

ORIGINAL ARTICLE

Bidirectional rescue of extreme genetic predispositions to anxiety: impact of CRH receptor 1 as epigenetic plasticity gene in the amygdala

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The continuum of physiological anxiety up to psychopathology is not merely dependent on genes, but is orchestrated by the interplay of genetic predisposition, gene x environment and epigenetic interactions. Accordingly, inborn anxiety is considered a polygenic, multifactorial trait, likely to be shaped by environmentally driven plasticity at the genomic level. We here took advantage of the extreme genetic predisposition of the selectively bred high (HAB) and low anxiety (LAB) mouse model exhibiting high vs low anxiety-related behavior and tested whether and how beneficial (enriched environment) vs detrimental (chronic mild stress) environmental manipulations are capable of rescuing phenotypes from both ends of the anxiety continuum. We provide evidence that (i) even inborn and seemingly rigid behavioral and neuroendocrine phenotypes can bidirectionally be rescued by appropriate environmental stimuli, (ii) corticotropin-releasing hormone receptor 1 (*Crhr1*), critically involved in trait anxiety, shows bidirectional alterations in its expression in the basolateral amygdala (BLA) upon environmental stimulation, (iii) these alterations are linked to an increased methylation status of its promoter and, finally, (iv) binding of the transcription factor Yin Yang 1 (YY1) to the *Crhr1* promoter contributes to its gene expression in a methylation-sensitive manner. Thus, *Crhr1* in the BLA is critically involved as plasticity gene in the bidirectional epigenetic rescue of extremes in trait anxiety.

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INTRODUCTION

Inborn anxiety is considered a polygenic, multifactorial trait whose continuum of physiological anxiety up to psychopathology is likely to be shaped by environmentally driven plasticity at the genomic level.¹ Epigenetic processes are capable of integrating environmental signals into the genome^{2,3} and indeed interact with the genetic predisposition of an individual,⁴ thereby creating a new category of disease etiology. Epigenetic mechanisms contribute to pathological alterations in brain regions important for the regulation of anxiety *inter alia* by adding⁵ or removing² methyl groups at CpG dinucleotides within regions denoted as CpG islands (CGi). The concept of plasticity genes⁶ emphasizes that a promising candidate should (i) be amenable to both beneficial and detrimental environmental influences and (ii) reflects the interplay of genetic load and epigenetic processes in the regulation of anxiety along the continuum. Plasticity genes thus represent promising candidates to elucidate how epigenetic processes might shape a (seemingly) rigid genetic predisposition in the development of pathological anxiety—a scenario that has largely been neglected so far.

We here took advantage of a mouse model of extremes in trait anxiety and tested whether environmental manipulation is capable of bidirectionally rescuing the genetically driven anxiety phenotype via epigenetic mechanisms. After >45 generations of selective inbreeding, HAB mice are supposed to accumulate risk

factors of pathological anxiety, thus resembling genetically predisposed psychiatric patients.⁷ Inbred LAB mice represent the other end of the phenotypic continuum. Environmental manipulations such as enriched environment (EE)—a combination of biologically relevant inanimate and social stimuli—and chronic mild stress (CMS) are promising tools to induce anxiolytic and anxiogenic effects, respectively, thereby shifting the anxiety phenotype of HAB and LAB mice bidirectionally toward 'normal'.

Recently, we have shown that differences in amygdala activity is a mechanism behind shifts in anxiety-related behavior after EE and CMS.⁸ *Crhr1*, which is one of the most important mediators of amygdala activity, is a candidate plasticity gene that possibly contributes or even underlies such environmentally driven behavioral and electrophysiological changes in the HAB/LAB model. Both the amygdala and the corticotrophin-releasing hormone system are well known to be involved in the regulation of behavioral and neuroendocrine correlates of anxiety.^{9,10}

We therefore aimed at testing whether even an extreme genetic predisposition toward high (HAB) or low (LAB) trait anxiety may be rescued toward 'normal' anxiety by applying either EE or CMS (Figure 1a). If so, does this bidirectional rescue involve *Crhr1* as a plasticity gene of anxiety whose differential expression profiles might be explained by epigenetic phenomena? Based on comprehensive phenotyping, we succeeded in identifying molecular-genetic mechanisms underlying the bidirectional epigenetic

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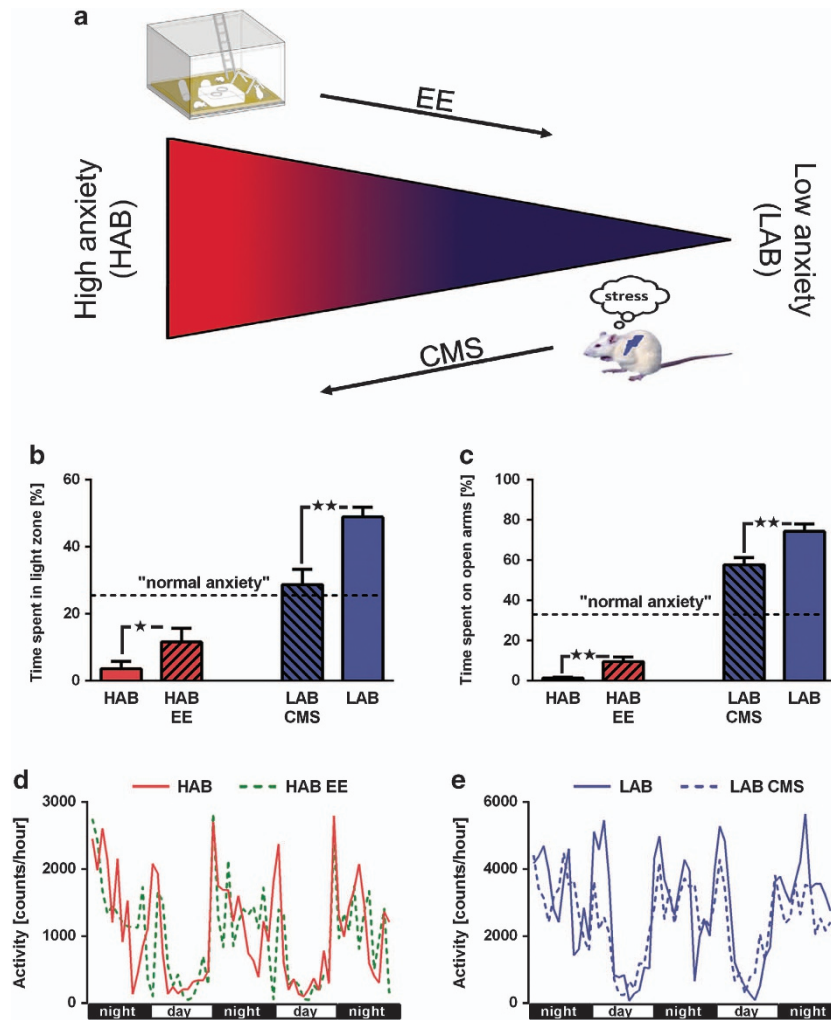


Figure 1. Conceptual framework and behavioral effects of environmental modifications. The triangle reflects the anxiety continuum with HAB and LAB representing its extremes and HAB-EE and LAB-chronic mild stress (CMS) the environmentally driven shifts toward 'normal' (a). 'Normal' anxiety (dashed line) indicates behavior of normal anxiety-related behavior mice (NAB), bred in parallel with LAB and HAB mice, with percent time spent on the open arms of the elevated plus-maze in the 35–45% range. Enriched environment (EE) significantly decreased and CMS increased percent time spent in the light compartment of the light–dark (LD) box (b) and open arms of the elevated plus maze (c) ($n = 8–12$ animals per group). High and low anxiety after environmental manipulations is not associated with decreased or increased home cage locomotion after EE (d) or CMS (e) ($n = 8$ animals per group). Bars represent mean \pm s.e.m. * $P < 0.05$, ** $P < 0.01$ (MWU).

rescue of genetically predisposed anxiety, including methylation of the *Crhr1* promoter and the epigenetic transcription factor YY1.

MATERIALS AND METHODS

Animals

Mice were selectively inbred from a CD-1 outbred population for high, 'normal' (NAB) or low anxiety-related behavior for >45 generations, with percent time spent on open arms as key criterion (HAB < 15%, NAB 35–45%, LAB >60%). They were kept under standard housing conditions and were provided with food and water *ad libitum* (details see in Supplementary Information).

EE and CMS

EE was applied to HAB mice and comprised two periods: from postnatal days 15–28, pups and their respective dam were transferred for 6 h per day to EE, whereas from postnatal days 28 pups were weaned, arranged in groups of three and transferred to EE permanently until postnatal days 42. CMS was applied to LAB mice exactly at the same time period as EE and comprised a series of alternating mild stressors such as maternal

separation, restrained stress, overcrowding and others. Detailed information on EE and CMS is described in the Supplementary Materials (Supplementary Information).

Anxiety-related behavior

To measure anxiety-related behavior, animals were tested in the open field, elevated plus-maze and light–dark (LD) box. For more details see Supplementary Information.

Home cage locomotion

Home cage activity was quantified via an automated system (Inframot; TSE, Bad Homburg, Germany) over a period of two light cycles and three dark cycles (details see in Supplementary Information).

HPA axis reactivity test (HPA-RT)

HPA axis reactivity, that is, increase of blood corticosterone (CORT) after stress, of HABs was evaluated by exposure to 15 min restraint stress. Five-minute forced swimming instead of immobilization stress was used as a stressor for LABs, since the former had regularly been used throughout the

CMS procedure. Blood CORT was compared before and after HPA-RT (details see in Supplementary Information).

Radio immunoassay

Ten microliters of plasma were used to determine the concentration of CORT via a RIA kit from MP Biomedicals (Solon, Ohio, USA) according to the manufacturer's instructions (details see in Supplementary Information).

Bilateral application of a CRHR1 antagonist

Male mice were fixed into a stereotaxic apparatus and anesthetized with an oxygen/Forene mixture. A guide cannula was implanted bilaterally in the basolateral amygdala (BLA) and fixed. Animals were injected three times within an interval of 36 h either with 2 μ l of CRHR1 antagonist (α -helical CRF 9–41, Sigma-Aldrich, Hamburg, Germany) or vehicle (Ringer's solution). To assess anxiety-related behavior, tests were conducted 40 min after injections (details see in Supplementary Information).

Quantitative real-time PCR (qPCR)

Total RNA was extracted from tissue punches collected from BLA or from mouse-neuro-2a (N2a) cells as previously described.¹¹ RNA was reverse transcribed to cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Expression of *Crrh1* and *YY1* was performed using QuantiFast SYBR Green PCR Kit (Qiagen). All samples were analyzed in duplicates and normalized to the housekeeping genes: *Polr2b*, *B2mg* and *Rpl13a* (details see in Supplementary Information).

Identification of CGI, primer design and pyrosequencing

The CGI of *Crrh1* was identified utilizing CpG Island Searcher (default settings, release 29.10.04). DNA for pyrosequencing was extracted from the other half of tissue punch or N2a cells lysate and processed by Varionostic (Ulm, Germany), which performed primer design and pyrosequencing (details see in Supplementary Information).

Construction of a promoter-luciferase reporter

Phusion DNA polymerase (New England Biolabs, Frankfurt, Germany) was used to generate a 1786 bp promoter part from LAB genomic DNA, which had been inserted between the *SpeI* and *HindIII* sites of a pCpG-free luciferase vector (provided by M. Rehli, University Hospital Regensburg, Germany). The plasmid carrying murine *YY1* cDNA was purchased from the DNA Resource CORE (Clone ID: MmCD00311470). All plasmids were sequenced before the reporter assays (details see in Supplementary Information).

In vitro methylation of DNA

Site-specific methylation was performed according to Martinowich *et al.*¹² Modified oligonucleotides were purchased from Sigma-Aldrich. Complete methylation of the *Crrh1* promoter was performed using *SssI* methylase (New England Biolabs) according to the supplier's instructions; a mock-methylated plasmid was treated in the absence of *S*-adenosylmethionine. Products were bisulfite converted (EpiTech Bisulfite Kit, Qiagen, Hilden, Germany) and sequenced to control their respective methylation status (details see in Supplementary Information).

Cell culture, transfection and reporter gene assay

N2a cells were cultured under standard conditions. Cells were plated in 96 well plates, transfected either with a methylated or unmethylated plasmid and pCMV-Gaussia vector as an internal control using Turbofect Transfection reagent (Thermoscientific, Braunschweig, Germany). Cells were lysed after 40 h. Firefly and gaussia luciferase activities were measured as described by Schulke *et al.*¹³ To evaluate if *YY1* exhibits a regulatory role on the *Crrh1* promoter, a control vector (SPORT6) or a vector expressing *YY1* cDNA (*YY1*) was co-transfected with plasmids carrying the *Crrh1* promoter and pCMV-Gaussia. The same approach was used to overexpress *YY1* in the presence of plasmids with modified methylation (more detailed see in Supplementary Information).

Western blotting

To verify that transfection with plasmid carrying *YY1* cDNA indeed induced a higher amount of *YY1* protein, the nuclear fraction was extracted from

transfected cells. Total protein amounts were measured in a BSA assay (Pierce BCA Protein Assay Kit, Thermoscientific, Braunschweig, Germany). Assessment of *YY1* protein was performed via Western Blot using an anti-*YY1* antibody (sc-1703, Santa Cruz Biotechnology, Heidelberg, Germany) (details see in Supplementary Information).

Immunofluorescent assay

To further visualize an increase in the amount of *YY1* protein in transfected cells, plasmid carrying *YY1* cDNA was co-transfected with GFP. Cells were mounted on cover slips, transfected, fixed with 4% paraformaldehyde and incubated overnight with anti-*YY1* antibody (sc-1703, Santa Cruz Biotechnology). Next day, cover slips were washed and stained with secondary antibody (ALEXA Fluor 594, Lifetechnologies, Darmstadt, Germany), followed by a 4',6-diamidino-2-phenylindole solution and finally analyzed by fluorescent microscopy (details see in Supplementary Information).

Electrophoretic mobility shift assay

EMSA probes were end-labeled with ³²P-dCTP (PerkinElmer, Rodgau, Germany). For the EMSA reaction, nuclear extracts from N2a cells were incubated for 5 min in binding buffer. Labeled fragments were diluted to 20 000 c.p.m., thereof 1 μ l was added to the EMSA reaction. Samples were electrophoresed on 6% polyacrylamide gel. Antibodies and a competitor for the shift assay were supplied 30 min before adding labeled oligonucleotides. To check the impact of CpG1 methylation on *YY1* binding affinity, a plasmid carrying the *Crrh1* promoter fragment was incubated with *SssI* methylase as described before. Gels were exposed to radiation-sensitive films (Kodak Biomax MR films, Eastman Kodak, Rochester, NY, USA) for 2 days (for details see Supplementary Information).

Statistics

Data were analyzed using either ANOVA (one-way, two-way or two-way with repeated measures, as appropriate) followed by Tukey's *post hoc* test. Comparisons of two independent groups were analyzed by Mann-Whitney *U* test. Statistical significance was achieved at $P < 0.05$ (for details see Supplementary Information).

RESULTS

Bidirectional rescue of extreme trait anxiety

EE exhibited a significant anxiolytic and CMS an anxiogenic effect in HAB and LAB mice, respectively (Figure 1b and c). While EE significantly increased percent time spent on the open arms of the elevated plus maze ($P < 0.01$) and in the light compartment of the LD ($P < 0.05$), CMS significantly decreased the same parameters ($P < 0.01$ for both elevated plus maze and LD).

Repeated measures ANOVA revealed that EE or CMS did not affect locomotion in the home cage of HABs ($F_{(1,59)} = 0.32$, $P = 0.58$) and LABs ($F_{(1,59)} = 0.87$, $P = 0.37$), respectively (Figure 1d and e).

HPA axis reactivity is associated with anxiety-related behavior

We utilized HPA-RT to reveal whether stress-induced CORT release is associated with the bidirectional shift of anxiety-related behavior. Indeed, ANOVA revealed an association between housing and CORT release ($F_{(1,31)} = 7.40$, $P < 0.01$ for HAB and $F_{(1,41)} = 45.5$, $P < 0.001$ for LAB; Figure 2a). The EE-induced decrease and CMS-induced increase in the CORT response to stressor exposure suggest that not only the behavioral but also the neuroendocrine phenotype could be shifted in a bidirectional manner.

Crrh1 in the amygdala modulates anxiety-related behavior

Earlier it was shown that anxiety-related behavior is associated with *Crrh1* expression in the amygdala.⁸ We analyzed *Crrh1* mRNA levels within the BLA by qPCR and, indeed, observed both a difference between HAB and LAB as well as a bidirectional modulation of gene expression dependent on the environmental manipulation used. HABs exhibited an overexpression of *Crrh1* in

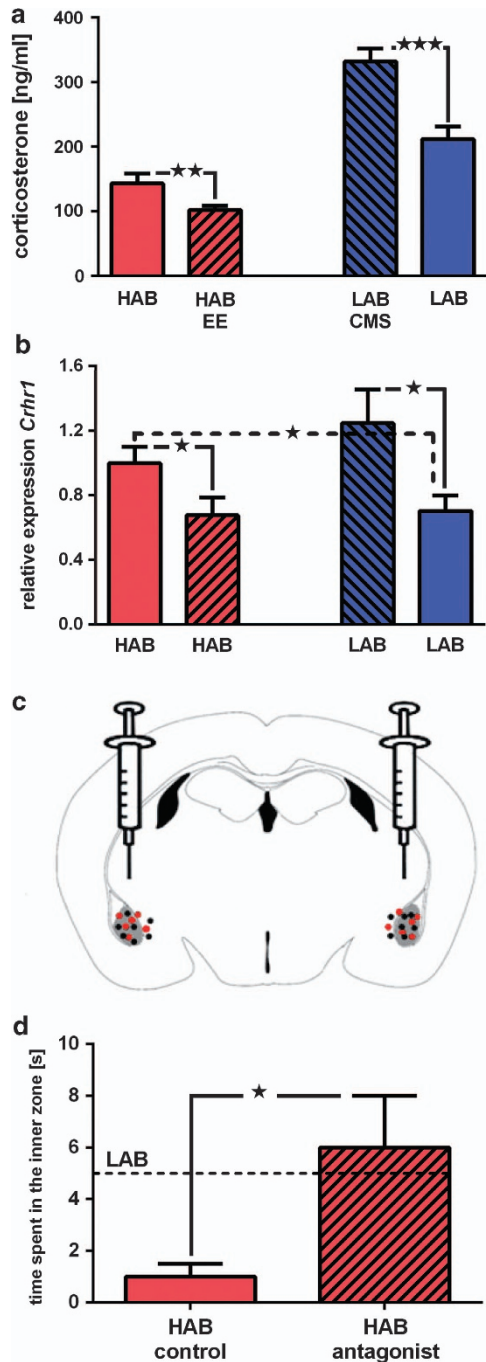


Figure 2. Effects of environmental modifications on stress-induced CORT release and involvement of *Crhr1* in the regulation of anxiety. Enriched environment (EE)-induced anxiolysis is associated with a significantly reduced (analysis of variance) corticosterone (CORT) response to stressor exposure (reactive CORT); in contrast, chronic mild stress (CMS) caused an anxiogenic effect associated with an increased CORT response (a). *Crhr1* in the basolateral amygdala (BLA) contributes to anxiety-related behavior; it is overexpressed in HAB compared to LAB mice (MWU), whereas EE and CMS are capable of bidirectionally shifting messenger RNA levels (b) ($n = 6-7$ per group). Bilateral application of a CRHR1 antagonist into BLA. Schematic illustration of the sites of BLA hits for animals used in CRHR1 antagonist group (black dots) and vehicle group (red dots) (c). Antagonist treatment significantly reduced (MWU) anxiety-related behavior of HABs in the open field test (d). Bars represent mean \pm s.e.m. $n = 6-7$ per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

comparison to LABs ($P < 0.05$), which could be shifted bidirectionally with EE significantly decreasing ($P < 0.05$) and CMS significantly increasing *Crhr1* mRNA levels ($P < 0.05$; Figure 2b). Importantly, no changes were observed in the expression of *Crh*, thereby minimizing the possibility of masking effects of the ligand.

Next, we injected the CRHR1 antagonist α -helical CRH₍₉₋₄₁₎ (Sigma-Aldrich) bilaterally into the BLA of HABs to verify the CRHR1 contribution to anxiety-related behavior (Figure 2c). Indeed, administration of the antagonist entailed an anxiolytic effect apparent as increase of percent time spent in the inner zone of the OF ($P < 0.05$; Figure 2d).

CpG1 methylation of *Crhr1* promoter suggests a critical role for epigenetic regulation

Assuming an epigenetic mechanism in the regulation of *Crhr1*, we performed *in silico* analyzes and identified a CGi in the promoter region of HAB and LAB mice (CpG island searcher), which was subsequently pyrosequenced (Figure 3a). We identified a differentially methylated region (DMR) 1348 bp upstream of the translation start site with both EE ($n = 4$ HAB, HAB-EE, $P < 0.05$) and CMS ($n = 4$ LAB, LAB-CMS, $P < 0.05$) significantly increasing methylation at the CpG1 site (Figure 3e). *In silico* analysis (Ensembl, NCBI37) based on whole genome bisulfite sequencing of two cell lines—embryonic stem cells and nasopharyngeal carcinoma cells—also revealed different methylation patterns at that position (≈ 50 and 100% methylation, respectively) (Figure 3b), suggesting a critical role of CpG1 for epigenetic regulation. A subsequent luciferase assay confirmed that methylation of the identified DMR indeed had an impact on promoter activity, since methylation induced a significant reduction of luciferase activity compared to the unmethylated plasmid ($P < 0.05$; Figure 3f). Complete methylation of the plasmid using *SssI* methylase caused comparable silencing of the reporter gene ($P < 0.01$; Figure 3f).

Transcription factor YY1 regulates *Crhr1* expression in a methylation-dependent manner

Further *in silico* analyzes (JASPAR CORE vertebrata database) demonstrated that the transcription factor YY1 can bind adjacent to the identified DMR (Figure 3c). Indeed, our EMSA experiments confirmed the ability of YY1 to bind at the predicted position (Figure 3d); YY1 overexpression (lanes 2–3) as well as higher protein input (lanes 7–9) increased band intensity, whereas addition of unlabeled probe in excess (competitor) or YY1-specific antibody (YY1 AB), but not unspecific (US AB), prevented band appearance (lanes 4–6). These data indicated that the amount of YY1 was important for the regulation of gene activity. Importantly, YY1 expression was found modulated bidirectionally with EE decreasing and CMS increasing ($P < 0.05$ both) YY1 mRNA levels in the BLA of HAB and LAB mice, respectively (Figure 4a). To further test whether YY1 is able to regulate *Crhr1* expression, we transfected N2a cells with a plasmid carrying YY1 cDNA, resulting in a higher accumulation of YY1 ($P < 0.001$; Figure 4b and c), similar to CMS exposure (Figure 4a). Indeed, overexpression of the transcription factor induced higher expression of *Crhr1* mRNA ($P < 0.05$; Figure 4d). Kim *et al.*¹⁴ found that the amount of YY1 is important for *de novo* methylation of DNA sequence around its binding sites. Thus, we evaluated the methylation of CpG1 after YY1 overexpression and found no difference between mock and YY1 cDNA transfected cells (Figure 4e) indicating that methylation could be a primary event potentially affecting the binding ability of YY1. We tested this hypothesis by using methylated and non-methylated probes for EMSA and indeed we succeeded in showing that methylation of CpG1 decreased the binding ability of YY1 ($P < 0.05$; Figure 5a). At the functional level, using luciferase assay, YY1 overexpression strongly enhanced *Crhr1* promoter activity ($P < 0.001$), whereas activity of the methylated plasmid was significantly lower ($P < 0.05$; Figure 5b).

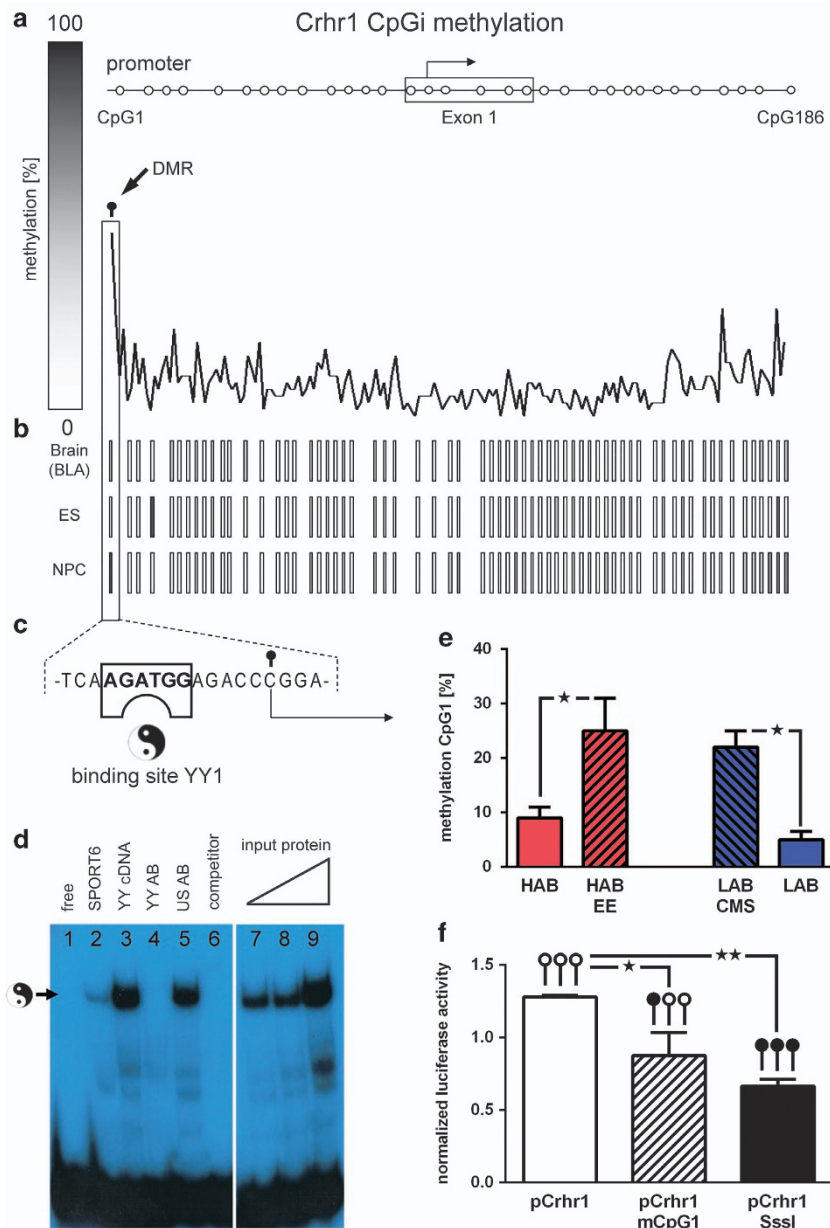


Figure 3. The relations of promoter methylation and YY1 to *Crhr1*. Methylation of 186 single CpG sites of the *Crhr1* promoter has been analyzed in the basolateral amygdala (BLA) of CD-1 mice (**a**). *In silico* analysis predicts CpG1 to be a differentially methylated region (DMR). Prediction is based on data from whole genome bisulfite sequencing of embryonic stem cells (ES) and neuronal primary cells (NPC) (**b**). Methylation-sensitive transcription factor YY1 was proposed to bind close to the DMR, thereby modulating gene expression (**c**). Electrophoretic mobility shift assay (**d**) confirmed binding of YY1 in the proximity to CpG1 of *Crhr1*: (i) YY1 overexpression increased band intensity (lanes 1–3), (ii) incubation with specific YY1-antibody, but not with unspecific antibody, prevented band formation (lanes 4–5), (iii) addition of unlabeled probe in excess (competitor) prevents band formation (lane 6), and (iv) increased amount of protein input induced higher band intensity (lanes 7–9). Both environmental manipulations (enriched environment (EE) and chronic mild stress (CMS)) significantly increased methylation of a CpG site within the *Crhr1* promoter; for details see text (**e**). Complete methylation of the *Crhr1* promoter (pCrhr1 Sssl) further decreases its activity as indicated by the luciferase assay; however, specific methylation of only CpG1 (pCrhr1 mCpG1) is already sufficient for reducing promoter activity (**f**). Bars represent mean+s.e.m. * $P < 0.05$, ** $P < 0.01$ (MWU).

DISCUSSION

Pathological anxiety originates from a complex interplay of genetic predisposition, environmental and epigenetic processes,¹ highlighting the necessity to identify epigenetic processes that alleviate or even rescue a detrimental genetic predisposition. In the present study, we provide evidence that environmental manipulations indeed can bidirectionally shift the extreme genetic

predispositions to high (HAB) and low (LAB) anxiety along the anxiety continuum toward ‘normal’ by epigenetic regulation of the *Crhr1* promoter in the BLA.

EE has repeatedly proven its anxiolytic effects,^{9,15} contrary to CMS that promotes anxiety-related^{16,17} and depression-like¹⁸ behaviors. We here confirm and extend these effects by illustrating that EE increased (HAB) and CMS decreased (LAB) percent time spent in the light compartment of the LD and on open arms

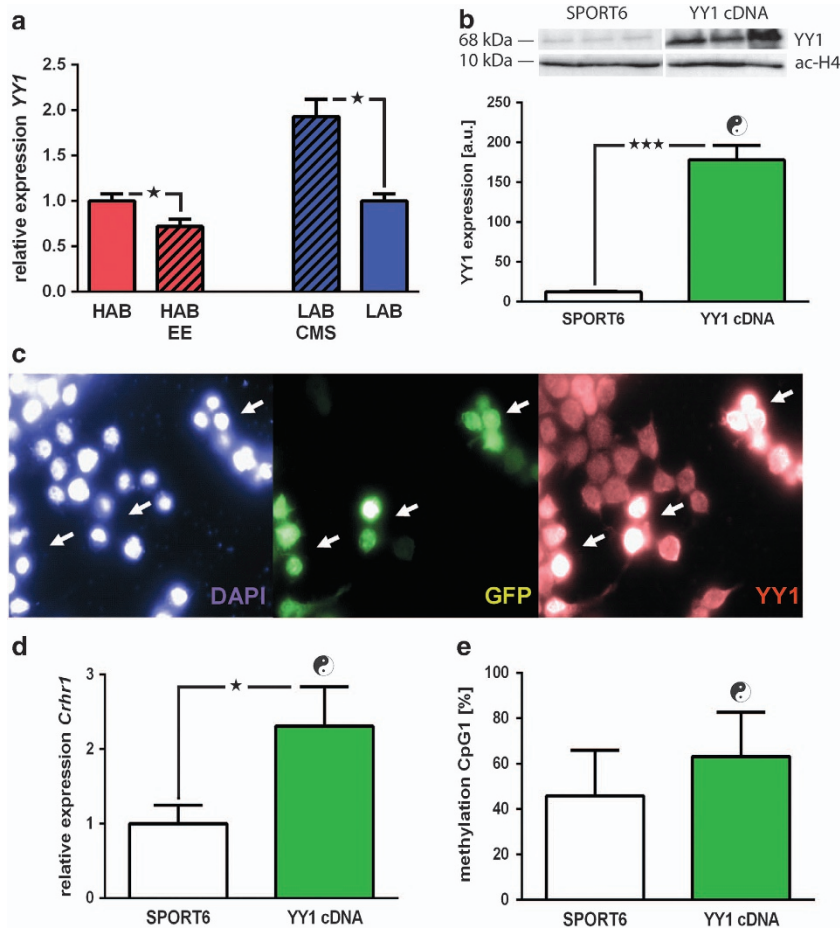


Figure 4. YY1 overexpression and effects on the CpG1 methylation. A bidirectional regulation of *YY1* expression in the basolateral amygdala (BLA) of HAB and LAB mice after enriched environment (EE) and chronic mild stress (CMS), respectively (**a**). Transfection of mouse-neuro-2a (N2a) cells with a plasmid carrying *YY1* cDNA increases YY1 expression as indicated by Western blot (**b**). Only cells transfected with *YY1*-expressing plasmid (green staining) accumulated higher YY1 (red staining) as shown using immunofluorescent microscopy (**c**). *YY1* overexpression up-regulates *Crhr1* messenger RNA (**d**), but does not alter methylation of CpG1 in the *Crhr1* promoter (**e**). Bars represent mean \pm s.e.m. * $P < 0.05$, *** $P < 0.001$ (MWU).

of the elevated plus maze (Figure 1a and b). Importantly, the environmentally driven shifts in anxiety are not masked by locomotion, as spontaneous activity in the home cage was not affected, neither by EE or by CMS (Figure 1d and e). Moreover, we could show that HPA axis reactivity is associated with anxiety-related behavior (Figure 2a). Thus, even robust behavioral and neuroendocrine phenotypes driven by a (seemingly) rigid genetic predisposition can be shifted bidirectionally by environmental manipulations depending on the respective type used. This is particularly striking in male HAB mice that turned out to be non-responsive even to anti-depressive/anxiolytic medication in adulthood.¹⁹ It is important to note here that, according to the mismatch hypothesis,^{20,21} exposure of LAB mice to EE and HAB mice to CMS can potentially lead to similar behavioral outcomes due to changes in their stress-sensitivity. Although our experiments did not support this suggestion (data not shown), the mismatch approach can be another hypothetical way of manipulating the phenotype of HAB and LAB mice, respectively.

Human studies indicate that amygdala activation positively correlates with the degree of trait anxiety.²² Our recent experiments also found higher amygdala activity in HAB compared with LAB mice, with changes in anxiety after EE and CMS followed by corresponding shifts in amygdala activity.⁸ However, the molecular mechanisms behind these phenomena are largely unknown. Accordingly, Rogers *et al.*²³ reported strong associ-

ations between anxiety-related behavior, amygdala activity and *Crhr1* in nonhuman primates. We here hypothesized that *Crhr1* in the amygdala is critically involved as plasticity gene in the bilateral behavioral shifts. Indeed, HABs compared with LABs exhibited an overexpression of *Crhr1* in the amygdala. Moreover, the *Crhr1* expression profile could be shifted bidirectionally to the better or the worse manner⁶ depending on the type of environmental manipulation (Figure 2b). A causal relation between anxiety and CRHR1 in the BLA was further established by bilateral application of the CRHR1 antagonist α -helical CRH₍₉₋₄₁₎ leading to significant anxiolytic effects in HABs (Figure 2d). The antagonist effect clearly suggests that alterations in expression have the potential to contribute to the bilateral phenotypic shifts. These data are in line with Sztainberg *et al.*⁹ who found reduced expression of amygdalar *Crhr1* as a mediator of anxiolytic effects of EE.

Based on the expression data in HAB vs LAB mice and the response to environmental manipulation, we further hypothesized that DNA methylation exerts regulatory control over *Crhr1* within the BLA to integrate environmental stimuli into the genome to adapt anxiety-related behavior to external demands. Previous studies showed that adding⁵ or removing² methyl groups at CpG dinucleotides within regions denoted as CGIs may silence or stimulate transcription of genes thought to be involved in the regulation of anxiety-related behavior. Therefore, we utilized

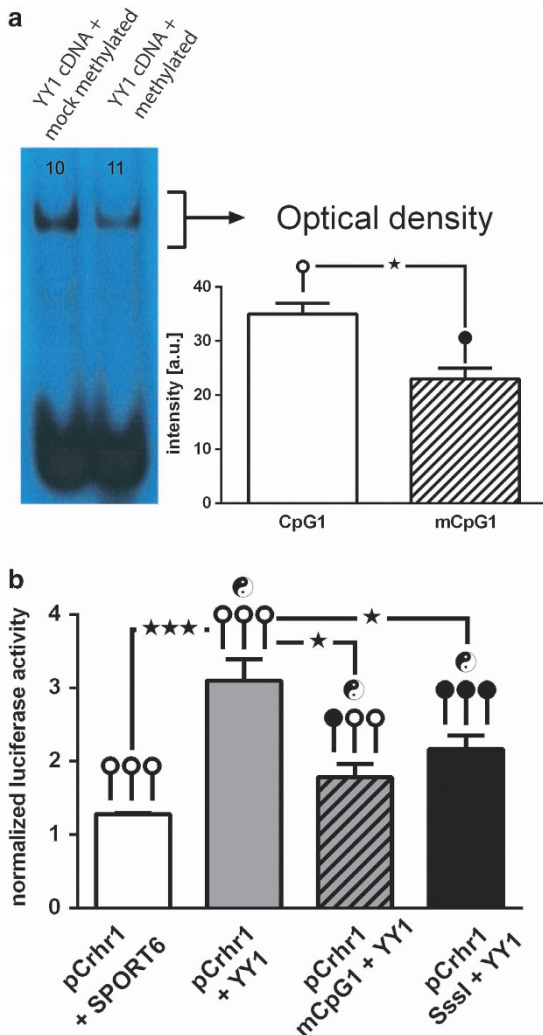


Figure 5. Methylation-sensitive binding of YY1 affects promoter activity. Methylation of CpG1 (mCpG1) (lane 11) decreases binding of YY1 as shown by EMSA (a). YY1 overexpression (e) induces promoter activation (pCrhr1+YY1), whereas a combination with either complete (pCrhr1 Sssl+YY1) or site-specific (pCrhr1 mCpG1+YY1) methylation reduces this effect (b). Bars represent mean+s.e.m. * $P < 0.05$, *** $P < 0.001$ (MWU).

pyrosequencing of bisulfite-treated DNA to assess methylation of the *Crhr1* CGI *in vivo* (Figure 3a). To our surprise, we identified that both EE and CMS significantly increased methylation at a CpG site 1348 bp (CpG1) upstream of the translational start site (Figure 3d). Moreover, data from whole genome bisulfite sequencing of embryonic stem cells (ES) and neuronal primary cells (NPC) also suggest CpG1 as DMR (Figure 3b), highlighting an implication of this site in epigenetic control. A subsequent luciferase assay confirmed that this DMR indeed exerted a regulatory role on *Crhr1* promoter with methylation promoting a decrease in activity (Figure 3f). To further elucidate this unidirectional increase in CpG1 methylation after EE and CMS, we performed an *in silico* analysis and identified the epigenetic transcription factor YY1, which binds adjacent to the DMR (Figure 3c). Surprisingly, YY1 is bidirectionally modulated with HAB-EE mice exhibiting lower and LAB-CMS mice higher expression compared with corresponding controls (Figure 4a). *In vitro* overexpression of YY1 positively regulated *Crhr1* (Figure 4d), thus suggesting that higher expression of YY1 *in vivo* after CMS mediates higher expression of *Crhr1*. The opposite effect could be true for HAB-EE mice expressing less

of this transcription factor. Interestingly, Kim *et al.*²⁴ found YY1 to have an important role in *de novo* methylation of CpGs around its binding sites during genomic imprinting. We did not observe a change in methylation of *Crhr1* CpG1 after YY1 overexpression (Figure 4e), suggesting another mechanism of regulation of this epigenetic mark in response to external environmental influences. The unidirectional increase in CpG1 methylation after EE and CMS seems to be a paradox; however, our data provide evidence of an importance of CpG1 methylation for YY1-induced regulation of *Crhr1* expression. The presence of methylation in the DMR affects transcription factor and DNA interaction, as indicated by weaker binding in EMSA (Figure 5a) and less activation of the complete and site-specific methylated (CpG1) promoters compared with the unmethylated control in the presence of YY1 (Figure 5b). These data are consistent with studies indicating a methylation-sensitive mechanism of YY1 interaction.^{24,25} Thus, methylation can facilitate (i) downregulation of *Crhr1* expression when YY1 is lacking (for example, HAB-EE) and, in turn, (ii) fine-tune an expression enhancement when YY1 is upregulated (for example, LAB-CMS).

Supporting and extending the concept of plasticity genes,⁶ we suggest that *Crhr1* is the first identified plasticity gene of anxiety which (i) is critically involved in the regulation of behavioral and neuroendocrine phenotypes by epigenetic mechanisms and (ii) may be regulated bidirectionally, depending on the type of environmental stimuli. This plasticity may epigenetically rescue extremes in anxiety-related behavior from both ends of the anxiety continuum. The CpG1 site has thus the potential to be used as a biomarker for 'normalization' of extreme anxiety-related behavior and highlights the role of transcription factor YY1 in the epigenetic regulation of *Crhr1* expression. It is plausible that the bidirectionality, as described here, gives rise to subtle and flexible regulation of anxiety-related behavior. Future experiments using whole genome assays will reveal plastic candidate genes in addition to *Crhr1*.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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