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

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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 genome2,3 and indeed interact with the genetic predisposition of an individual,3 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.8,9 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 corticotropin-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 involves 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
rescue of genetically predisposed anxiety, including methylation of the \textit{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 the 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 \textit{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 stresor 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 μl of CRHR1 antagonist (o-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 Crhr1 and YY1 was performed using Quantifast SYBR Green PCR Kit (Qiagen). All samples were analyzed in duplicates and normalized to the housekeeping genes: Polr2b, B2m and Rpl13a (details see in Supplementary Information).

Identification of CGI, primer design and pyrosequencing
The CGI of Crhr1 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 was inserted between the SpeI and HindIII sites of a pcPfG-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. Modified oligonucleotides were purchased from Sigma-Aldrich. Complete methylation of the Crhr1 promoter was performed using SsIl methylase (New England Biolabs) according to the supplier’s instructions; a mock-methylated plasmid was treated in the absence of 5-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 (Thermoscience, Braunschweig, Germany). Cells were lysed after 40 h. Firefly and gauussia luciferase activities were measured as described by Schulke et al. To evaluate if YY1 exhibits a regulatory role on the Crhr1 promoter, a control vector (SPORT6) or a vector expressing YY1 cDNA (YY1) was co-transfected with plasmids carrying the Crhr1 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 μl 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 Crhr1 promoter fragment was incubated with SsIl 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 and LAB mice, 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(1,33) = 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.

Crhr1 in the amygdala modulates anxiety-related behavior
Earlier it was shown that anxiety-related behavior is associated with Crhr1 expression in the amygdala.5 We analyzed Crhr1 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 Crhr1 in
CpG1 methylation of Crh1 promoter suggests a critical role for epigenetic regulation

Assuming an epigenetic mechanism in the regulation of Crh1, 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, NCBI/M37) 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 Crh1 expression in a methylation-dependent manner

Further in silico analyzes (JASPAR CORE verterbata 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 Crh1 expression, we transfected N2a cells with a plasmid carrying YY1 CDNAs, 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 Crh1 mRNA (P < 0.05; Figure 4d). Kim et al. 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 Crh1 promoter activity (P < 0.001), whereas activity of the methylated plasmid was significantly lower (P < 0.05; Figure 5b).
DISCUSSION

Pathological anxiety originates from a complex interplay of genetic predisposition, environmental and epigenetic processes, 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, contrary to CMS that promotes anxiety-related and depression-like 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 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 SssI) 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).
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.6 However, the molecular mechanisms behind these phenomena are largely unknown. Accordingly, Rogers et al.23 reported strong associations 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 overexpression of Crhr1 messenger RNA (d), but does not alter methylation of CpG1 in the Crhr1 promoter (e). Bars represent mean +s.e.m. *P < 0.05, ***P < 0.001 (MWU).

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 +s.e.m. *P < 0.05, ***P < 0.001 (MWU).
of this transcription factor. Interestingly, Kim et al.\textsuperscript{24} found YY1 to have an important role in \textit{de novo} methylation of CpGs around its binding sites during genomic imprinting. We did not observe a change in methylation of \textit{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 \textit{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.\textsuperscript{24,25} Thus, methylation can facilitate (i) downregulation of \textit{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,\textsuperspt{6} we suggest that \textit{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 \textit{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 \textit{Crhr1}.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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