oscillator that supports movement of MZ B cells between MZ and follicle (fig. S6). A general implication of these findings is that GRK2 antagonists may suppress lymphocyte migration from blood into tissue and thus could have therapeutic potential as immunosuppressants.

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### Supporting Online Material

www.sciencemag.org/cgi/content/full/333/6051/1898/DC1
Materials and Methods
Figs. S1 to S6
References (*30*–*36*)

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# Glutamatergic and Dopaminergic Neurons Mediate Anxiogenic and Anxiolytic Effects of CRHR1

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The corticotropin-releasing hormone receptor 1 (CRHR1) critically controls behavioral adaptation to stress and is causally linked to emotional disorders. Using neurochemical and genetic tools, we determined that CRHR1 is expressed in forebrain glutamatergic and γ-aminobutyric acid–containing (GABAergic) neurons as well as in midbrain dopaminergic neurons. Via specific CRHR1 deletions in glutamatergic, GABAergic, dopaminergic, and serotonergic cells, we found that the lack of CRHR1 in forebrain glutamatergic circuits reduces anxiety and impairs neurotransmission in the amygdala and hippocampus. Selective deletion of CRHR1 in midbrain dopaminergic neurons increases anxiety-like behavior and reduces dopamine release in the prefrontal cortex. These results define a bidirectional model for the role of CRHR1 in anxiety and suggest that an imbalance between CRHR1-controlled anxiogenic glutamatergic and anxiolytic dopaminergic systems might lead to emotional disorders.

Corticotropin-releasing hormone (CRH) and its type 1 high-affinity receptor (CRHR1) are widely distributed throughout the brain (*1*, *2*). Together they orchestrate the neuroendocrine and behavioral adaptation to stress (*3*–*5*). Chronic stress-associated hyperfunction of the CRH/CRHR1 system has been implicated in the onset of mood and anxiety disorders (*3*–*5*). Mutant CRHR1 mice have provided crucial information in this regard. Constitutive CRHR1 knockout (KO, *Crhr1*$^{KO}$) mice show reduced anxiety-related behavior (*6*, *7*), and a similar phenotype has been observed in *Crhr1*$^{Camk2aCre}$ (Camk2aCre, Cre driven by the calcium/calmodulin-dependent protein kinase type II alpha chain promoter) conditional KO (CKO) mice lacking the CRHR1 in all principal neurons of the forebrain (*8*). Nevertheless, two fundamental questions are still unsolved. First: On which kind of neurons is CRHR1 expressed? Second: Which are the underlying neurotransmitter (NT) circuits controlled by CRH that modulate anxiety-like behavior?

Regarding the expression analysis, this gap of knowledge can be ascribed to (i) the low expression levels of CRHR1 that challenge its detection by double in situ hybridization (ISH) approaches and (ii) the apparent (but never systematically tested) lack of reliable antibodies to CRHR1. Moreover, available CRHR1 KO mice are limited in their value to be used as appropriate negative controls [see the supporting online material (SOM) and fig. S1] because they might express truncated versions of the receptor.

We therefore generated a new CRHR1 knockin mouse line (*Crhr1*$^{\Delta Egfp}$) (EGFP, enhanced green fluorescent protein), which on the one hand reports CRHR1 expression via GFP and on the other hand fully abolishes the *Crhr1* transcript (figs. S1 and S2). By using cell cultures and *Crhr1*$^{\Delta EGFP}$, mice we demonstrated that seven antibodies to CRHR1 tested were inappropriate to reliably detect CRHR1 (SOM and fig. S3). Therefore, we established a sensitive double ISH method (SOM and table S1). We found that *Crhr1* mRNA was present in glutamatergic (Glu) neurons of the cortex and hippocampus (Fig. 1, A and B); in γ-aminobutyric acid–containing (GABAergic) neurons of the reticular thalamic nucleus (RTN), globus pallidus (GP), and septum (Fig. 1B and fig. S4); and in dopaminergic (DA) neurons of the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) (Fig. 1C). Just a very few serotonergic [5-hydroxytryptamine (5-HT)] neurons of the dorsal and median raphe nuclei expressed *Crhr1* (Fig. 1D).

This expression pattern was entirely recapitulated by applying double immunohistochemistry using antibodies raised against neuronal identity markers and against GFP in *Crhr1*$^{\Delta Egfp}$ mice (Fig. 1, A to D). These results offer a systematic neurochemical map of CRHR1 expression in different NT systems.

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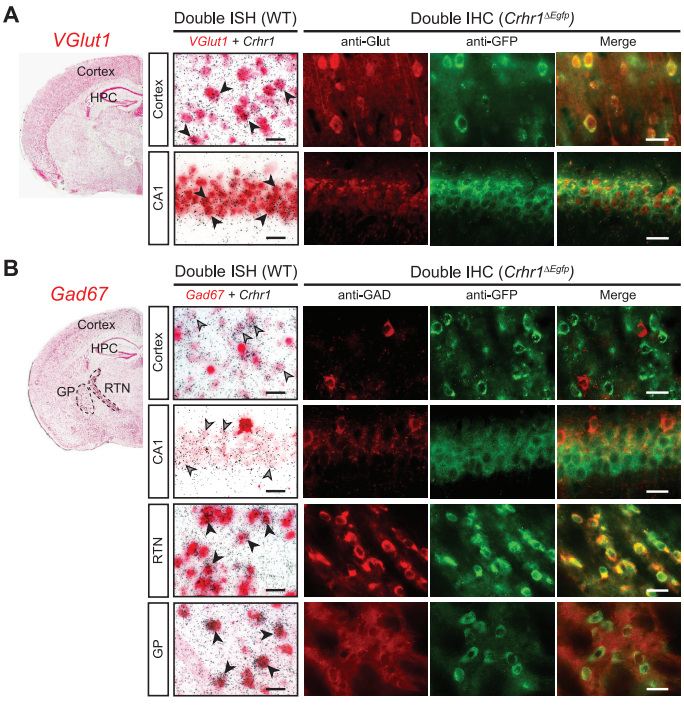


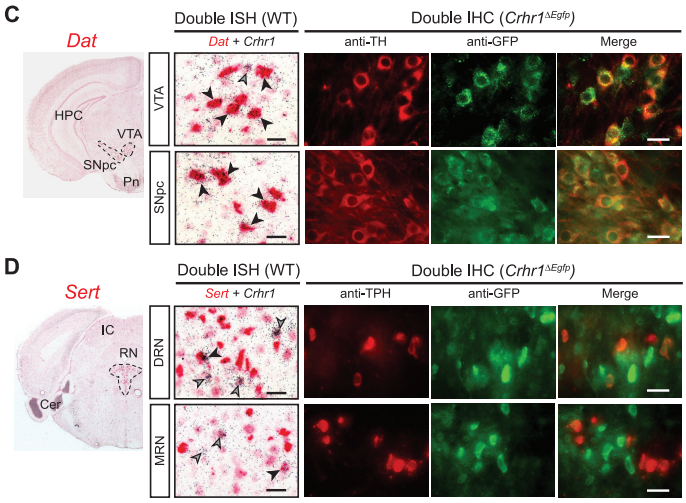
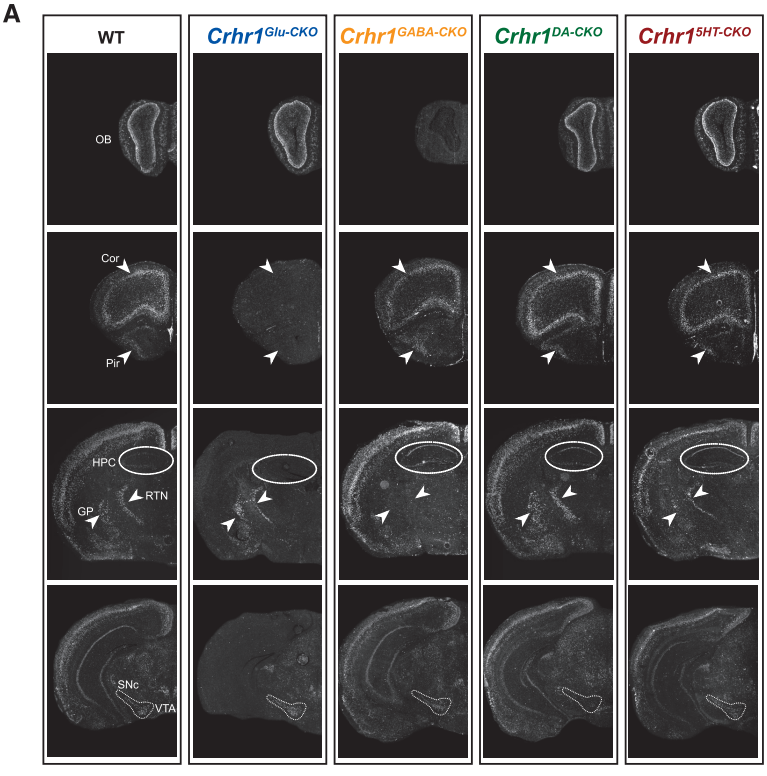
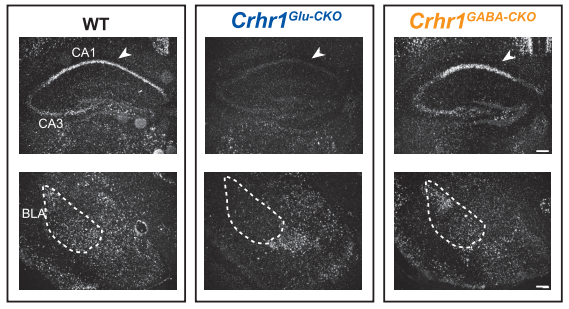




**Fig. 1.** CRHR1 is expressed in neurons of different neurotransmitter systems. CRHR1 was colocalized with neurotransmitter-specific markers by double ISH and double immunohistochemistry (IHC) using wild-type and *Crhr1*$^{\Delta EGFP}$ mice respectively. (**A**) In the cortex and hippocampus (HPC), CRHR1 is expressed in glutamatergic (VGlut1, glutamate) neurons. (**B**) CRHR1 is not expressed in GABAergic (GAD67) neurons of the cortex or of the hippocampus but in GABAergic neurons of the RTN and the GP. (**C**) CRHR1 is expressed in dopaminergic (Dat, TH) neurons of the VTA and the SNpc. (**D**) CRHR1 is scarcely expressed in serotonergic (Sert, TPH) neurons of the dorsal (DRN) and median raphe (MRN). Gray arrowheads indicate cells expressing only CRHR1 (silver grains). Black arrowheads indicate cells coexpressing CRHR1 and the respective markers (red staining).



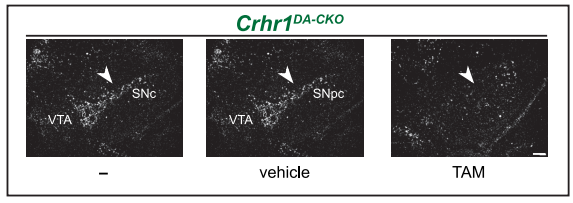




**Fig. 2.** Neurotransmitter-specific *Crhr1*-CKO lines lack *Crhr1* expression in a cell type–specific manner. Expression of *Crhr1* mRNA was assessed by ISH in wild-type and neurotransmitter-specific *Crhr1*-CKO lines. (**A**) Dark-field photomicrographs of *Crhr1* mRNA expression pattern in brain sections of wild-type, *Crhr1*$^{Glu\text{-}CKO}$, *Crhr1*$^{GABA\text{-}CKO}$, *Crhr1*$^{DA\text{-}CKO}$, and *Crhr1*$^{5HT\text{-}CKO}$ mice. Areas of interest are highlighted with arrowheads and dashed lines. OB, olfactory bulb; Cor, cortex; Pir, piriform cortex; Scale bar, 500 μm. (**B**) Higher-magnification dark-field photomicrographs indicate a specific lack of *Crhr1* expression in the HPC and BLA of *Crhr1*$^{Glu\text{-}CKO}$ mice. Scale bar, 200 μm. (**C**) Normal *Crhr1* mRNA expression in the VTA/SNpc is not affected by vehicle injections but is completely absent 3 weeks after tamoxifen (TAM) administration in the *Crhr1*$^{DA\text{-}CKO}$ line carrying an inducible Cre-ERT2. Scale bar, 200 μm.

Central administration of CRHR1 antagonists elicits anxiolytic responses (*3*, *5*). Accordingly, constitutive $Crhr1^{KO}$ and forebrain-restricted $Crhr1^{CamK2aCre}$ CKO mice exhibit reduced anxiety-like behavior (*6*–*8*). However, neither region-specific KO mice nor direct infusion of CRHR1 agonists or antagonists can dissect CRHR1 functions on neurotransmitter-specific neuronal subpopulations.

We thus used conditional mutagenesis to genetically dissect the specific involvement of CRHR1 in distinct neuronal populations. We crossed $Crhr1^{flox/flox}$ mice with *Nex-Cre*, *Dlx5/6-Cre*, *ePet-Cre*, and *Dat-CreERT2* mice to generate, respectively, the following lines: $Crhr1^{Glu\text{-}CKO}$, where *Crhr1* is deleted in forebrain glutamatergic neurons; $Crhr1^{GABA\text{-}CKO}$, carrying a *Crhr1* deletion in forebrain GABAergic neurons; $Crhr1^{DA\text{-}CKO}$, lacking *Crhr1* in midbrain DA neurons; and $Crhr1^{5HT\text{-}CKO}$, with a *Crhr1* deletion in brainstem serotonergic neurons.

The pattern of *Crhr1* deletion in all CKO lines perfectly mirrored the expression maps traced with the histochemical mapping (Fig. 2, fig. S5, and table S2) and underscores the extraordinarily selective neurotransmitter type–specific deletion properties of these animals.

To functionally dissect the neuronal subpopulations mediating the effects of the CRH/CRHR1 system on emotional behavior, neurotransmitter type–specific CKO mice were subjected to a series of tests to assess anxiety-like behavior, locomotion, forced swimming behavior, and fear memory.

In the dark-light box test, $Crhr1^{Glu\text{-}CKO}$ mice showed reduced anxiety-like behavior as compared to control littermates ($Crhr1^{Glu\text{-}Ctrl}$), which is depicted in an increase in lit compartment time and number of entries (Fig. 3A). $Crhr1^{Glu\text{-}CKO}$ mice also showed a reduced latency to enter the lit compartment ($Crhr1^{Glu\text{-}CKO}$ = 42.4 ± 6 s versus $Crhr1^{Glu\text{-}Ctrl}$ = 108.2 ± 21 s; Mann-Whitney *U* test, $U = 32.00$; $P < 0.01$). The low-anxiety phenotype of $Crhr1^{Glu\text{-}CKO}$ mice was confirmed in three additional tests assessing anxiety-like behavior (fig. S6). Along these lines, $Crh\text{-}COE^{CamCreERT2}$ mice, in which limbic CRH overexpression is induced in adulthood, showed increased anxiety-like behavior (fig. S7). These results mirror the low anxiety phenotype previously found in $Crhr1^{Camk2aCre}$ mice (*8*), in which Cre-mediated deletion of *Crhr1* expression starts after the second week of postnatal life (fig. S8). These results suggest that manipulation of the CRH/CRHR1 system during adulthood is responsible for the behavioral changes observed in $Crhr1^{Glu\text{-}CKO}$ mice.

No changes in anxiety-related behavior were observed in $Crhr1^{GABA\text{-}CKO}$ mice (Fig. 3B). Similarly $Crhr1^{5HT\text{-}CKO}$ mice did not show any phenotype with respect to anxiety-related behavior (Fig. 3D). This indicates that the reported interactions between the CRH and 5-HT systems (*9*, *10*) are not directly exerted by CRHR1 on serotonergic neurons but take place at the postsynaptic level on target neurons where CRHR1 and 5-HT receptors are coexpressed (*10*). An increased anxiety-like phenotype was observed in $Crhr1^{DA\text{-}CKO}$ mice in the dark-light box (Fig. 3C), the elevated plus maze, the novel object exploration, and the modified hole board tests (fig. S6). Besides, $Crhr1^{DA\text{-}CKO}$ mice showed decreased locomotion only in the first 5 min of the open field test, suggesting

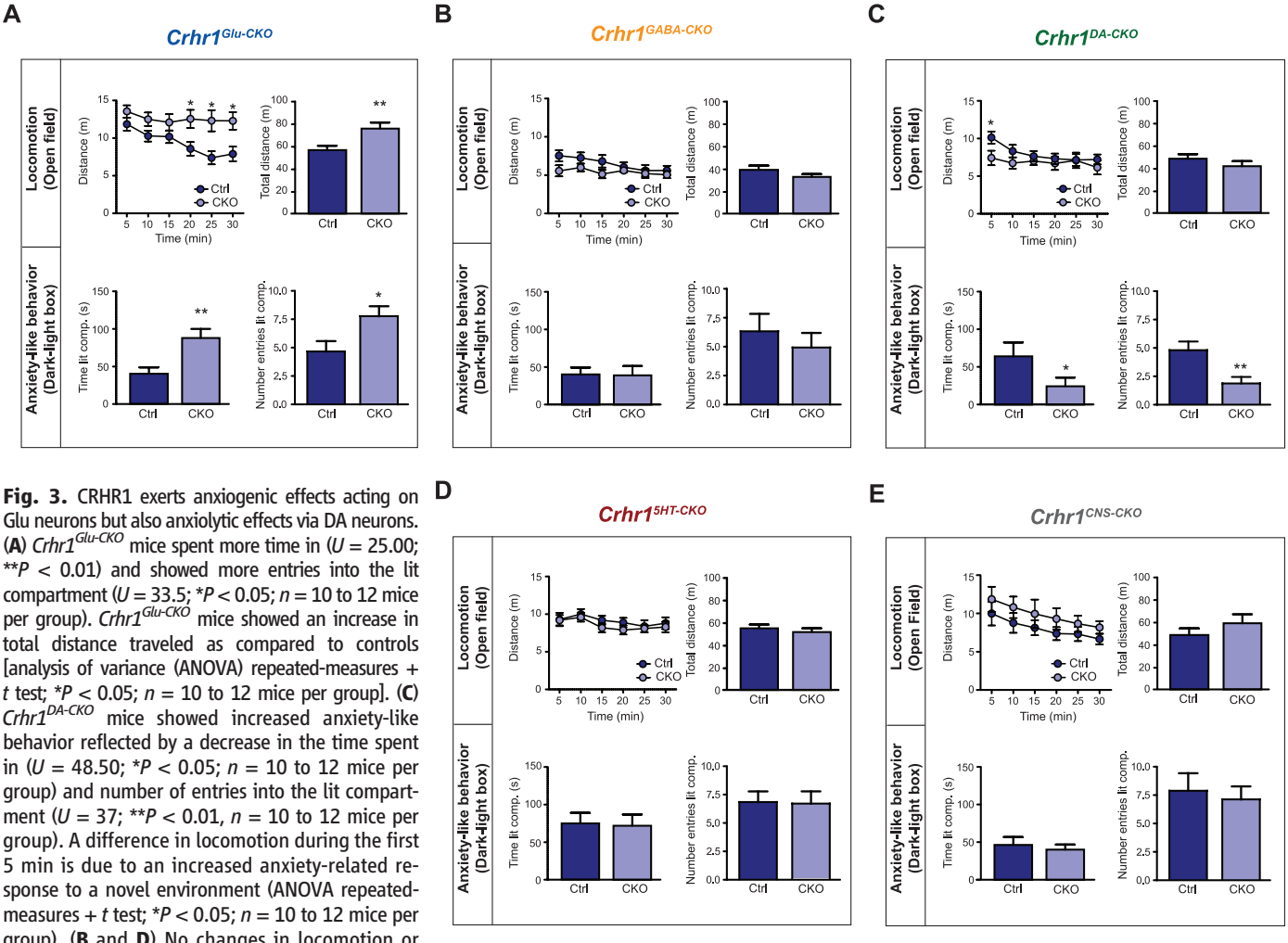



**Fig. 3.** CRHR1 exerts anxiogenic effects acting on Glu neurons but also anxiolytic effects via DA neurons. (**A**) $Crhr1^{Glu\text{-}CKO}$ mice spent more time in ($U = 25.00$; $^{**}P < 0.01$) and showed more entries into the lit compartment ($U = 33.5$; $^{*}P < 0.05$; $n = 10$ to 12 mice per group). $Crhr1^{Glu\text{-}CKO}$ mice showed an increase in total distance traveled as compared to controls [analysis of variance (ANOVA) repeated-measures + *t* test; $^{*}P < 0.05$; $n = 10$ to 12 mice per group]. (**C**) $Crhr1^{DA\text{-}CKO}$ mice showed increased anxiety-like behavior reflected by a decrease in the time spent in ($U = 48.50$; $^{*}P < 0.05$; $n = 10$ to 12 mice per group) and number of entries into the lit compartment ($U = 37$; $^{**}P < 0.01$, $n = 10$ to 12 mice per group). A difference in locomotion during the first 5 min is due to an increased anxiety-related response to a novel environment (ANOVA repeated-measures + *t* test; $^{*}P < 0.05$; $n = 10$ to 12 mice per group). (**B** and **D**) No changes in locomotion or anxiety-related behavior were observed in $Crhr1^{GABA\text{-}CKO}$ or $Crhr1^{5HT\text{-}CKO}$ mice. (**E**) Locomotion and anxiety-related behavior were unchanged between $Crhr1^{CNS\text{-}CKO}$ and $Crhr1^{CNS\text{-}Ctrl}$ animals.

increased novelty-induced anxiety-like behavior (Fig. 3C). These results describe a defined anxiolytic effect of CRHR1, which coexists with its anxiogenic properties and was probably masked by the inability of the genetic and pharmacological tools used up to now to inactivate the receptor in specific types of neurons.

No changes in the total distance traveled were observed in the mutant lines except for *Crhr1*$^{Glu\text{-}CKO}$, mice which showed increased locomotor activity (Fig. 3). Nevertheless, this does not represent a confounding factor for interpreting their anxiety-like behavior, because it manifested 20 min after the test started. Anxiety-like behavior was evaluated during the first 5 min after exposing the mice to the new test environment (except for the novel object exploration test, which lasted 15 min).

In line with previous studies using *Crhr1* mutant lines (*8*, *11*, *12*), behavior in the forced swim test was not affected in any of the *Crhr1*-CKO lines investigated (fig. S9). An influence of corticosteroids on the behavioral differences observed (*3*) can be excluded, because none of the mutant lines showed alterations in either basal or stress-induced corticosterone levels (fig. S10). Conventional *Crhr1* null mutant mice do not exhibit alterations in fear conditioning (*13*), but the low glucocorticoid levels of this mutant line might obscure the interpretation of those results (*6*, *7*). Behavioral analysis of tone-dependent fear conditioning failed to demonstrate any significant difference between NT-selective *Crhr1*-CKOs and their controls (fig. S11), further highlighting the specific role of CRHR1 in anxiety.

The fact that *Crhr1*$^{Glu\text{-}CKO}$ was the only mouse line tested that showed less anxiety-like behavior clearly points toward the central role of Glu neurotransmission in stress-induced anxiety. In addition, *Crhr1*$^{Glu\text{-}CKO}$ mice lack CRHR1 expression in the hippocampus and amygdala, two critical limbic regions in the neurobiology of mood disorders (*14*–*16*). Hence, these animals should also show impairments in CRH-induced changes on excitatory neurotransmission in these limbic structures. Indeed, a comparison of CRH effects on evoked field potentials (FPs) in the basolateral amygdala (BLA) of *Crhr1*$^{Glu\text{-}CKO}$ mice versus control mice revealed that CRH increases excitatory FPs via CRHR1 on glutamatergic neurons (Fig. 4A).

Single-cell physiology studies or FP recordings are very valuable tools to address particular molecular events occurring during neurotransmission in single synapses. However, they do not provide any information on the dynamics of neuronal networks, which might be a closer neurophysiological correlate of behavior (*17*). Using voltage-sensitive dye imaging (VSDI), changes in hippocampal neuronal dynamics have been linked to behavior in an animal model of depression (*18*). We developed a VSDI assay which allows the investigation of neuronal activity propagation through the entire hippocampal formation (HF) in brain slices (Fig. 4, B and C) (*19*). We found that CRH was enhancing neuronal activity propagation from the classical hippocampal input region (dentate gyrus, DG) to the CA1 output area, in slices from *Crhr1*$^{Glu\text{-}Ctrl}$ mice but not from *Crhr1*$^{Glu\text{-}CKO}$ mice (Fig. 4, D and E). This effect of CRH was not due to increased neuronal excitation within the DG-input region but to an amplification of neuronal excitation on its passage through the HF, a phenomenon that was completely abolished in slices from *Crhr1*$^{Glu\text{-}CKO}$ mice (Fig. 4, E and F). These results are in line with previous studies showing that CRH, more than affecting synaptic efficacy or strength, facilitates action potential firing (*20*–*22*). Thus we conclude that the activation of CRHR1 (as it would take place in response to stressors) specifically modulates glutamatergic neurotransmission, producing an amplification of neuronal excitation in the DG-CA3-CA1 network.

The interaction between CRH and DA circuits has been extensively studied in the context of addiction (*23*–*25*), but its role in emotional behavior is far from clear. The prefrontal cortex (PFC) is at the same time a critical structure in

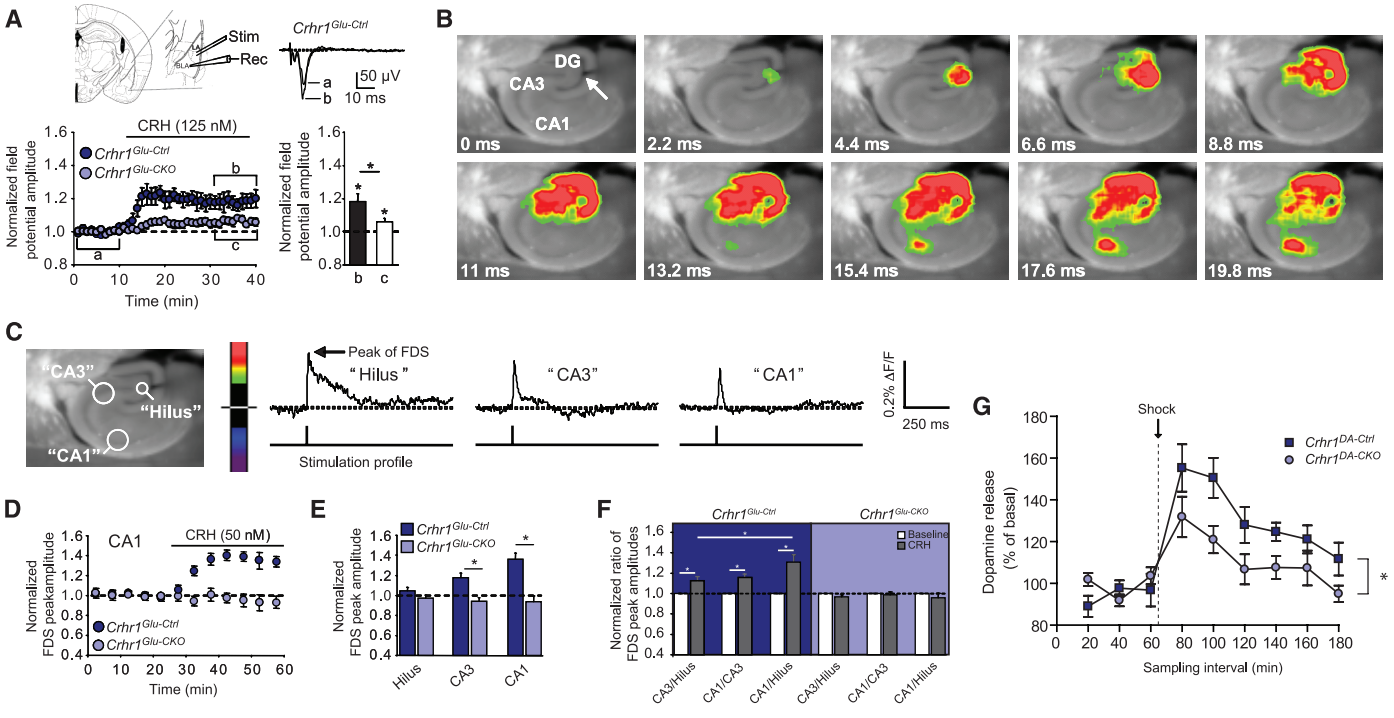



**Fig. 4.** CRHR1 in Glu neurons facilitates excitatory neurotransmission in the amygdala and activity propagation in the HF, whereas CRHR1 in DA cells controls dopamine release in the prefrontal cortex. (**A**) CRH increases evoked FPs in the BLA via CRHR1 on Glu neurons ($n$ = 14 slices from 7 animals per group, $t$ test, *$P < 0.05$). (**B**) Representative filmstrip depicting the propagation of a DG-evoked VSDI signal through the HF. (**C**) Illustration of ROIs used for the calculation of neuronal population activity. The peak amplitude of the fast, depolarization-mediated, VSDI signal (FDS) was used as a quantifier of neuronal population activity. (**D** to **F**) CRH enhances neuronal activity propagation via CRHR1 on Glu neurons by amplifying neuronal excitation on its passage through the HF. (D) Time courses of experiments depicted for CA1 ($n$ = 7 slices from 5 animals per group). (E) Quantification of CRH effects. (F) Ratios of FDS peak amplitudes normalized to the respective ratios under baseline conditions. (**G**) Effect of footshock stress on DA release in the PFC of *Crhr1*$^{DA\text{-}CKO}$ versus *Crhr1*$^{DA\text{-}Ctrl}$ mice. ANOVA with repeated measures revealed a significant effect for the factor sampling interval ($F_{6,8} = 6.19$, *$P < 0.05$) as well as for genotype ($F_{1,13} = 4.71$, *$P < 0.05$).

circuits controlling anxiety (*15*) and the main target of mesocortical DA neurons. The pattern of *Crhr1* deletion in $Crhr1^{DA\text{-}CKO}$ animals indicates that CRHR1 in the VTA/SNpc is present in DA neurons, and indeed CRH increases the action potential firing rate in VTA neurons via CRHR1 (*26*). Using in vivo microdialysis, we observed that $Crhr1^{DA\text{-}CKO}$ mice display a decreased response to stress-induced dopamine release in the PFC as compared to littermate controls (Fig. 4G), indicating that CRHR1 targets DA cells to control PFC dopamine release under stress conditions.

Recent findings point toward a role of the DA system in emotional disorders (*27*). However, the precise role of dopamine and the underlying mechanisms have not been elucidated yet. The most plausible reason why is that specific subpopulations of DA neurons, which are physically intermingled in the VTA/SNpc (*28*, *29*), play different roles, and as such cannot be functionally dissected with classical pharmacological compounds, which block receptors irrespective of the type of neurons. Our findings, based on more defined genetic tools, certainly support an anxiolytic role for precisely the subpopulation of DA neurons expressing CRHR1. In fact, mesencephalic DA neurons are less homogeneous than thought before (*28*), and two distinct types of DA neurons in the VTA/SNpc differentially respond to aversive stimuli (*29*). Hence, the CRHR1-positive DA cells controlling anxiolysis might represent a subset of DA neurons, and the identification of their postsynaptic targets will be mandatory in the future.

The observed dual role of CRHR1 suggests that under physiological conditions, CRH/CRHR1-controlled Glu and DA systems might function in a concerted but antagonistic manner to keep adaptive anxiety responses to stressful situations in balance. The fact that $Crhr1^{CNS\text{-}CKO}$ animals ($Crhr1^{flox/flox} \times Nestin^{Cre}$) carrying deletions in both neurotransmitter systems in parallel do not show alterations in anxiety-like behavior (Fig. 3E) supports this notion. These results foster the hypothesis that the CRH hyperactivity, present in many patients suffering from emotional disorders (*3*–*5*), might not be general but restricted to particular neuronal circuits, triggering symptoms by generating an imbalance between CRHR1-controlled glutamatergic and dopaminergic neuronal circuits involved in emotional behavior.

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### Supporting Online Material

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References


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