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ORIGINAL INVESTIGATION

Lifelong CRF overproduction is associated with altered gene expression and sensitivity of discrete GABA_A and mGlu receptor subtypes

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Abstract

Rationale Repeated activation of corticotropin-releasing factor (CRF) receptors is associated with increased anxiety and enhanced stress responsivity, which may be mediated via limbic GABAergic and glutamatergic transmission.

Objective The present study investigated molecular and functional alterations in GABA_A receptor (GABA_AR) and metabotropic glutamate receptor (mGluR) responsivity in transgenic mice that chronically overexpress CRF.

Methods CRF₁ receptor, GABA_AR, and mGluR sensitivity were determined in CRF-overexpressing mice using the stress-induced hyperthermia (SIH) test. In addition, we measured mRNA expression levels of GABA_AR α subunits and mGluRs in the amygdala and hypothalamus.

Results CRF-overexpressing mice were less sensitive to the anxiolytic effects of the CRF₁ receptor antagonists CP154,526 and DMP695, the GABA_AR α_3 -selective agonist TP003 (0–3 mg/kg) and the mGluR_{2/3} agonist LY379268 (0–10 mg/kg) in the SIH test. The hypothermic effect of the non-selective GABA_AR agonist diazepam (0–4 mg/kg) and the α_1 -subunit-selective GABA_AR agonist zolpidem (0–10 mg/kg) was reduced in CRF-overexpressing mice. No genotype differences were found using the GABA_AR α_5 -subunit preferential compound SH-053-2'F-R-CH₃ and mGluR₅ antagonists MPEP and MTEP. CRF-overexpressing mice showed decreased expression levels of GABA_AR α_2 subunit and mGluR₃ mRNA levels in the amygdala, whereas these expression levels were increased in the hypothalamus. CRF-overexpressing mice also showed increased hypothalamic mRNA levels of α_1 and α_5 GABA_AR subunits.

Conclusions We found that lifelong CRF overproduction is associated with altered gene expression and reduced functional sensitivity of discrete GABA_A and mGluR receptor subtypes. These findings suggest that sustained over-activation of cerebral CRF receptors may contribute to the development of altered stress-related behavior via modulation of GABAergic and glutamatergic transmission.

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Introduction

The neuropeptide corticotropin-releasing factor (CRF) was initially characterized as the principal HPA-axis modulator in response to stress (Vale et al. 1981). However, CRF has

been found to orchestrate autonomic, immune, and behavioral stress-related responses via central CRF₁ and CRF₂ receptors (Heinrichs and Koob 2004). These non-endocrine CRF brain circuits extend outside the hypothalamus and include cortical, limbic, striatal, and brainstem areas (Hauger et al. 2006). In line with a pivotal role in modulating the stress system, CRF dysfunction appears to be present in various psychiatric disorders including anxiety disorders, drug addiction, major depressive disorder, and schizophrenia (Binder and Nemeroff 2010; Hauger et al. 2009; Kehne and Cain 2010; Logrip et al. 2011; Millan 2003; Reul and Holsboer 2002).

A transgenic mouse model of long-term CRF overexpression has been proposed to model CRF dysfunction. In these animals, chronically elevated CRF levels in the central nervous system are associated with behavioral, neurochemical, autonomic, and physiological changes, including altered HPA axis activity, dexamethasone non-suppression, and reduced heart rate variability (Groenink et al. 2003). These data suggest that chronic postnatal CRF overproduction in these mice leads to alterations mimicking findings that are associated with anxiety disorders and major depression (Binder and Nemeroff 2010; Licht et al. 2009; Linthorst 2005). Clearly, exogenous CRF administration has been found to elicit arousal and anxiety, putatively through CRF₁ receptor stimulation (Bijlsma et al. 2011; Heinrichs et al. 1997; Kehne and Cain 2010; Millan 2003). However, other neurotransmitter systems such as the GABA_A and glutamate system may adjust in response to (sub)chronic CRF receptor activation. In support, CRF has been shown to increase GABA release in the amygdala (Bagosi et al. 2008; Kash and Winder 2006; Nie et al. 2004) and striatum (Sirinathsinghji and Heavens 1989), while also affecting excitatory glutamatergic transmission depending on localization (pre/postsynaptic) and the CRF receptor type (Liu et al. 2004). Moreover, repeated central infusion of the CRF receptor agonist urocortin into the basolateral amygdala (BLA) resulted in long-lasting deficits in fast GABA_A receptor-mediated inhibitory transmission which could be prevented by NMDA receptor antagonists (Rainnie et al. 2004). Therefore, the present study aimed to investigate the putative link between chronic central CRF overexpression and subsequent alterations in GABA_A and glutamate receptor sensitivity. To this end, we used the stress-induced hyperthermia (SIH) test. This test is based on the fact that stress-induced increases in body temperature can be reversed by putative anxiolytics, including (non-)selective GABA_A receptor agonists, metabotropic glutamate receptor (ant)agonists, and CRF₁ receptor antagonists (Bouwknicht et al. 2007; Vinkers et al. 2008). If chronic CRF release elicits long-lasting changes in fast neurotransmitter systems, this would provide a putative mechanism by which

CRF dysfunction could contribute to the emergence of stress-related disorders.

Materials and methods

Animals

Transgenic mice overexpressing neural CRF were generated as described previously (Dirks et al. 2002). Briefly, the CRF transgene was composed of the complete coding sequence of rat CRF cDNA (.6-kb fragment), which was inserted into an 8.2-kb genomic DNA fragment encompassing the murine Thy-1.2 gene, including regulatory regions and polyadenylation signal sequence. The Thy-1 regulatory sequences drive constitutive transgene expression in postnatal and adult neurons. Subsequent breeding at the local breeding facilities (Utrecht, the Netherlands) consisted of matings between heterozygous transgenic males (C57BL/6J background) and C57BL/6Jco females (Charles River, the Netherlands).

Male transgenic CRF-overexpressing mice (15th generation) were used in these experiments. Littermate WT mice served as control mice. Animals were group-housed at constant room temperature (21±2°C) and relative humidity (50–60%) with PVC tubing as cage enrichment. Standard rodent food pellets (Special Diet Services, Witham, Essex, United Kingdom) and water were freely available. Mice were maintained on a 12-h light–dark cycle (lights on at 6 AM). All experiments were performed in accordance with the governmental guidelines for care and use of laboratory animals and approved by the Ethical Committee for Animal Research of the Faculties of Sciences, Utrecht University, The Netherlands.

Drugs

Diazepam (base) and zolpidem (tartaric acid salt) were obtained from Sigma Aldrich. MPEP HCl (2-methyl-6-(phenylethynyl)pyridine) and MTEP (3-((2-methyl-4-thiazolyl)ethynyl)pyridine) were obtained from Alexis Biochemicals. LY379268 (1*R*,4*R*,5*S*,6*R*)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate was obtained from Tocris. SH-053-2'F-R-CH₃ (the (*R*) stereoisomer of 8-ethynyl-4-methyl-6-phenyl-4*H*-2,5,10*b*-triazabenz[e]azulene-3-carboxylic acid ethyl ester) was synthesized by the laboratory of Dr. J.M. Cook (University of Wisconsin-Milwaukee, USA). TP003 (4,2'-difluoro-5'-[8-fluoro-7-(1-hydroxy-1-methylethyl)imidazo[1,2-*a*]pyridin-3-yl]biphenyl-2-carbonitrile) was synthesized according to published methods (Dias et al. 2005; Humphries et al. 2006). CP154,526 HCl (butyl-ethyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7*H*-pyrrolo[2.3-*d*]pyrimidin-4-*y*]amine) and DMP695 mesy-

late (*N*-(2-chloro-4,6-dimethylphenyl)-1-[1-methoxy-methyl-(2-methoxyethyl)-6-methyl-1*H*-1,2,3,4-triazolo[4,5-*c*]pyridin-4-amine) were gifts from Institut de Recherche, Servier, Croissy/Seine France. An injection volume of 10 ml/kg was used for intraperitoneal injections of all drugs. All drugs were suspended in gelatine–mannitol 0.5%/5%. Fresh solutions and suspensions were prepared each testing day.

The stress-induced hyperthermia procedure

The SIH procedure was carried out according to standard procedures (Groenink et al. 2009). Briefly, animals ($n=10$ – 13) were injected intraperitoneally with vehicle or drug 60 min before the first temperature measurement (T_1). The temperature was again measured 10 min later (T_2), representing the stress-induced body temperature. The stress-induced hyperthermia response was calculated by subtracting T_1 from T_2 . A within-subject design was used, and cages were randomly and evenly allocated. The body temperature of mice was measured by rectally inserting a thermistor probe by a length of 2 cm. Digital temperature recordings were obtained with an accuracy of 0.1°C using a Keithley 871A digital thermometer (NiCr–NiAl thermocouple). The probe, dipped into silicon oil before inserting, was held in the rectum until a stable rectal temperature had been obtained for 20 s.

Quantitative PCR analysis

GABA_A receptor subunit levels as well as mGlu receptor mRNA levels were determined in drug-naïve wildtype and transgenic mice. Mice were decapitated, brains were removed and stored at –80°C until further use. Sections of 0.5 mm were made using a cryostat and were kept frozen while the hypothalamus and amygdala were dissected under a binocular microscope. We chose to study the hypothalamus and amygdala for mRNA expression because these brain areas have been shown to be pivotal in the autonomic stress responses such as the stress-induced hyperthermia response (Ulrich-Lai and Herman 2009; Vinkers et al. 2008). Tissue samples were homogenized in Trizol reagent (Invitrogen, the Netherlands) followed by a clean-up with NucleoSpin® RNA Clean-up XS (Machery Nagel, Germany). Reverse transcriptase was performed using the RevertAid™ kit (Fermentas, Germany) and an oligo(dT) primer. The following protocol was used for all PCR reactions, carried out in 20-μl reactions in an ABI PRISM 700 (Applied Biosystems, the Netherlands): 15 min at 95°C, followed by 40 cycles consisting of 15 s at 95°C and 60 s at 60°C. Each reaction mix contained 0.1 μM of each of the gene specific primers and ABsolute™ QPCR SYBR® Green mix (Abgene, Epsom, United Kingdom). The efficiency of a given PCR reaction was determined

using five two-fold dilutions of a mixture of the obtained cDNA samples. Each reaction had an efficiency close to 100%. Measured cDNA levels were normalized against the levels of GAPDH. The following primers were used (FP, RP): GAPDH (GCACCCTGCATTATTTTGTCAC, CTCCAGGAGCGAGACCCCA), GABA_A receptor α₁ subunit (CCACACCCCATCAATAGGTTCT, AATTCTCGGTGCAGAGGACTGAA), GABA_A receptor α₂ subunit (GGGACGGGAAGAGTGTAGTCAA, CCGCATA GGCGTTGTTCTGT), GABA_A receptor α₃ subunit (GCCACTGAAGTTTGGAAGCTAT, CATCCTGTGCTACTTCCACAGATT), GABA_A receptor α₅ subunit (GCCCAGAGAGTCTCTGGAGCT, GGGCCACCTCTCCAAAGTAAAC), mGlu2 receptor (AACCTTGGTCAAGGGTCTG, AAGCGACGATGTTGTTGAG), mGlu3 receptor (CCTGGATGGAAAGAAGTTG, TTGAATGGAGCTGTGAAG), and the mGlu5 receptor (TACTTCTGGGCAGTGATG, GACAGCTTCTCGCTGATAC).

Data analysis

For SIH experiments, a basal temperature (T_1), an end temperature (T_2), and the difference (SIH response= T_2-T_1) was determined for each individual mouse. Treatment effects on the SIH response and basal body temperature (T_1) were evaluated using a repeated-measures analysis of variance with “drug” as within-subject factor and “genotype” as between-subject factor. For post-hoc comparisons, Dunnett’s *t* tests were applied. mRNA levels were analyzed using a univariate analysis of variance with genotype (WT/CRF-OE) as a fixed factor. A probability level of $p<0.05$ was set as statistically significant.

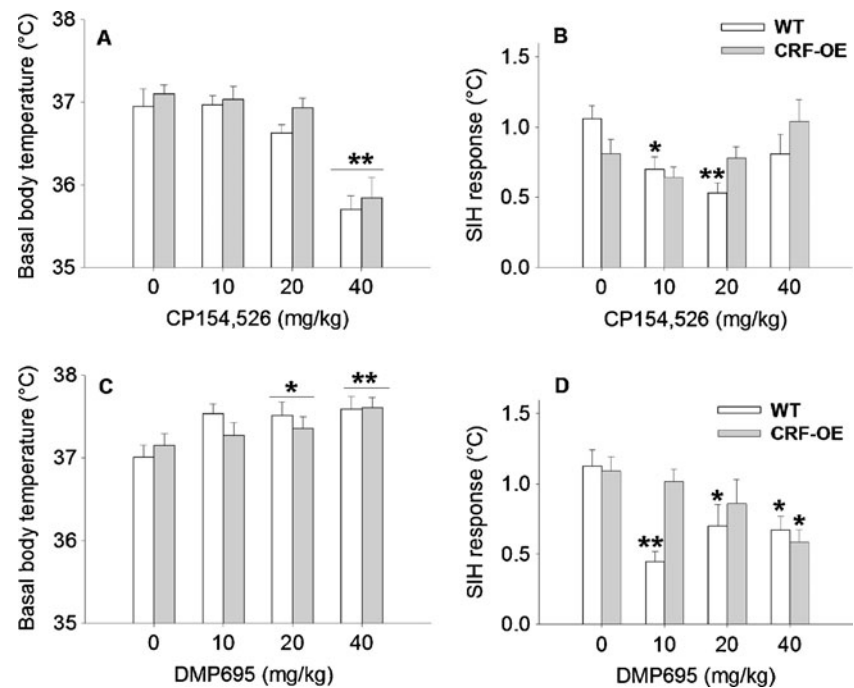
Results

CP154,526 (0–40 mg/kg, IP)

CP154,526 affected the SIH response differently in WT animals compared to CRF-OE mice (CP154,526 × genotype interaction $F_{3,60}=3.60$, $p<0.05$) (Fig. 1b). Separate analysis of the genotypes revealed that CP154,526 reduced the SIH response in WT animals ($F_{3,33}=8.12$, $p<0.001$) but not in CRF-OE animals ($F_{3,30}=2.59$, $p=0.11$). In WT animals, post hoc analysis showed that this effect was attributable to the 10 and 20 mg/kg doses of CP154,526.

CP154,526 lowered basal body temperature regardless of genotype (CP154,526 effect $F_{3,60}=38.57$, $p<0.01$; CP154,526 × genotype interaction, $F_{3,60}=0.54$, $p=0.66$, NS; genotype effect $F_{1,20}=0.66$, $p=0.43$, NS) (Fig. 1a). Post hoc analysis revealed that this effect was significant only at the 40 mg/kg dose of CP154,526.

Fig. 1 Effects of the CRF₁ receptor antagonists CP154,526 (0–40 mg/kg, IP, **a–b**) and DMP 695 (0–40 mg/kg, IP, **c–d**) on basal body temperature and the SIH response in wildtype mice (WT) and CRF-overexpressing mice (CRF-OE). Asterisk drug effect relative to vehicle (* $p < 0.05$; ** $p < 0.01$)



DMP695 (0–40 mg/kg, IP)

DMP695 reduced the SIH response to a larger extent in WT animals compared to CRF-OE mice (DMP695 \times genotype interaction $F_{3,63}=5.63$, $p < 0.01$) (Fig. 1d). Separate analysis of the genotypes showed that DMP695 reduced the SIH response in WT animals ($F_{3,30}=10.41$, $p < 0.01$) as well as CRF-OE animals ($F_{3,33}=6.26$, $p < 0.01$). Post hoc analysis indicated that in WT mice, all DMP695 doses significantly reduced the SIH response, whereas in CRF-OE animals, only the highest dose of DMP695 reduced the SIH response.

DMP695 increased body temperature regardless of genotype (DMP695 effect $F_{3,63}=6.33$, $p < 0.01$; DMP695 \times genotype interaction, $F_{3,63}=1.07$, $p = 0.37$, NS) (Fig. 1c). Post hoc analysis revealed that compared to vehicle, this increase was significant at the 20 and 40 mg/kg doses of DMP695.

Diazepam (0–4 mg/kg, IP)

The hypothermic effect of diazepam was dependent on genotype (diazepam \times genotype interaction, $F_{3,63}=2.77$, $p < 0.05$) (Fig. 2a). Separate analysis of the genotypes indicated that diazepam reduced basal body temperature in WT animals ($F_{3,36}=7.77$, $p < 0.001$) but not in CRF-OE animals ($F_{3,27}=0.10$, $p = 0.96$, NS). In WT animals, post hoc analysis showed that only the 4 mg/kg dose of diazepam significantly reduced basal body temperature.

Diazepam reduced the SIH response regardless of genotype (diazepam effect $F_{3,63}=17.25$, $p < 0.001$; diaze-

pam \times genotype interaction $F_{3,63}=0.17$, $p = 0.92$, NS; genotype effect $F_{1,21}=0.03$, $p = 0.86$, NS) (Fig. 2b). Post hoc analysis revealed that diazepam significantly reduced the SIH response at all doses compared to vehicle.

Zolpidem (0–10 mg/kg, IP)

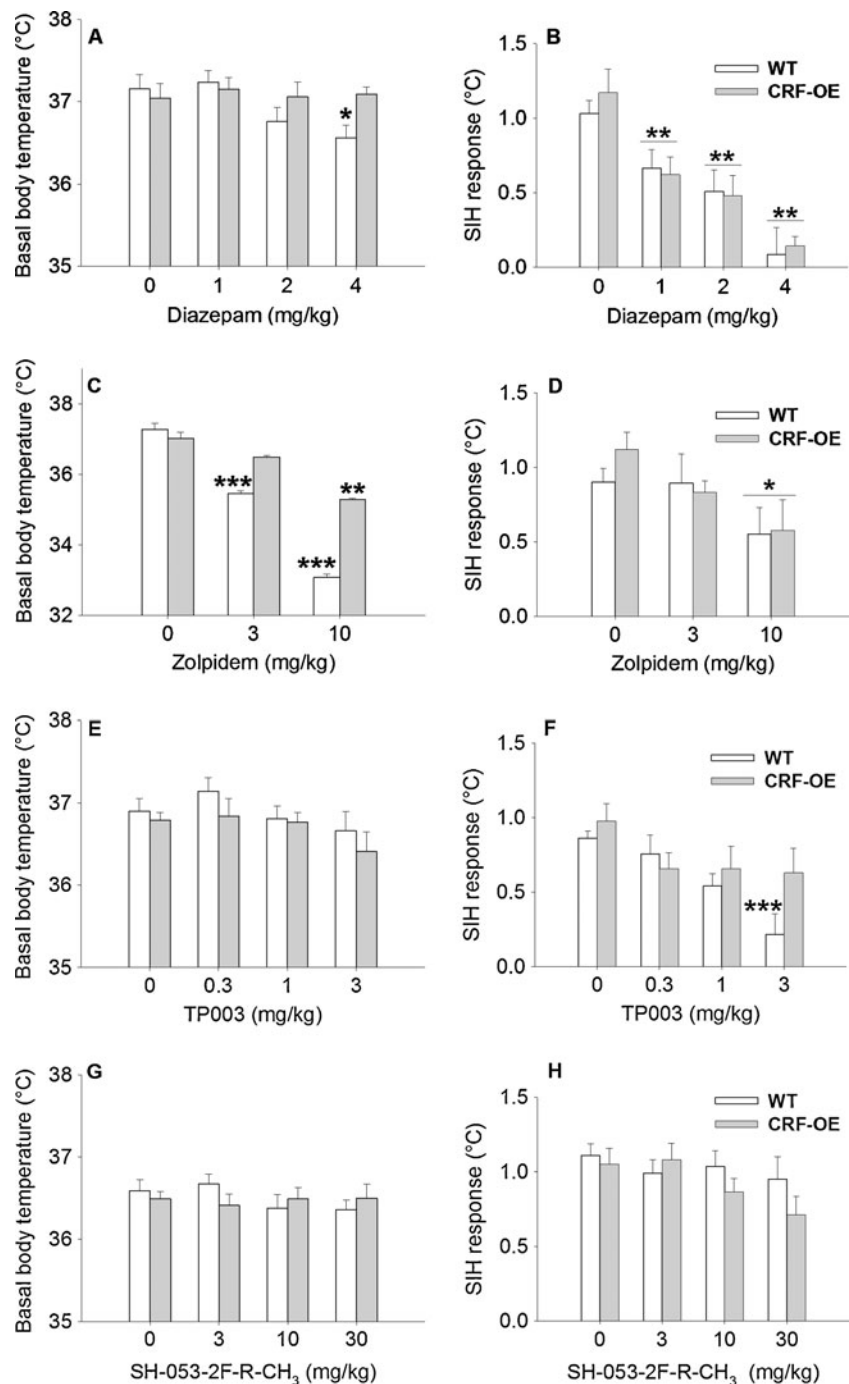
Zolpidem affected the SIH response regardless of genotype (zolpidem effect $F_{2,40}=5.21$, $p = 0.01$; zolpidem \times genotype interaction $F_{2,40}=0.52$, $p = 0.60$, NS; genotype effect $F_{1,20}=0.17$, $p = 0.69$, NS) (Fig. 2d). Post hoc analysis showed that zolpidem reduced the SIH response only at the 10 mg/kg dose.

Zolpidem reduced basal body temperature more in WT animals than in CRF-OE mice (zolpidem \times genotype interaction, $F_{2,40}=6.39$, $p < 0.01$) (Fig. 2c). Separate analysis of the genotypes showed that zolpidem reduced basal body temperature in WT animals ($F_{2,22}=27.86$, $p < 0.001$) as well as in CRF-OE animals ($F_{2,18}=21.93$, $p < 0.001$). Post hoc analysis showed that in WT animals, all zolpidem doses had a strong hypothermic effect, whereas in CRF-OE animals, only the 10 mg/kg dose of zolpidem significantly induced hypothermia.

TP003 (0–3 mg/kg, IP)

TP003 reduced the SIH response, and a trend for a genotype effect was present (TP003 effect $F_{3,60}=5.42$, $p < 0.01$; TP003 \times genotype interaction $F_{3,60}=1.99$, $p = 0.09$) (Fig. 2f). Separate analysis of the genotypes showed that TP003 reduced the SIH in WT animals ($F_{3,33}=5.16$, $p < 0.01$)

Fig. 2 Effects of the non-subunit selective GABA_A receptor agonist diazepam (0–4 mg/kg, IP, **a–b**), the preferential α_1 subunit GABA_A receptor agonist zolpidem (0–10 mg/kg, IP, **c–d**), the GABA_A receptor α_3 subunit agonist TP003 (0–3 mg/kg, IP, **e–f**) and the GABA_A receptor α_5 subunit agonist SH-053-2'F-R-CH₃ (0–30 mg/kg, IP, **g–h**) on basal body temperature and the stress-induced hyperthermia (SIH) response in wildtype (WT) and CRF-overexpressing mice (CRF-OE). Asterisk drug effect relative to vehicle (* p <0.05; ** p <0.01; *** p <0.001)



but not in CRF-OE animals ($F_{3,27}=1.84$, $p=0.16$, NS). Post hoc analysis indicated that in WT animals, only the 3 mg/kg dose of TP003 significantly reduced the SIH response.

TP003 reduced basal body temperature regardless of genotype (TP003 effect $F_{3,60}=3.21$, $p<0.05$; TP003 \times genotype interaction, $F_{3,60}=0.36$, $p=0.78$, NS; genotype effect $F_{1,20}=1.41$, $p=0.25$, NS) (Fig. 2e). After post hoc analysis, it appeared that this effect was not significant at any separate TP003 dose.

SH-053-2'F-R-CH₃ (0–30 mg/kg, IP)

SH-053-2'F-R-CH₃ did not affect the SIH response regardless of genotype (SH-053-2'F-R-CH₃ effect $F_{3,60}=2.03$, $p=0.12$, NS; SH-053-2'F-R-CH₃ \times genotype interaction $F_{3,60}=0.67$, $p=0.57$, NS; genotype effect $F_{1,20}=0.36$, $p=0.56$, NS) (Fig. 2h).

SH-053-2'F-R-CH₃ did not affect body temperature (SH-053-2'F-R-CH₃ effect $F_{3,60}=0.76$, $p=0.52$, NS; SH-053-2'F-R-CH₃ \times genotype interaction, $F_{3,60}=1.20$, $p=0.32$, NS; genotype effect $F_{1,20}=0.08$, $p=0.78$, NS) (Fig. 2g).

MPEP (0–30 mg/kg, IP)

MPEP reduced the SIH response regardless of genotype (MPEP effect $F_{3,63}=16.55$, $p<0.001$; MPEP×genotype interaction $F_{3,63}=0.31$, $p=0.82$, NS; genotype effect $F_{1,21}=0.95$, $p=0.34$, NS) (Fig. 3b). Post hoc analysis indicated that MPEP significantly reduced the SIH response at the 10 and 30 mg/kg doses compared to vehicle-treated mice.

MPEP increased body temperature regardless of genotype (MPEP effect $F_{3,63}=5.63$, $p<0.01$; MPEP×genotype interaction, $F_{3,63}=0.65$, $p=0.58$, NS; genotype effect $F_{1,21}=1.66$, $p=0.21$, NS) (Fig. 3a). Post hoc analysis revealed that this difference was significant at the 30 mg/kg MPEP dose.

MTEP (0–30 mg/kg, IP)

MTEP reduced the SIH response regardless of genotype (MTEP×genotype interaction $F_{3,63}=0.03$, $p=0.99$, NS; MTEP effect $F_{3,63}=21.87$, $p<0.001$; genotype effect $F_{1,21}=0.04$,

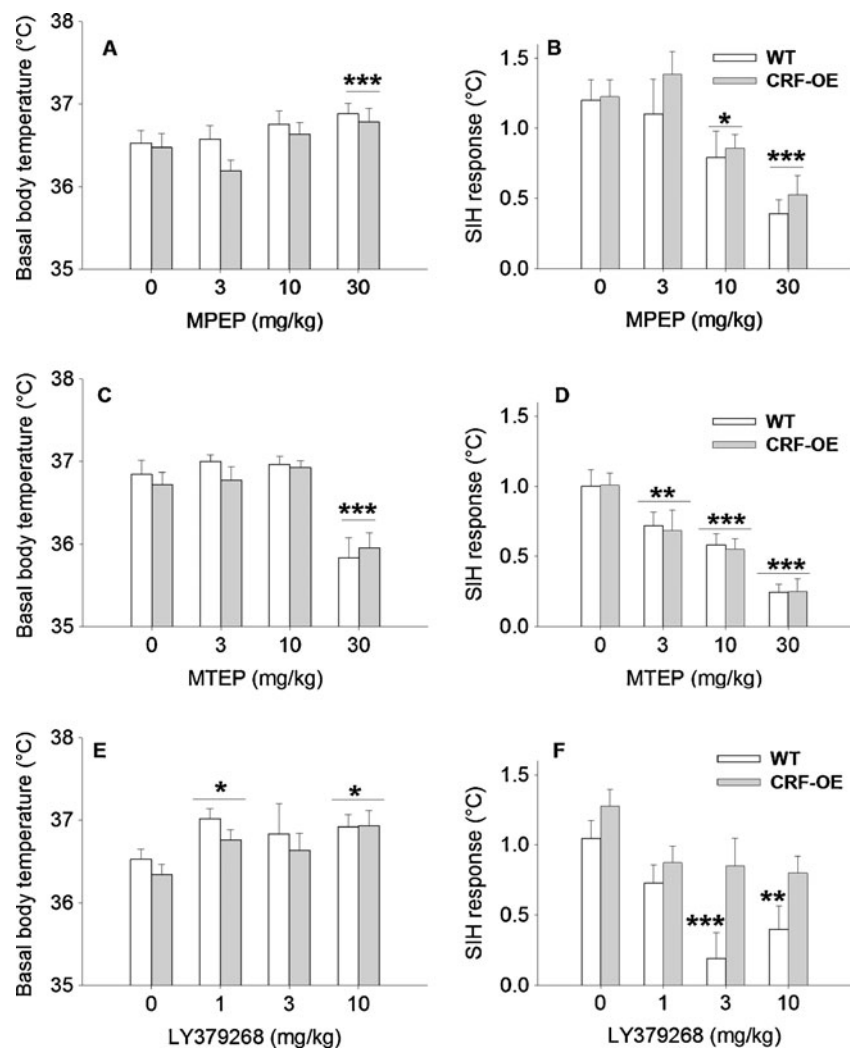
$p=0.85$, NS) (Fig. 3d). Post hoc analysis showed that MTEP significantly reduced the SIH response at all doses compared to vehicle-treated mice.

MTEP overall reduced body temperature regardless of genotype (MTEP effect $F_{3,63}=19.04$, $p<0.001$; MTEP×genotype interaction, $F_{3,63}=0.42$, $p=0.74$, NS; genotype effect $F_{1,21}=0.42$, $p=0.53$, NS) (Fig. 3c). Post hoc analysis showed that this effect was significant at the 30 mg/kg MTEP dose ($p<0.001$).

LY379268 (0–10 mg/kg, IP)

The effect of LY379268 on the SIH response was dependent on the genotype in which it was tested (LY379268×genotype interaction $F_{3,60}=3.08$, $p<0.05$) (Fig. 3f). Separate analysis of the genotypes showed that LY379268 reduced the SIH in WT animals ($F_{3,27}=8.85$, $p<0.001$) but not in CRF-OE animals ($F_{3,27}=2.30$, $p=0.14$, NS). Post hoc analysis indicated that in WT mice, the 3 and 10 mg/kg LY379268 doses significantly reduced the SIH response.

Fig. 3 Effects of the mGluR₅ antagonists MPEP (0–30 mg/kg, IP, a–b), MTEP (0–30 mg/kg, IP, c–d) and the mGluR_{2/3} agonist LY379268 (0–10 mg/kg, IP, e–f), on basal body temperature and the stress-induced hyperthermia (SIH) response in wildtype (WT) and CRF-overexpressing mice (CRF-OE). *Asterisk* drug effect relative to vehicle (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)



LY379268 increased body temperature regardless of genotype (LY379268 effect $F_{3,60}=3.59$, $p<0.05$; LY379268 \times genotype interaction, $F_{3,60}=0.22$, $p=0.89$, NS; genotype effect $F_{1,21}=0.81$, $p=0.38$, NS) (Fig. 3e). Post hoc analysis revealed that this effect was significant at the 1 and 10 mg/kg doses of LY379268.

Quantitative PCR analysis

Results of the PCR analysis showed increased GABA_AR α_1 , α_2 , α_5 subunit, and mGluR₃ mRNA levels in the hypothalamus in CRF-overexpressing group, whereas no changes were found in α_3 subunit, mGluR₂, and mGluR₅ mRNA levels (Fig. 4a). In contrast, decreased GABA_AR α_2 subunit and mGluR₃ mRNA levels were present in the amygdala of CRF-overexpressing mice compared to WT mice (Fig. 4b). All mRNA levels were normalized against levels of GAPDH.

Discussion

The present studies investigated the putative link between chronically elevated CRF levels and subsequent alterations in GABA_A and glutamate receptor responsivity using transgenic mice that overexpress CRF in the brain. To this end the effect of CRF₁ receptor, GABA_AR, and mGluR ligands were studied in the SIH test. In WT mice, the CRF₁ receptor antagonists CP154,526 and DMP695 reduced the SIH response, which is indicative for an anxiolytic effect of these compounds (Kehne and Cain 2010; Millan et al. 2001; Zorrilla and Koob 2010). The fact that DMP695 induced a mild hyperthermia of around 0.5°C most probably did not interfere with the capability to induce a SIH response because stress-induced rectal temperature is capable of rising over 39°C, and is even present in interleukin-induced fever (Vinkers et al. 2009a). Our data confirm previous anxiolytic effects of the CRF₁ receptor antagonist SSR125543A using the SIH paradigm (Griebel et al. 2002). In contrast to the effects observed in WT animals, CRF-OE mice showed an impaired anxiolytic response to the CRF₁ receptor antagonists CP154,526 and DMP695 (Fig. 1). However, no apparent rightward shift in responsivity to CRF₁ receptor antagonists was found, suggesting that elevated CRF levels do not induce a straightforward receptor desensitization or downregulation. In fact, there is evidence that no prominent CRF receptor mRNA downregulation is present in CRF-OE mice (Korosi et al. 2006). Impaired sensitivity to CRF₁ receptor antagonists in CRF-OE mice could also be explained by a more brain-structure-specific change of CRF receptor distribution in these mice, with decreased CRF₁ receptor mRNA and increased CRF₂ receptor mRNA in specific

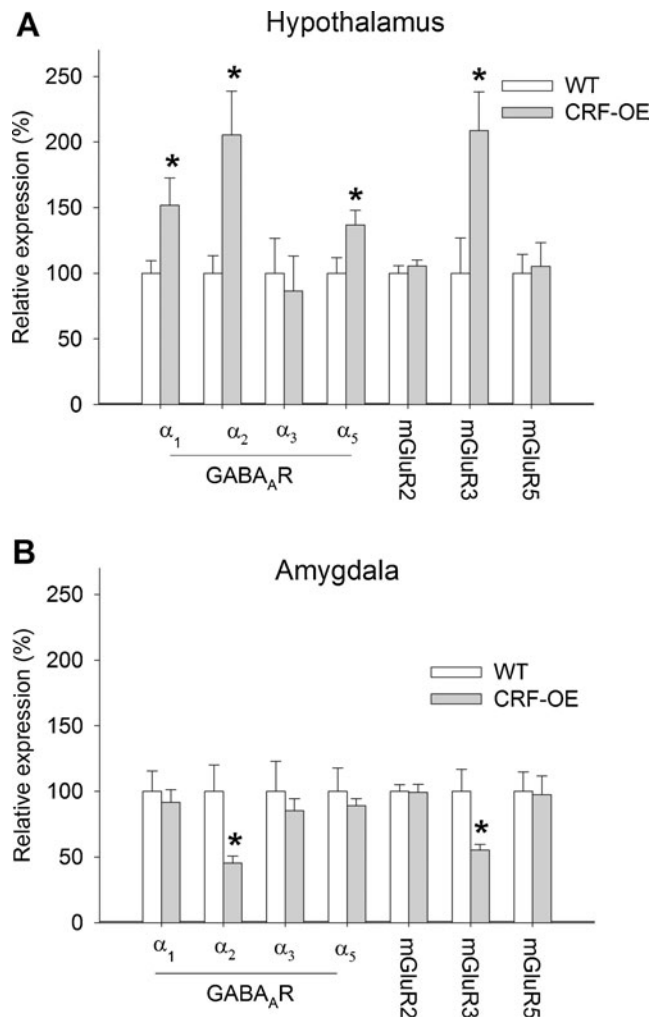


Fig. 4 mRNA levels of GABA_A receptor subunits and mGluR receptors (mean \pm SEM) in the hypothalamus **a** and the amygdala **b** of wildtype (WT) on CRF-overexpressing mice (CRF-OE) mice. The mRNA expression was normalized against GAPDH level. * $p<0.05$

brain structures (Korosi et al. 2006). Irreversible and specific changes in the CRF system in CRF-OE mice seem to contrast with the fact that prepulse inhibition deficits in these animals are readily reversed after administration of CRF₁ receptor antagonists (Groenink et al. 2008). However, a possible caveat is that these PPI deficits—in contrast to anxiety-related processes—are only present during lasting activation of CRF receptors (Bijlsma et al. 2011). Here, CRF₁ receptor antagonists either decreased (CP154,526) or increased basal body temperature (DMP695). We do not have a good explanation for this difference as both drugs show high affinity for human (h)CRF₁ (K_i , 3.3 nM) and native rat CRF₁ (K_i , 4.6 nM) receptors and low affinity for CRF₂ α and other classes of receptors (Millan et al. 2001). Possible explanations include reduced bioavailability of CP154-526, or partial agonist activity at and differential involvement of various CRF₁ receptor isoforms and

binding sites. These drug-induced changes in basal body temperature were similar across both genotypes, and basal body temperature was comparable between WT and CRF-OE mice, suggesting that the role of CRF₁ receptors in stress-induced hyperthermia is different from that in basal thermoregulatory processes. Acute central CRF administration increases basal body temperature, suggesting that the CRF system may directly affect thermoregulatory processes (Heinrichs et al. 2001). Although Dirks and colleagues reported increased body temperature in CRF-OE mice in the late afternoon (maximum 0.5°C) in CRF-OE mice (Dirks et al. 2002), we found no overall effects of genotype on basal body temperature. This is probably due to the fact that the present experiments were carried out between 9 AM and 2 PM, whereas body temperature in CRF-OE mice was only found to be moderately increased from 2–5 PM.

Chronic CRF overproduction not only resulted in reduced sensitivity to the anxiolytic effects of CRF₁ receptor antagonists, but also in reduced sensitivity to the anxiolytic effects of the GABA_A $\alpha_{2/3}$ subunit-selective agonist TP003 (Dias et al. 2005) and the mGlu_{2/3}R agonist LY379268. Both TP003 and LY379268 reduced the SIH response to a lesser extent in CRF-OE animals than in WT mice but did not result in hypothermia. The SIH-reducing effect of these compounds is in line with known anxiolytic effects of both $\alpha_{2/3}$ selective GABAergic modulators (Atack 2010; Dias et al. 2005; Mirza et al. 2008) and mGluR (ant)agonists (Niswender and Conn 2010). We also report that the hypothermic effect of the non-selective GABA_AR agonist diazepam and the α_1 -selective GABA_AR agonist zolpidem (Petroski et al. 2006) were less pronounced in CRF-OE than in WT mice (Fig. 2). The impaired GABAergic and glutamatergic sensitivity in CRF-OE mice appeared to be specific, as genotype did not affect responsiveness to the α_5 -subunit preferential GABA_A receptor agonist SH-053-2'F-R-CH₃ (Fig. 2) or the mGluR₅ antagonists MPEP and MTEP (Fig. 3). In contrast to our results, previous studies did not show a significant increase of basal body temperature after administration of MPEP up to oral doses of 30 mg/kg in mice (Nordquist et al. 2007; Spooren et al. 2002; Spooren et al. 2000). However, the MPEP-induced hyperthermia is minor with a maximum MPEP-induced hyperthermia of around 0.3°C occurring at the highest dose. In support, MPEP-induced basal body temperature as reported by Spooren and colleagues display an apparent trend for body temperature increases although this does not reach statistical significance (Spooren et al. 2000). In support, we administered MPEP intraperitoneally (compared to oral administration in the other studies), leading to increased CNS drug levels. Moreover, the present study used a within-subject design which may be more sensitive to result in statistical significance.

Our results confirm and extend the SIH-reducing effects of (non-)selective GABAergic modulators diazepam, zolpidem, and TP003 (for review, see (Vinkers et al. 2010b)) as well as those of the glutamatergic compounds (mGluR_{2/3} agonists and mGluR₅ antagonists) (Nordquist et al. 2007; Spooren et al. 2002). The fact that the α_5 -subunit preferential GABA_A receptor agonist SH-053-2'F-R-CH₃ did not affect basal body temperature nor the SIH response compared to vehicle-treated mice may suggest that the α_5 subunit is not involved in the anxiolytic and hypothermic effects of benzodiazepines, which is in line with the already published findings (Atack 2011; Rudolph and Mohler 2006; Savic et al. 2010). However, the use of α_5 -selective (silent) antagonists is warranted to further characterize the role of this subunit in hypothermia and anxiolysis.

Long-lasting CRF hyperactivity is thus associated with a decreased GABAergic and glutamatergic receptor sensitivity at adult age. In our results, either the SIH response (TP003 and LY379268) or basal body temperature (diazepam and zolpidem) were affected in CRF-OE mice, suggesting that neurocircuitry involved in these processes may have adapted in these transgenic mice. These results may indicate that a blunted SIH attenuation without concomitant basal body temperature differences reflect specific dysfunction involving stress-related circuitry in CRF-OE animals. In support, basal body temperature and the autonomic stress response have been found to be regulated independently with a putative role for the GABA_AR α_1 subunit in basal thermoregulation and a role for α_2 and α_3 subunits in the SIH response (Vinkers et al. 2009b). Our current results are in the line with such a distinction in α subunit function in the SIH paradigm.

Besides alterations in functional receptor sensitivity, CRF-OE mice display molecular alterations in GABA_AR and mGluR circuitry in the amygdala and hypothalamus. These brain areas were chosen since they have been shown to be pivotal in autonomic stress responsiveness (Ulrich-Lai and Herman 2009; Vinkers et al. 2010a; Vinkers et al. 2008). Also, central thermoregulation is eventually regulated in the hypothalamus, and a connection between the anxiety-involved limbic system and the hypothalamic temperature execution areas is generally assumed (Dimicco and Zaretsky 2007). Amygdaloid GABA_AR α_2 subunit and mGluR₃ mRNA levels were decreased in CRF-OE mice (Fig. 4), whereas opposing effects were found in the hypothalamus. The hypothalamic increase in α_5 subunit expression may reflect a compensatory mechanism in which changes in α_5 -dependent GABAergic transmission would lead to increased tonic (extra)synaptic activity (Caraiscos et al. 2004). In support, chronic administration of anabolic androgenic steroids which can affect mood and induce anxiety also resulted in increased mRNA expression of the α_5 subunit in the hypothalamus, (Penatti et al. 2009).

Moreover, chronic stress led to up-regulation of the α_5 subunit expression in the hypothalamic paraventricular nucleus (Verkuyl et al. 2004). Surprisingly, the impaired sensitivity to the GABA_AR α_3 subunit-selective agonist TP003 was not accompanied by changes in α_3 subunit mRNA expression. This may be due to the fact that α_3 subunit expression is relatively low in the amygdala and hypothalamus. The expression of the α_3 subunit is highest in the cortex, hippocampus, thalamus, and brainstem (including monoaminergic neurons such as the raphe nuclei and the locus coeruleus) (Mohler et al. 2002). Also, the α_3 -selective nature of TP003 can be questioned. Although TP003 appears to possess somewhat lower in vitro efficacy for the α_2 subunit compared to the α_3 subunit (Dias et al. 2005), these differences may not be so pronounced in vivo. Moreover, subtype selective GABA compounds that distinguish between the α_2 and the α_3 subtype have not been developed yet (Atack 2010). Together, TP003 may prove to be functionally $\alpha_{2/3}$ subtype selective rather than α_3 selective. In support of specific changes in α subunits, mRNA expression of the γ_2 subunit was not altered in the hypothalamus and amygdala of CRF-overexpressing mice compared to wildtype mice (data not shown). We did not investigate mRNA expression of other γ subunits because the γ_1 and γ_3 subunits characterize a small population of GABA_A receptors with a reduced affinity for classical benzodiazepines (McKernan and Whiting 1996).

The fact that CRF-OE mice display more subtle region-specific differences in CRF₁ and CRF₂ receptor mRNA expression suggest that adaptations in the GABA_A and glutamate receptor systems are probably not directly related to concomitant changes in CRF receptor expression (Korosi et al. 2006). The finding that chronic CRF exposure exerts opposing effects on hypothalamic and amygdaloid GABA_AR and mGluR₃ mRNA levels may be related to the differential effects of CRF₁ and CRF₂ receptors on the fast neurotransmitter systems. In support, CRF₁ receptor activation in the amygdala decreased glutamate transmission, whereas CRF₂ receptor activation opposed these CRF₁ receptor-mediated effects on glutamate transmission (Liu et al. 2004). However, the applied methodology has important limitations. We cannot exclude the possibility that changes in the GABA and glutamate system are present in other brain regions of CRF-overexpressing mice such as the prefrontal cortex or the hippocampus. Also, no mRNA expression of amygdaloid and hypothalamic subnuclei was determined since punching out separate nuclei of these brain areas is technically very challenging in mice, and RNA isolation from extremely small tissue samples may suffer from the low input. Thus, our results do not pinpoint the exact subnuclei within the hypothalamus or amygdala that represent the overall changed gene expression. However, the observed changes are strong indications for

changes in these areas that should be further explored using other methods such as immunohistochemistry or in situ hybridisation.

A number of studies support the notion that a disruption of the balance between glutamatergic excitation and GABAergic inhibition could underlie CRF-induced anxiety-like behavior. Repeated administration of urocortin, a CRF-related ligand into the basolateral amygdala induced long-lasting anxiety-like responses that were dependent on NMDA receptor activation but resulted in concomitant specific loss of GABA_A receptor-mediated inhibition (Rainnie et al. 2004). Also, CRF enhanced GABA_AR inhibitory postsynaptic currents IPSCs in central amygdala neurons from wild-type and CRF₂ receptor knockout mice but not CRF₁ receptor knockout mice (Nie et al. 2004). In that study, CRF₁ but not CRF₂ receptor antagonists blocked these effects in wild type mice, a finding that was later confirmed using in vitro techniques (Bagosi et al. 2008). CRF enhanced GABA_A-mediated transmission via postsynaptic activation of CRF₁ receptors in the bed nucleus of the stria terminalis (Kash and Winder 2006), and CRF has been shown to increase GABA release in the striatum (Sirinathsinghji and Heavens 1989). In serotonergic dorsal raphe neurons, CRF elevated presynaptic GABA release and increased GABA_A receptor-mediated miniature IPSCs that were mediated by both CRF₁ and CRF₂ receptors (Kirby et al. 2008). In addition to CRF effects on the GABA_A neurotransmission, Liu et al. (2004) showed concentration-dependent and opposing effects of CRF on fast excitatory glutamatergic transmission in the central nucleus of the amygdala and the lateral septum mediodorsal nucleus. Moreover, CRF potentiated NMDA receptor-mediated excitation in the ventral tegmental area which was mediated by CRF₂ receptors (Ungless et al. 2003). Although we found changes in mRNA expression levels that may suggest alterations at the receptor level associated with CRF overexpression, we cannot exclude the possibility that changes in GABAergic and glutamatergic neurotransmitter systems in CRF-OE mice may be the result of changes in intracellular signaling pathways. In support, CRF is implicated in protein kinase C and Ca²⁺/calmodulin-dependent kinase II-dependent long-term neuronal potentiation and depression (Blank et al. 2003; Miyata et al. 1999), and CRF increases calcium currents in central amygdala neurons (Yu and Shinnick-Gallagher 1998). Also, CRF-induced changes could be mediated through the serotonergic system (Lukkes et al. 2008; Price et al. 1998; Tan et al. 2004). To our knowledge, no GABA_A receptor subunit levels have been assessed in transgenic CRF mice lines or in rodents that have repeatedly been exposed to CRF or urocortin infusions.

In conclusion, we show that chronic CRF overproduction is accompanied by reduced functional GABA_A and

metabotropic glutamate_{2/3} receptor sensitivity in parallel with opposing changes in mRNA expression of these receptors in the amygdala and hypothalamus. These data provide evidence that CRF exerts a modulatory role on GABAergic and glutamatergic pathways in at least the amygdala and hypothalamus. Thus, these observations contribute to evidence linking an increased central CRF drive to the emergence of dysfunctional GABAergic and glutamatergic transmission which could play a role in stress-related disorders. Specifically, CRF-induced changes in activity at GABA_A α_3 subunits and mGlu_{2/3} receptors may be of particular significance. If chronic CRF-induced changes in GABAergic and glutamatergic transmission contribute to the development of anxiety, depressed mood, drug-seeking, psychosis and other stress-related behavioral changes associated with sustained over-activation of CRF, ligands targeting specific classes of GABA_A subunits and mGluR receptors may prove beneficial in the management of these disorders (Atack 2011; Millan 2003; Nicoletti et al. 2010; Yasuhara and Chaki 2011).

Conflicts of interest The authors declare no financial disclosures or conflicts of interest.

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