *in situ* hybridization. *Crhr1*$^{loxP/loxP}$ animals showed the normal widespread expression pattern of *Crhr1* in the central nervous system as previously described[22,25] (**Fig. 2**), whereas in *Crhr1*$^{loxP/loxP}$ *Camk2a-cre* conditional mutants, Cre recombination takes place in the anterior forebrain, including the hippocampal pyramidal and granule cell layers, neocortex, striatum and amygdala, leading to an almost complete inactivation of *Crhr1* expression in these regions (**Fig. 2**). *Crhr1* mRNA expression was unaffected in the anterior lobe of the pituitary gland (data not shown).

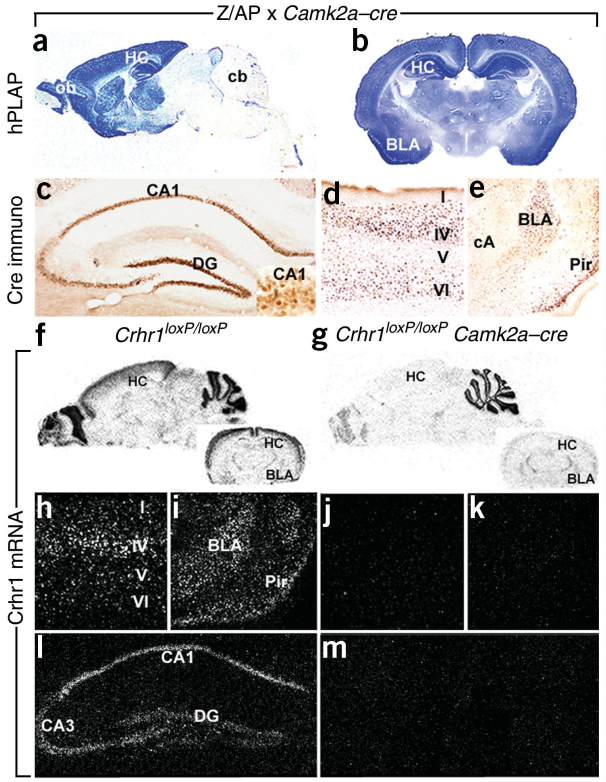

Figure 2 Verification of the region-specific *Crhr1* knockout. (**a**,**b**) The transgenic line *Camk2a-cre* was crossed to a double-transgenic reporter mouse, the *lacZ*/human placental alkaline phosphatase (hPLAP) reporter mouse (Z/AP), in which hPLP is activated following *cre*-mediated excision of the *lacZ* gene[24]. The first reporter gene (*lacZ*) is expressed only before *cre*-mediated recombination (data not shown), whereas after *cre*-mediated excision, *lacZ* expression is replaced with a second reporter gene, hPLAP. Sagittal (**a**) and coronal (**b**) brain sections following AP-staining show a specific, intense labeling of the mouse anterior forebrain, including the hippocampal formation (HC), the cortex and the amygdaloid complex (BLA = basolateral amygdaloid nucleus), indicating *Camk2a*-driven *cre* expression in these brain regions (Ob, olfactory bulb; cb, cerebellum). (**c**–**e**) Cre immunohistochemistry in the *Camk2a-cre* transgenic line shows strong expression of Cre recombinase in hippocampal pyramidal neurons (insert, CA1 pyramidal cells) and the granule neurons of the dentate gyrus (**c**). Strong Cre-like immunoreactivity was also observed in the cortex (**d**; I–VI refer to the cortical layers) and the amygdaloid nuclei (**e**; BLA, basolateral nucleus of the amygdala; cA, central amygdaloid nucleus; Pir, piriform cortex). (**f**–**m**) Verification of conditional inactivation of *Crhr1* by *in situ* hybridization. $Crhr1^{loxP/loxP}$ animals show the normal widespread expression pattern of *Crhr1* in the central nervous system (**f**), whereas in $Crhr1^{loxP/loxP}$*Camk2a-cre* conditional mutants, *Crhr1* expression is selectively inactivated in the anterior forebrain, including the hippocampal formation (HC) and the amygdaloid nuclei (BLA, basolateral nucleus of the amygdala). (**g**) *Crhr1* expression in the conditional mutants was not affected in the cerebellum, where *Camk2a* is absent. Details of *Crhr1 in situ* hybridization in control animals in the cortex (**h**), amygdaloid complex (**i**) and the hippocampal formation (**l**). In $Crhr1^{loxP/loxP}$*Camk2a-cre* mutants, no specific signal was detected after hybridization with the *Crhr1* probe in the same anatomical regions (panels **j**, **k** and **m** show corresponding sections at the same anatomical level).

Less anxiety-related behavior in *Crhr1* conditional mutants

To test our hypothesis that limbic *Crhr1* has a crucial role in mediating anxiety-like behavior independently of HPA system function, we used two behavioral methods: the light-dark box[26] and the elevated plus-maze test[27]. In the light-dark box experiment, we found a significant effect of genotype, caused mainly by significant differences between the two groups (**Fig. 3**), in the percentage of time spent in the lit compartment (univariate *F*-tests, $F_{1,39} = 9.85$, $P = 0.003$), latency to enter the lit compartment ($F_{1,39} = 13.9$, $P = 0.0006$) and percentage of entries into the lit compartment ($F_{1,39} = 12.82$, $P = 0.0009$). However, the number of entries into the dark as an activity-related parameter was similar in wild-type mice and conditional mutants ($F_{1,39} = 1.1$, $P = 0.3$; **Fig. 3**). In the elevated plus-maze test, $Crhr1^{loxP/loxP}$*Camk2a-cre* conditional mutants spent more time in the open arms of the plus-maze and had a higher percentage of entries into the open arms than their wild-type littermates, although this difference did not reach statistical significance ($P = 0.065$). The total number of head-dips ($F_{1,17} = 7.9$, $P < 0.05$) was significantly greater in the mutants, as was the number of head-dips in the unprotected arms ($F_{1,17} = 4.66$, $P = 0.45$; data not shown). Concomitantly, the percentage of head-dips that were in the protected arms (compared to the total number of head dips) was significantly decreased in $Crhr1^{loxP/loxP}$*Camk2a-cre* conditional mutants ($F_{1,17} = 4.93$, $P = 0.04$; **Fig. 3**). There were no differences in the frequency of rearings or stretched attend postures between $Crhr1^{loxP/loxP}$*Camk2a-cre* conditional mutants and $Crhr1^{loxP/loxP}$ littermates (data not shown).

In the open-field paradigm for the assessment of general locomotor activity, $Crhr1^{loxP/loxP}$*Camk2a-cre* conditional mutants showed an increase in locomotor activity (factor genotype: $F_{1,39} = 9.7$, $P < 0.05$; factor time: $F_{5,195} = 14.9$, $P < 0.05$; interaction genotype × time: $F_{5,195} = 1.1$; $P = 0.39$; **Fig. 3c**).

Crhr1 conditional mutants are hypersensitive to stress

Basal plasma ACTH and corticosterone levels in $Crhr1^{loxP/loxP}$*Camk2a-cre* conditional mutants were similar to wild-type levels (**Figs. 4** and **5**). We did not observe any alterations in the circadian pattern of stress hormone secretion in $Crhr1^{lox/loxP}$*Camk2a-cre* mice (**Figs. 4** and **5**). Therefore, in further investigations we took the averages of the morning and evening levels as baseline values.

We further analyzed plasma ACTH and corticosterone levels immediately after 2, 5 and 10 min of restraint stress, as well as 0, 30 or 90 min after a 5-min period of acute immobilization stress. For the first design, a two-factor MANOVA with repeated measurements revealed a significant treatment effect (Wilks multivariate test of significance; effect of treatment, $F_{6,13} = 774.84$, $P < 0.0001$) for both plasma ACTH and corticosterone (univariate *F*-tests, $P < 0.05$). For the second design, we detected a significant treatment effect and a significant 'genotype × treatment' interaction effect (Wilks multivariate test of significance; effect of treatment, $F_{4,15} = 52.01$, $P < 0.0001$; effect of genotype, $F_{2,17} = 20.61$, $P < 0.0001$; genotype × treatment, $F_{4,15} = 6.56$, $P = 0.003$) for both ACTH and corticosterone levels as well (univariate *F*-tests, $P < 0.05$). There were no differences in stress-associated increases in plasma ACTH between wild-type animals and conditional knockouts immediately after the 5-min restraint stress. However, in $Crhr1^{loxP/loxP}$*Camk2a-cre* conditional mutants, plasma ACTH levels remained significantly elevated for 30 and 90 min following a short period of acute immobilization stress (tests with contrasts, $P < 0.05$; **Fig. 4**). Likewise, plasma corticosterone levels were also significantly higher in $Crhr1^{loxP/loxP}$*Camk2a-cre* conditional mutants when compared to controls 30 and 90 min following a short period of acute immobilization stress (tests with contrasts, $P < 0.05$; **Fig. 5**). Female animals showed the same time course of stress hormone activation as males (see **Supplementary Fig. 1** online).

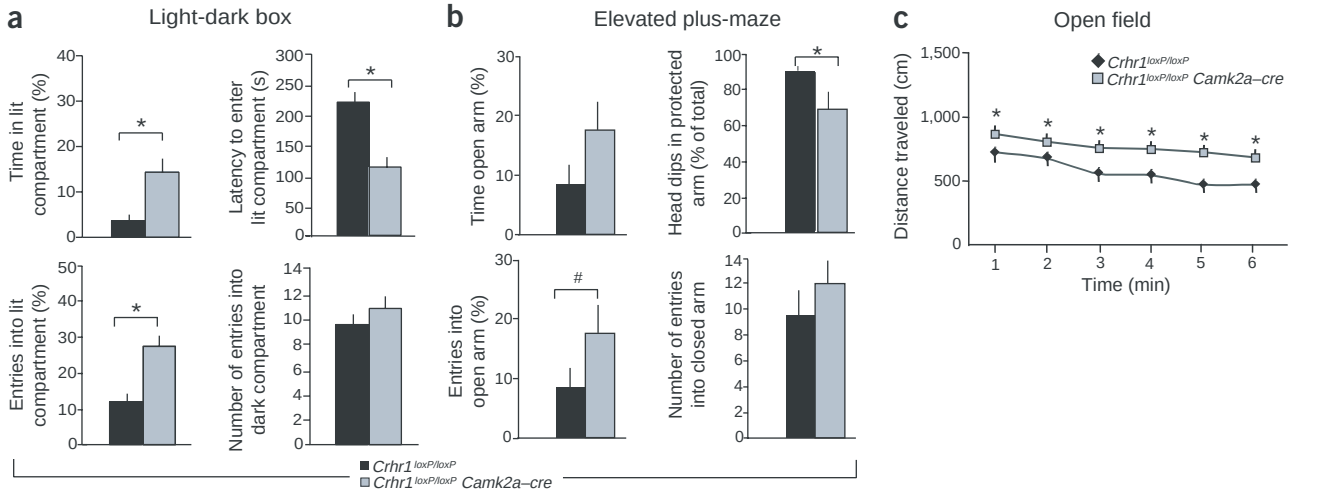

Figure 3 Reduced anxiety-related behavior in *Crhr1*$^{loxP/loxP}$*Camk2a-cre* conditional mutants (males, age 3–5 months). (**a**) Conditional mutants show significantly reduced anxiety-related behavior in the light-dark box paradigm. There was a significant effect of genotype for percentage of time spent in the lit compartment, latency to enter the lit compartment and percentage of entries into the lit compartment. However, the number of entries into the dark as an activity-related parameter was similar in both groups. The anxiolytic phenotype of *Crhr1*$^{loxP/loxP}$*Camk2a-cre* conditional mutants could be confirmed in the elevated plus-maze test. (**b**) As a parameter of increased exploratory behavior, conditional mutants showed significantly fewer head-dips in the protected arms as a percentage of total head dips, and an increase in the percentage of entries into the open arm in the elevated plus-maze test. However, the number of entries into the closed arm as a general activity-related parameter was similar in both groups. (**c**) In the open field protocol, *Crhr1*$^{loxP/loxP}$*Camk2a-cre* conditional mutants showed a slight increase in general locomotor activity. *, $\alpha = 0.05$; #, $P = 0.065$.

Evidence for functional interaction between *Crhr1* and MR

To investigate the functional interaction between hippocampal *Crhr1* and the mineralocorticoid receptor (MR, encoded by gene *Nr3c2*), we performed *in situ* hybridization analyses for MR mRNA, both under basal conditions and following stress (mean gray levels for *Crhr1*$^{loxP/loxP}$ (basal): CA1, 16.9 ± 9; CA3, 27.3 ± 13.5; dentate gyrus, 18.6 ± 10.7; mean gray levels (stress): CA1, 154.5 ± 44.8; CA3, 118.9 ± 29; dentate gyrus, 83.6 ± 24; *Crhr1*$^{loxP/loxP}$*Camk2a-cre* basal: CA1, 68.1 ± 39; CA3, 55.1 ± 32; dentate gyrus, 57 ± 28.7; stress: CA1, 41.6 ± 12; CA3, 59.2 ± 17; dentate gyrus, 42 ± 7.3). A two-factor ANOVA revealed a significant interaction effect (genotype × treatment; Wilks multivariate test of significance: $F_{3,14} = 7.65$, $P = 0.003$) for the MR expression in all hippocampal subfields (univariate *F*-tests, $P < 0.05$). By further analyzing the simple factor effects on MR mRNA expression, we did not observe any significant difference in MR mRNA expression between wild-type animals and conditional mutants under basal conditions (**Fig. 6**). However, after stress, conditional mutants showed significantly lower MR mRNA levels than wild-type animals (tests with contrasts, $P < 0.05$): *Crhr1*$^{loxP/loxP}$*Camk2a-cre* mutants did not show an increase in hippocampal MR mRNA following stress ($F_{3,15} = 1.77$, $P = 0.194$), whereas in control mice, the stress-induced increase in hippocampal MR expression was significant in all hippocampal subfields examined ($F_{3,15} = 9.81$, $P = 0.001$; tests with contrasts, $P < 0.05$).

We also found a significant genotype × treatment interaction for CRH mRNA levels (mean gray levels, PVN (basal): *Crhr1*$^{loxP/loxP}$, 29.5 ± 3.3; *Crhr1*$^{loxP/loxP}$*Camk2a-cre*, 38.6 ± 2; PVN (stress): *Crhr1*$^{loxP/loxP}$, 49.2 ± 4; *Crhr1*$^{loxP/loxP}$*Camk2a-cre*, 38.8 ± 3.2) in the hypothalamic PVN ($F_{1,16} = 27.38$, $P < 0.0001$; **Fig. 6**). Under basal conditions, conditional mutants showed higher (marginally significant) CRH mRNA levels in the hypothalamic PVN than did *Crhr1*$^{loxP/loxP}$*Camk2a* control mice (**Fig. 6**). CRH mRNA expression in the hypothalamic paraventricular nucleus shows a stress-induced increase over basal levels in control animals (tests with contrasts, $P < 0.05$; **Fig. 6e,f**), whereas in *Crhr1*$^{loxP/loxP}$*Camk2a-cre* conditional mutants, there was no stress-induced increase in CRH mRNA expres-

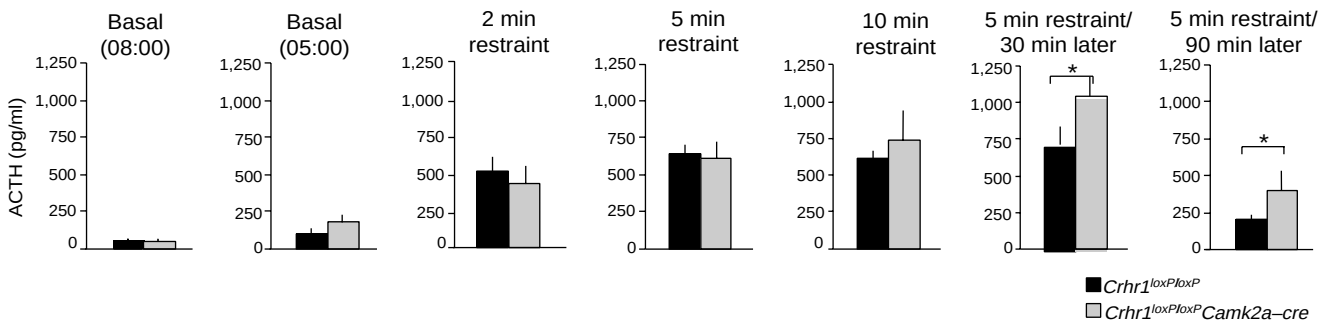

Figure 4 Plasma ACTH levels under basal conditions and following different durations of restraint stress. A two-factor MANOVA with repeated measurements design revealed significantly higher plasma ACTH levels in the *Crhr1*$^{loxP/loxP}$*Camk2a-cre* conditional mutants 30 and 90 min after a 5-min restraint stress in comparison to controls (for more details, see Results). *, $\alpha = 0.05$.

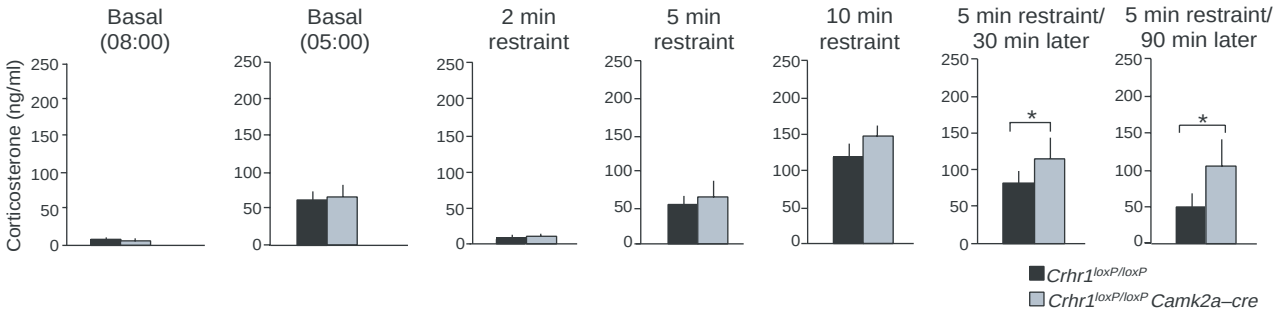

Figure 5 Plasma corticosterone levels under basal conditions and following different durations of restraint stress. Similar to plasma ACTH levels, plasma corticosterone levels at 30 and 90 min after a 5-min restraint stress were significantly higher in *Crhr1*$^{loxP/loxP}$*Camk2a-cre* conditional mutants than in control animals (for more details, see Results). *$\alpha = 0.05$.

sion over already-increased baseline levels.

DISCUSSION

To genetically distinguish neuronal CRH/*Crhr1* pathways modulating behavior from those regulating HPA system function, we generated a *Crhr1* conditional knockout mouse line in which *Camk2a*-driven, *cre*-mediated inactivation of *Crhr1* takes place selectively in behaviorally relevant limbic neuronal circuitries, including the hippocampal pyramidal and granule cell layers, the amygdala and the neocortex. *Crhr1* mRNA expression in these mutants is unaffected in the anterior lobe of the pituitary gland, leaving basal and stress-associated activation of the HPA system intact.

Limbic Crhr1 mediates anxiety-related behavior

Selective disruption of CRH/*Crhr1* signaling pathways in behaviorally relevant limbic neuronal circuitries significantly reduces anxiety-related behavior. The anxiety-reduced phenotype of *Crhr1*$^{loxP/loxP}$*Camk2a-cre* conditional mutants was confirmed in two different behavioral paradigms based on the natural avoidance behavior of mice, the light-dark box paradigm[26] and the elevated plus-maze test[27]. In the light-dark box paradigm, *Crhr1*$^{loxP/loxP}$*Camk2a-cre* mutants showed a significantly reduced latency to enter the lit compartment, they had a higher percentage of entries into the lit compartment and spent significantly more time there. However, the absolute number of entries into the dark compartment of the test box did not differ between wildtype and mutant mice. It is, therefore, unlikely that the observed anxiolytic effect is attributed to a general increase in locomotor activity during the phase of testing. We could confirm the anxiolytic effect of region-specific inactivation of *Crhr1* in the elevated plus-maze test. *Crhr1*$^{loxP/loxP}$*Camk2a-cre* conditional mutants spent more time in the open arms of the plus-maze and had a higher percentage of entries into the open arms than their wild-type littermates. A more detailed analysis of ethological measures of anxiety in the elevated plus-maze test according to the work of Rodgers *et al.*[27,28] revealed that the total number of head-dippings was significantly increased in *Crhr1*$^{loxP/loxP}$*Camk2a-cre* conditional mutants, which was due to the higher number of head dippings in the open (= unprotected) arms. These parameters have been suggested to be a sensitive indicator of anxiety, with diazepam increasing the number of exploratory head-hippings specifically in the unprotected area, as observed in our *Crhr1*$^{loxP/loxP}$*Camk2a-cre* conditional mutants[28]. Consistent with the findings in our conventional *Crhr1* knockout mouse line[8], *Crhr1*$^{loxP/loxP}$*Camk2a-cre* conditional mutants displayed increased locomotor activity in an open field test. Activity-related parameters in the light-dark box paradigm and the elevated plus-maze test, however, were not significantly different between the two groups, indicating that the behavioral assessment of anxiety-related measures in these particular paradigms has not been systematically influenced by a general increase in locomotor acitivity.

The behavioral effects of CRH administration in laboratory animals resemble some of the keys symptoms of human affective disorders (i.e. anxiety and mood disorders), including increased anxiety-related behavior and altered locomotor activity, increased grooming, decreased food intake and disruption of sexual behavior[11]. Blockade of the Crhr1 subtype, in contrast, by either selective antagonists or by functional downregulation via antisense technology has been shown to produce anxiolytic effects[13,29,30]. In addition, the behavioral phenotype of *Crhr1* null mutants previously suggested this receptor to play a crucial role in mediating anxiety-related behavior[8,14]. However, the behavioral analysis of conventional *Crhr1* knockout mice is hampered by the fact that, first, in these animals, absence of *Crhr1* during embryonic development activates

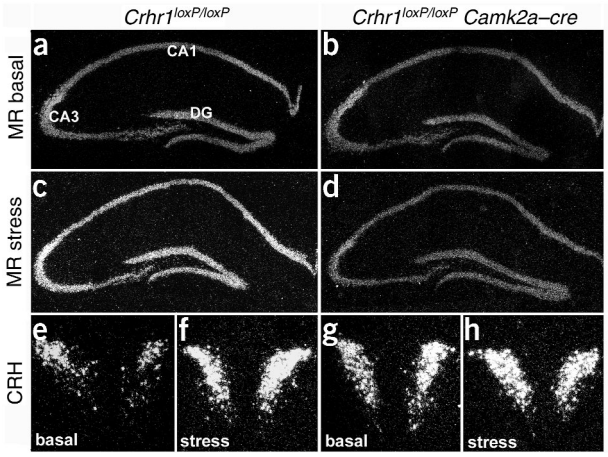

Figure 6 Expression of mineralocorticoid receptor (MR; **a**–**d**) and *Crh* (**e**–**h**) under basal conditions and after stress in *Crhr1*$^{loxP/loxP}$ controls and conditional *Crhr1*$^{loxP/loxP}$*Camk2a-cre* mutants. Hippocampal MR expression under basal conditions was similar in both groups (**a**,**b**). Control animals showed an increase in hippocampal (all subfields) MR mRNA following stress (**c**), whereas *Crhr1*$^{loxP/loxP}$*Camk2a-cre* mutants did not (**d**). Expression of *Crh* in the hypothalamic PVN shows a stress-induced increase over basal levels in control animals (**e**,**f**). *Crhr1*$^{loxP/loxP}$*Camk2a-cre* mutants show higher basal *Crh* mRNA levels than *Crhr1*$^{loxP/loxP}$*Camk2a* control mice (**g**).

compensatory mechanisms[10] that might complicate interpretation of discrete behavioral effects. *cre*-mediated conditional inactivation of *Crhr1* in *Crhr1*$^{loxP/loxP}$*Camk2a-cre* mutants, in contrast, takes place after postnatal day 20 (ref. 20), thus allowing for physiological development of neuronal circuitries and neuropeptidergic pathways. Second, *Crhr1* null mutants show severe glucocorticoid deficiency[8,10,14]. Given the potential of corticosteroid hormones to modulate principal eletrophysiological properties of limbic neurons[31], it becomes evident that corticosteroids play important roles in modulating fear and anxiety-related behavior[17]. The mechanisms by which corticosteroids exert their effects on behavior are often indirect, by modulating particular sets of neurons or neurotransmitter systems. CRH-containing neurons of the amygdala, in particular, can be directly modulated by alterations in circulating glucocorticoids through glucocorticoid receptors, which are expressed with Crhr1 in these CRH-containing neurons[32]. The anxiolytic effect observed in conventional *Crhr1* knockout mice may, therefore, result from either *Crhr1* deficiency itself or be influenced by a marked reduction in circulating glucocorticoid hormone levels in these animals, as disruption of glucocorticoid receptor signaling in the central nervous system also has been shown to reduce anxiety-related behavior in mice[18].

In *Crhr1*$^{loxP/loxP}$*Camk2a-cre* conditional mutants, in contrast, plasma ACTH and corticosterone levels under basal conditions are similar to wildtype levels. We can, therefore rule out the possibility that the behavioral phenotype of conditional *Crhr1* mutants might have been influenced by central nervous system effects of circulating stress hormones. We conclude that limbic *Crhr1* is crucial for modulating anxiety-related behavior, and that this anxiolytic effect occurs independent of circulating stress hormone levels.

Limbic Crhr1 is required for central control of stress adaptation

To further assess in detail whether limbic *Crhr1* plays a role in modulating stress-induced activation or the recovery phase of the HPA response, we determined plasma ACTH and corticosterone levels following different durations of restraint stress as well as 30 or 90 min. following a 5 min. period of acute immobilization stress. There was no significant difference in the time course of stress hormone activation between the genotypes in both males and females. However, plasma ACTH and corticosterone levels remained significantly elevated in *Crhr1*$^{loxP/loxP}$*Camk2a-cre* conditional mutants 30 and 90 min after a short period of acute immobilization stress (**Figs. 4** and **5**). These data provide the first evidence that limbic *Crhr1* is required for central control of HPA-system feedback and hormonal adaptation to stress. The importance of maintaining stress-induced HPA system activation and glucocorticoid secretion within tolerable limits requires efficient mechanisms for feedback inhibition. Lack of control and persistent uncertainty in prolonged stressful situations can lead to a chronic state of 'distress,' resulting in chronically elevated levels of circulating corticosteroid hormones. This is believed to enhance vulnerability to a variety of diseases (for review, see ref. 33). This feedback inhibition is accomplished by multiple neuroanatomical pathways, involving direct glucocorticoid negative feedback on hypothalamic parvocellular neurons, but also a number of indirect mechanisms. A key inhibitory role for the hippocampus in HPA-system regulation is supported by lesion studies, which indicate that hippocampal damage potentiates stress-induced glucocorticoid secretion in rats and primates[34,35]. Hippocampal fibers reach the PVN either through the medial hippocampal tract, or by fibers relayed in the lateral septum. Hippocampal mineralocorticoid receptors (MR), in particular, are known to exert a tonic inhibitory influence on stress hormone regulation, restraining HPA system activity indirectly via stimulation of inhibitory interneurons (for review, see ref. 36).

Indirect evidence linking CRH to stress-associated regulation of limbic MRs comes from a recent study in which CRH was identified as an important regulator of hippocampal MR expression[37]. Our endocrine data clearly support an involvement of the CRH/*Crhr1* signaling pathway in mediating central control of HPA system feedback. To further investigate the functional interaction between hippocampal Crhr1 and MR, we performed *in situ* hybridization analyses for MR mRNA both under basal conditions and following stress. MR mRNA expression was significantly activated following stress in wildtype animals. In contrast, in mutants, no stress-induced increase in MR mRNA expression could be observed in any of the hippocampal subfields. Our data, therefore, provide genetic evidence for a functional interaction between the limbic CRH/*Crhr1* pathway and MR regulation. CRH mRNA expression in the hypothalamic paraventricular nucleus was slightly elevated under basal conditions in the conditional mutants, providing further evidence for an impaired central negative feedback on HPA-system activity in these animals. Human postmortem studies show an increased expression of hypothalamic CRH mRNA in depressed patients[38,39], as well as a significant reduction in hippocampal MR mRNA in suicide victims[40]. These findings, together with data from numerous clinical studies, suggest that impaired central corticosteroid receptor feedback mechanisms might have a causal role in the pathophysiology of affective disorders (for review, see ref. 41).

In summary, conditional inactivation of *Crhr1* showed that limbic *Crhr1* neuronal circuitries mediate anxiety-related behavior, and that this anxiolytic effect is independent of HPA-system activity. Our results, therefore, genetically distinguish CRH/*Crhr1* neuronal pathways modulating behavior from those regulating neuroendocrine function. Moreover, we provide the first genetic evidence that limbic Crhr1 is required for proper feedback control of the HPA system and hormonal adaptation to stress. Finally, our data underline the importance of limbic CRH/CRHR1 neuronal pathways as a promising pharmacological target for the treatment of human affective disorders such as major depression[2] or the consequences of early-life stress[3–5].

METHODS

Targeting vector. A previously identified 35.8-kb cosmid clone was used to construct a targeting vector[8]. As a 5′-homology arm, a 3.1-kb *Xho*I/*Hind*III *lox*P fragment containing exons 9–13 of the *Crhr1* gene was inserted in front of the *lox*P site of pKSloxPNT (ref. 42). This homology arm was extended by a 5.4-kb *Spe*I/*Xho*I fragment encompassing exons 5–8, which was inserted between the thymidine-kinase cassette and the first *lox*P site. In addition, 3′ to the second *lox*P site, a 6.85-kb *Hind*III/*Bgl*II fragment from PGT-3/PT-1 (ref. 43) was introduced. This fragment contained the engrailed 2 splice-acceptor site with some exon sequence fused in-frame to a β-galactosidase with its own polyadenylation signal and a PGK-neomycin cassette, which was followed by the 3′-homology arm encompassing the 2.9–kb *Hind*III/*Bam*HI fragment located downstream of exon 13 and of the polyadenylation site.

Generation of conditional mutants. TBV2 (129/SvP) ES cells were electroporated with the targeting vector, and mutant ES cells were identified by genomic Southern blot as described previously[8]. Mutant ES cells were used to generate chimeric mice by blastocyst injection. Chimeras were bred with C57BL/6 mice to obtain F1 offspring, and germ-line transmission of the mutant allele was determined by Southern blot analysis using the 3′ external probe after *Hind*III digestion (data not shown). For the conditional inactivation of *Crhr1* in the limbic system, we used transgenic mice carrying the gene encoding Cre recombinase under the control of the *Camk2a-cre* promoter[20]. The deletion pattern and efficiency of *Camk2a-cre* was evaluated using Z/AP reporter mice[24]. To generate *Crhr1*$^{loxP/loxP}$*Camk2a-cre* and *Crhr1*$^{loxP/loxP}$ control mice, we crossed

mice harboring a *Crhr1*^{loxP} allele with *Camk2a-cre* mice. Mice used for this study were kept on a mixed 129/Sv × C57BL/6 background. Genotyping was performed by Southern blot analysis of *Xba*I-digested tail DNA using an internal *Crhr1* probe and a Cre recombinase–specific probe.

Histological analysis, immunohistochemistry and *in situ* hybridization. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Bavaria, Germany. Animals (n = 4–5 per genotype) were deeply anesthetized and transcardially perfused with 4% paraformaldehyde. hPLAP stainings were performed as previously described[24]. Immunohistochemistry for Cre was carried out as previously described, using an anti-Cre antibody (developed by G. Schütz, German Cancer Research Center). For the analysis of basal and stress-induced CRH and MR mRNA, male animals were housed singly. Five animals per genotype were either killed under basal conditions or subjected to a 30-min restraint stress and killed 3 h later (between 08:00 and 11:30). *In situ* hybridization was performed as described[44] using cRNA probes transcribed from plasmids containing fragments of cDNAs encoding mouse *Crh*, *Crhr1* and rat MR. Semiquantitative analysis of mRNA levels was performed blind to genotype using NIH Image (http://rsb.info.nih.gov/nih-image/). At least three serial tissue sections per animal and region were analyzed. In the case of MR mRNA, CA1, CA3 and the granule cell layer of the dentate gyrus were analyzed separately.

Behavioral studies. Male mice (3–5 months) were housed individually. All behavioral studies were performed during the light period (09:00–13:00; lights on 07:00–19:00). Exploratory behavior was assessed in a light-dark box (dark compartment 15 × 20 × 25 cm, light compartment 30 × 20 × 25 cm, 650 lux, connected by a 4-cm long tunnel). For the assessment of anxiety-related behavior in the light-dark box, animals (n = 20–21 per group) were placed in the dark compartment, and we recorded the time spent in each compartment and the number of entries into each compartment for 5 min. The elevated plus-maze comprises two opposing open arms (30 × 5 × 0.5 cm) and two opposing enclosed arms (30 × 5 × 15 cm) that are connected by a central platform (5 × 5 cm), forming the shape of a plus sign[27]. The results were calculated as mean ratios of the time spent in, or entries made into, the open arms to the total time spent in both open and closed arms. Further parameters scored were total number of head dips, head dips on the protected and unprotected arms, stretched attend postures on the protected and unprotected arms, rearings and end exploration of the open arms. General locomotor activity was assessed in an open field (30 × 30 × 40 cm, 20 lux). Locomotor activity was recorded for 30 min by a video tracking system (TSE).

Endocrine analyses. Two weeks before the experiments, animals (age 3–5 months) were separated and housed singly to avoid uncontrolled stress reactions. All experiments and data analyses were performed separately for male and female animals. Only data from male mice are presented here; data from female animals are in **Supplementary Fig. 1** online. To determine the basal hormone plasma levels, mice (n = 5–10 per genotype) were left undisturbed throughout the night before the experiment. Blood sampling was performed in the early morning (07:00–08:00) and afternoon (17:00–18:00) by rapid retro-orbital bleeding, with the time from first handling of the animal to completion of bleeding not exceeding 45 s. For evaluation of the endocrine response to stress, we collected blood samples from the same animals immediately after 2, 5 or 10 min of restraint stress for which animals were placed in a 50-ml conical tube (plastic conical tube with the bottom removed). For evaluation of HPA system feedback, we bled the animals at 30 or 90 min after a 5-min restraint stress period. Stress experiments were performed in the morning (08:00–10:00), and the interval between the different experiments was 2 weeks. Plasma corticosterone and ACTH concentrations were measured in duplicate by commercially available radioimmune assay kits (ICN Biomedicals).

Statistics. Behavioral parameters were analyzed by an one-factor multivariate analysis of variance (MANOVA) with genotype as the between-subjects factor. Plasma concentrations of corticosterone and ACTH at baseline and after restraint stress were analyzed by two-factorial MANOVAs with two different repeated-measures designs: in the first design, the within-subjects factor 'treatment' had four levels (baseline and measurements immediately after 2-, 5- and 10-min restraint stress); in the second design, the within-subjects factor 'treatment' had three levels (measurements immediately after and 30 or 90 min after a 5-min restraint stress). Hippocampal MR and CRH mRNA expression was analyzed by a two-factor ANOVA with 'genotype' and 'treatment' as between-subjects factors with two levels each. If significant factor effects in the MANOVAs were detected, univariate F-tests were used to identify the parameters (variables) that contributed significantly to these effects. In addition, tests with contrasts were performed to test for significant pairwise differences in the factor levels. Behavioral and endocrine parameters were transformed with the $y = \ln(x + c)$ transformation before analysis of variance for approximating normality and homogeneity for this analysis. A nominal level of significance, $\alpha = 0.05$, was accepted and corrected (Bonferroni adjustment) for all *post hoc* tests (univariate F-tests and tests with contrasts) to keep the type-I error ≤0.05. Results are presented as means + s.e.m.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTEREST STATEMENT

The authors declare that they have no competing financial interests.

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