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Metabotropic Glutamate Receptor Subtype 7 Ablation Causes Dysregulation of the HPA Axis and Increases Hippocampal BDNF Protein Levels: Implications for Stress-Related Psychiatric Disorders

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Regulation of neurotransmission via group-III metabotropic glutamate receptors (mGluR4, -6, -7, and -8) has recently been implicated in the pathophysiology of affective disorders, such as major depression and anxiety. For instance, mice with a targeted deletion of the gene for mGluR7 (mGluR7$^{-/-}$) showed antidepressant and anxiolytic-like effects in a variety of stress-related paradigms, including the forced swim stress and the stress-induced hyperthermia tests. Deletion of mGluR7 reduces also amygdala- and hippocampus-dependent conditioned fear and aversion responses. Since the hypothalamic–pituitary–adrenal (HPA) axis regulates the stress response we investigate whether parameters of the HPA axis at the levels of selected mRNA transcripts and endocrine hormones are altered in mGluR7-deficient mice. Over all, mGluR7$^{-/-}$ mice showed only moderately lower serum levels of corticosterone and ACTH compared with mGluR7$^{+/+}$ mice. More strikingly however, we found strong evidence for upregulated glucocorticoid receptor (GR)-dependent feedback suppression of the HPA axis in mice with mGluR7 deficiency: (i) mRNA transcripts of GR were significantly upregulated in the hippocampus of mGluR7$^{-/-}$ animals, (ii) similar increases were seen with 5-HT$_{1A}$ receptor transcripts, which are thought to be directly controlled by the transcription factor GR and finally (iii) mGluR7$^{-/-}$ mice showed elevated sensitivity to dexamethasone-induced suppression of serum corticosterone when compared with mGluR7$^{+/+}$ animals. These results indicate that mGluR7 deficiency causes dysregulation of HPA axis parameters, which may account, at least in part, for the phenotype of mGluR7$^{-/-}$ mice in animal models for anxiety and depression. In addition, we present evidence that protein levels of brain-derived neurotrophic factor are also elevated in the hippocampus of mGluR7$^{-/-}$ mice, which we discuss in the context of the antidepressant-like phenotype found in those animals. We conclude that genetic ablation of mGluR7 in mice interferes at multiple sites in the neuronal circuitry and molecular pathways implicated in affective disorders.

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INTRODUCTION

Stress-related psychiatric disorders such as depression and anxiety disorders are an enormous public health concern (Wong and Licinio, 2001, 2004). Further, current treatments which largely target monoamine or GABAergic neurotransmission, are of limited efficacy in a significant proportion of patients and are associated with a troublesome side-effect burden in many others resulting in a huge need for the development of novel treatment strategies. Accumulating evidence suggests a role for glutamatergic systems and in particular metabotropic glutamate receptors (mGluRs) in the pathophysiology of stress-related behavioral disorders (Krystal *et al*, 2002; Paul and Skolnick, 2003; Bergink *et al*, 2004; Swanson *et al*, 2005). Using genetically modified mice we and others have recently implicated a role for group-III mGluR subtypes (including mGluR4, -6, -7, and -8) in anxiety and depression (Masugi *et al*, 1999; Linden *et al*, 2002; Cryan *et al*, 2003).

mGluR7, which is the most highly conserved of all mGluR subtypes across different mammalian species (Makoff *et al*, 1996; Flor *et al*, 1997), is abundant in brain regions that are known to be critical for the manifestation of anxiolysis and antidepressant action, such as the hippocampus, the

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amygdala, and the locus coeruleus (Kinoshita *et al*, 1998). Mice lacking mGluR7 have deficits in amygdala-dependent behaviors (fear response and conditioned taste aversion), but show no alterations in locomotor activity or pain sensitivity (Masugi *et al*, 1999). Electrophysiological analysis in mGluR7$^{-/-}$ mice further suggests that this receptor is a frequency-dependent regulator of neurotransmitter release (Sansig *et al*, 2001), and modulates short-term synaptic plasticity in the hippocampus (Bushell *et al*, 2002). Moreover, we have demonstrated that mGluR7 ablation in mice is associated with changes in animal behavioral paradigms predictive of antidepressant and anxiolytic action, which suggests that drugs acting at mGluR7 may provide novel treatments for psychiatric disorders such as depression and anxiety (Cryan *et al*, 2003). These tests, including the forced swim test, tail suspension test, light–dark box test, and the stress-induced hyperthermia test, all involve behaviorally observing the animal's response to a novel stressful situation. Hence our data suggest that mGluR7 may play a role in the integration of the stress response to aversive stimuli.

The hypothalamic–pituitary–adrenal (HPA) axis is the key regulator of the stress reaction. Dysregulation of this axis is thought to play a central role in the pathophysiology of anxiety and depressive disorders (Plotsky *et al*, 1998; Steckler *et al*, 1999; Holsboer, 2000; Posener *et al*, 2000; Lopez *et al*, 1998; De Kloet *et al*, 2005). During stress, the synthesis of corticotrophin-releasing factor (CRF) in the hypothalamic paraventricular nucleus (PVN) increases and when CRF is released and reaches the anterior pituitary gland, it promotes the release of adrenocorticotropic hormone (ACTH), which, in turn, induces the synthesis and release of glucocorticoids from the adrenal cortex. Two types of glucocorticoid receptors (GRs) are well known in brain; the mineralcorticoid receptor (MR) and the GR, the latter having less affinity to corticosterone than MR (De Kloet *et al*, 1998). During stress the main function of GR in the brain appears to suppress stress-induced hyperactivity of the HPA axis at the level of the PVN, the anterior pituitary, but also at the hippocampal level (reviewed in Jacobson and Sapolsky, 1991; Barden, 2004).

Furthermore, the serotonin (5-HT) system and the HPA axis have complex inter-relationships (Porter *et al*, 2004). In particular, the 5-HT$_{1A}$ receptor (5-HT$_{1A}$R) is very susceptible to modulation by stress and HPA-axis activation and is well known to play an important role in the pathophysiology of mood disorders (Lopez *et al*, 1998; Cryan and Leonard, 2000; Leitch *et al*, 2003). Accumulating evidence points also to an important role for the neurotrophin brain-derived neurotrophic factor (BDNF) in both the pathophysiology of mood disorders and in the therapeutic effects of antidepressants (Duman, 2002; Castren, 2004; Hashimoto *et al*, 2004). Further, hippocampal BDNF levels are modified by many types of chronic stress (Smith *et al*, 1995; Vaidya *et al*, 1997; MacQueen *et al*, 2003).

Given that mGluR7$^{-/-}$ mice have a phenotype that is indicative of an altered behavioral response to stress, we hypothesized that mGluR7 affects stress-related systems at different levels. Therefore, we used mGluR7$^{-/-}$ mice and tested the effects on levels of stress-related genes and hormones, and also examined the effects on the negative feedback system of the HPA axis by using the dexamethasone suppression test. To further test the possibility that BDNF is involved in antidepressant and anxiolytic phenotype of mGluR7$^{-/-}$ mice, we investigated BDNF mRNA and protein levels. These studies reveal HPA-axis dysregulation in mGluR7$^{-/-}$ mice, which may provide a physiological correlate for the behavioral phenotype of these mice.

MATERIALS AND METHODS

Animals

mGluR7$^{-/-}$ mice were generated as described previously from E14 (129/Ola) embryonic stem cells (Sansig *et al*, 2001). Larger age-matched groups of mGluR7$^{-/-}$ and mGluR7$^{+/+}$ mice were generated using a specific pathogen-free (SPF) breeding colony of 30 F14, B6-mGluR7$^{+/-}$ males mating with 60–90 F14, B6-mGluR7$^{+/-}$ females. All the mice in the studies reported here carried wild-type or mutant mGluR7 alleles on a 14th generation C57BL/6 genetic background. Mice were weaned at the age of 3 weeks, when tail biopsies were also taken for genotyping. Subsequently, genotyped mice were moved to a non-SPF conventional facility in which male animals were used for experiments at the ages of 3–9 month. Housing was at room temperature, in a 12 h light:dark cycle, with lights on at 0600. Male animals were used in all experiments. Food pellets and tap water were available *ad libitum* unless stated otherwise. Following decapitation of mice (mGluR7$^{-/-}$ and mGluR7$^{+/+}$ mice in a randomized order), bloods were collected and brains were immediately removed and frozen on dry ice and stored at -80°C. All animal experiments were subject to institutional review and conducted in accordance with the Veterinary Authority of Basel-Stadt, Switzerland.

Blood Sample Collection for Basal and Stress-Induced Conditions

In order to investigate the effects of mGluR7$^{-/-}$ mice on basal corticosterone and ACTH levels, male mGluR7$^{-/-}$ ($n=10$) and littermate mGluR7$^{+/+}$ ($n=10$) mice were decapitated and the blood was collected. Two batches of mice were killed, one between 0700 and 0800 and the other between 1700 and 1730. For the stress-induced hormone levels, male mGluR7$^{-/-}$ and littermate mGluR7$^{+/+}$ mice were subjected to swim stress (6 min) into plexiglass cylinders (24 cm tall $\times$ 21 cm in internal diameter) filled with water (23–25°C). Mice were decapitated 5 or 90 min after the stress session ($n=10$ per genotype and per time point) in another room and the blood was collected between 0800 and 1100. Simultaneously, nonstressed male mice ($n=10$ per genotype) were killed, which constituted control groups.

Blood Sample Collection following Dexamethasone Suppression Test

The dexamethasone suppression test was carried out essentially as described by Groenink *et al* (2002). Male mGluR7$^{-/-}$ and littermate mGluR7$^{+/+}$ mice were injected subcutaneously (SC) with saline, 0.03 or 0.1 mg/kg dex-

amethasone-21-phosphate disodium salt (Fluka, Buchs, Switzerland). After 6 h, mice were decapitated rapidly (within 30 s after first touching the cage) in an adjacent room, and trunk blood was collected ($n = 10$ per genotype).

Hormone Measurements

Plasma corticosterone and ACTH concentrations were measured using commercially available radioimmunoassay kits (ICN Biomedicals, Costa Mesa, CA, USA). The inter- and intra-assay coefficients of variability for ACTH and corticosterone were similar to the manufacturer's reported values with a detection limit of 5–10 pg/ml.

Extraction of BDNF from Tissues

Various brain regions were freshly dissected, weighed, collected on dry ice, and stored at −80°C, or dissected as soon as the frozen brains started to thaw as specified below. The extraction of BDNF was performed essentially as described previously (Kolbeck *et al*, 1999). Briefly, 20–40 vol/wt of extraction buffer (0.05 M sodium acetate, pH 4.0, 1.0 M sodium chloride, 1% (vol/wt) BSA (A-9418 of Sigma, Buchs, Switzerland), 1% Triton X-100, protease inhibitor cocktail tablets (Cat.No. 1 836 153, Roche, Rotkreuz, Switzerland)) was added and the tissues sonicated to homogeneity. The homogenates were kept on ice for 1 h and the sonication repeated three times. The homogenates were centrifuged (15 min, $10^5 g$), the supernatants (first extraction) collected, and the pellets resuspended in 20–40 volumes of extraction buffer. After another centrifugation (15 min, $10^5 g$), the supernatants were collected (second extraction) and the pellets resuspended in 20–40 volumes of extraction buffer. After another centrifugation (15 min, $10^5 g$), the supernatants were collected again (third extraction).

BDNF Immunoassay

Quantification of BDNF from tissue extracts was carried out essentially as described by Kolbeck *et al* (1999). Plates (MaxiSorp plates, Nunc, Rosklide, Denmark) were coated with carbonate buffer, pH 9.7 containing 400 ng per well of biotinylated first anti-BDNF antibody (courtesy of Y.-A. Barde, Basel University, Switzerland) overnight at 4°C and incubated with blocking buffer (phosphate-buffered saline, 2% BSA, 0.1% Triton X-100) for 1 h at room temperature. Standard amounts of BDNF were diluted in extraction buffer (0.1–6.25 ng/ml), and 25 µl per well of each concentration was applied. A measure of 25 µl per well of the extraction buffer alone (negative control) was used to check background activity. Similarly, 25 µl per well of different dilutions of the tissue-extract supernatants was added to the wells. Subsequently, 175 µl per well of incubation buffer (0.1 M potassium/sodium phosphate, pH 8.0, 1% BSA, protease inhibitor cocktail tablets (Cat.No. 1 836 153, Roche, Rotkreuz, Switzerland)) containing 100 mU/ml peroxidase (POD)-conjugated anti-BDNF antibody (courtesy of Y.-A. Barde, Basel University, Switzerland) was applied and incubated overnight at 4°C. Plates were washed with washing buffer (phosphate-buffered saline, 0.1% Triton X-100) three times and incubated with 200 µl per well of the BM blue POD substrate (Roche, Rotkreuz, Switzerland) for 30 min at room temperature. The reaction was stopped with 50 µl of 1 M sulfonic acid per well, and the intensity of the reaction product was measured in a luminometer at 490 nm. BDNF levels in tissue extracts were determined by comparison with the standard curve.

'Real-Time' Reverse Transcriptase (RT) PCR Assays

Various brain regions were freshly dissected, weighed, collected on dry ice, and stored at −80°C, or dissected as soon as the frozen brains started to thaw. Mouse brain regions were disrupted (2 × 2 min at 20 Hz) by using a Mixer Mill MM 300 (Retsch GmbH & Co. KG, Haan, Germany). Total RNA was isolated from disrupted brain tissue using the commercially available 'Absolutely RNA™' RT-PCR Miniprep Kit (Stratagene, Amsterdam, The Netherlands). The quantity of RNA was determined by OD measurement using a NanoDrop®ND-1000 Spectrophotometer. To remove traces of genomic DNA contamination, an aliquot of 400 ng total RNA was digested with RNAse-free DNAse I (RNAse-Free DNase Set, Qiagen, Hilden, Germany). The enzyme was inactivated by addition of EDTA and by heating up to 65°C. DNAse I-treated total RNA was reverse transcribed into cDNA by random priming using StrataScript™ Reverse Transcriptase (Stratagene, Amsterdam, The Netherlands) at 42°C for 60 min. Primers and probes were designed with the aid of the Primer Express software (Version 1.0; Applied Biosystems, Foster City, CA, USA). The probes were labeled at the 5′ end with the reporter fluorophore 6-carboxyfluorescein (FAM) and at the 3′ end with the quencher 6-carboxytetramethylrhodamine (TAMRA). Primers and probes were synthesized commercially (Microsynth, Balgach, Switzerland). The sequences of the primer pairs and probes were as shown in the table of sequences (below). 'Real-Time' RT PCR (TaqMan®) assays were performed using the qPCR™ Mastermix (Eurogentec, Seraing, Belgium) on an ABI Prism® 7700 sequence Detection System (Applied Biosystems, Foster City, CA, USA). In all assays for the target genes, primers and probes were used at 300 and 175 nM, respectively. Assays for the internal control gene 18S rRNA were performed by using the '18S Genomic Endogenous Control Kit' (Eurogentec, Seraing, Belgium). Relative levels of the target mRNA are reported after normalization to 18S rRNA, which was also detected by 'Real-Time' RT PCR. The normalized raw data, representing the average values of two independent determinations, were analyzed by using the comparative C_T method, as described in details by Livak and Schmittgen (2001).

Statistical Analysis

Relative gene and protein expression data are reported as means ± SEM. Values of mGluR7$^{-/-}$ mice were compared with those of mGluR7$^{+/+}$ mice by using the two-tailed Student's *t*-test. The level of significance was set at $p < 0.05$.

RESULTS

Analysis of Transcript Levels of Stress-Related Genes

In an attempt to address the physiological basis of the behavioral phenotype of mGluR7$^{-/-}$ mice, which showed

Table of Sequences: Primers and Probes Used in 'Real-Time' RT PCR Assays for Assessing Relative Expression Profiles of Target Genes

Gene product	GenBank/ RefSeq accession number	Sequences of primer and probe
BDNF	BC034862	5′-CCA TAA GGA CGC GGA CTT GT-3′ 5′-GAG GCT CCA AAG GCA CTT GA-3′ 5′-FAM-CAC TTC CCG GGT GAT GCT CAG CA-TAMRA-3′
CRF	AY128673	5′-AGC AGT TAG CTC AGC AAG CTC A-3′ 5′-GGC CAA GCG CAA CAT TTC-3′ 5′-FAM-TCC CGA TAA TCT CCA TCA GTT TCC TGT TGC T-TAMRA-3′
CRF1	X27305	5′-TCC GGT GCC TGA GAA ACA TC-3′ 5′-CGT GGC GTT GCG TAG GA-3′ 5′-FAM-TCC ACT GGA ACC TCA TCT CGG CTT TC-TAMRA-3′
GR	X04435	5′-CGG GAC CAC CTC CCA AA-3′ 5′-CCC CAT AAT GGC ATA CCG AA-3′ 5′-FAM-TCT GCC TGG TGT GCT CCG ATG AAG-TAMRA-3′
5-HT_{1A}	NH012585	5′-GCT CAT GCT GGT TCT CTA CGG-3′ 5′-GAC AGT CTT GCG GAT TCG GA-3′ 5′-FAM- CGC ATC TTC AGA GCC GCA CGC-TAMRA-3′
mGluR7	NM000844	5′-GCG CTC GAA CAG TCG CTT A-3′ 5′-CGC ACG TCG GAG GTG TC-3′ 5′-FAM-TTT CGT CCA GGC GCT CAT CCA GA-TAMRA-3′
MR	XM356093	5′-TGG ATG TGG TTG GAT GTA GGG-3′ 5′-TCT ACC TGT TGC AGC GCT TG-3′ 5′-FAM-CCA CGA CAA CCC TGC TGC GGA G-TAMRA-3′

reduced anxiety- and depression-like features, we started with an analysis of gene expression levels associated with the stress system of the brain. The genes chosen were those encoding GR, MR, CRF, CRF1 receptor and the 5-HT_{1A}R. Figure 1 shows expression levels of mGluR7 and these five stress-related genes in the hippocampus, the hypothalamus and the prefrontal cortex of mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice. As expected, there was no detectable level of mGluR7 mRNA either in the hippocampus, in the hypothalamus or in the prefrontal cortex of mGluR7$^{-/-}$ mice. In the hippocampus, mGluR7$^{-/-}$ mice showed an increased GR level compared to mGluR7$^{+/+}$ mice, which was highly significant in p.m. killed animals ($p = 0.007$; Figure 1d) but did not reach statistical significance in a.m. killed animals ($p = 0.093$; Figure 1a). 5-HT_{1A}R levels were dramatically increased in the hippocampus and in the prefrontal cortex of both a.m. ($p = 0.002$ and 0.016, respectively) and p.m. ($p = 0.002$ and 0.038, respectively) killed mGluR7$^{-/-}$ mice, when compared to mGluR7$^{+/+}$ mice (Figure 1a, c, d, f). The elevated levels of 5-HT_{1A}R mRNA in the hypothalamus of mGluR7$^{-/-}$ mice did not quite reach the level of statistical significance ($p = 0.055$ and 0.064; for a.m. and p.m. killed animals respectively; Figure 1b, e). Expression levels of MR, CRF, and CRF receptor type 1 (CRFR1) remained unchanged in the hippocampus, the hypothalamus and the prefrontal cortex between the two genotypes. Interestingly, there was a trend towards a significant increase in CRFR1 ($p = 0.076$) and GR ($p = 0.12$) in the prefrontal cortex at the afternoon time point only.

Basal Conditions of Stress Hormone Levels

Basal levels of serum corticosterone and ACTH concentrations were measured in mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice; although mGluR7$^{-/-}$ mice showed lower levels of both corticosterone and ACTH, especially in the morning (Figure 2), this did not reach statistical significance presumably due to the large variation between animals. Body weight and adrenal gland weight of mGluR7$^{-/-}$ mice were somewhat lower than those of mGluR7$^{+/+}$ mice; however adrenal gland weight normalized to body weight showed no difference between mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice (Table 1). This was also the case in female mice (data not shown).

Effects of Swim Stress on Stress Hormone Levels

In a next experiment, we used mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice (10 male animals each) and tested the level of corticosterone and ACTH in response to swim stress. When blood samples were taken between 0800 and 1100, mGluR7$^{-/-}$ mice showed remarkably lower levels of corticosterone (Figure 3a) and ACTH (Figure 3b) compared to mGluR7$^{+/+}$ mice (corticosterone; 9.55 ± 1.86 *vs* 27.57 ± 6.86 ng/ml, $p = 0.029$, ACTH; 33.65 ± 3.00 *vs* 75.55 ± 18.78 ng/ml, $p = 0.054$). These basal levels without swim stress showed less animal to animal variation than data of Figure 2. Immediately after swim stress, mGluR7$^{-/-}$ mice showed an increase in the levels of corticosterone (Figure 3c) and ACTH (Figure 3d) comparable to mGluR7$^{+/+}$ mice, and there was no difference between the two genotypes. In addition, 90 min after swim stress, the stress-induced increase of corticosterone and ACTH levels were recovered at least partially in both genotypes (Figure 3e, f), however interestingly the levels of both stress hormones in mGluR7$^{-/-}$ mice were lower than in mGluR7$^{+/+}$ mice, which may lead to the suggestion that mGluR7$^{-/-}$ mice may affect the recovery of the hormonal system from the stress, although these differences did not reach statistical significance.

Dexamethasone Suppression Test

We also examined levels of plasma corticosterone and ACTH when mice were injected with the synthetic

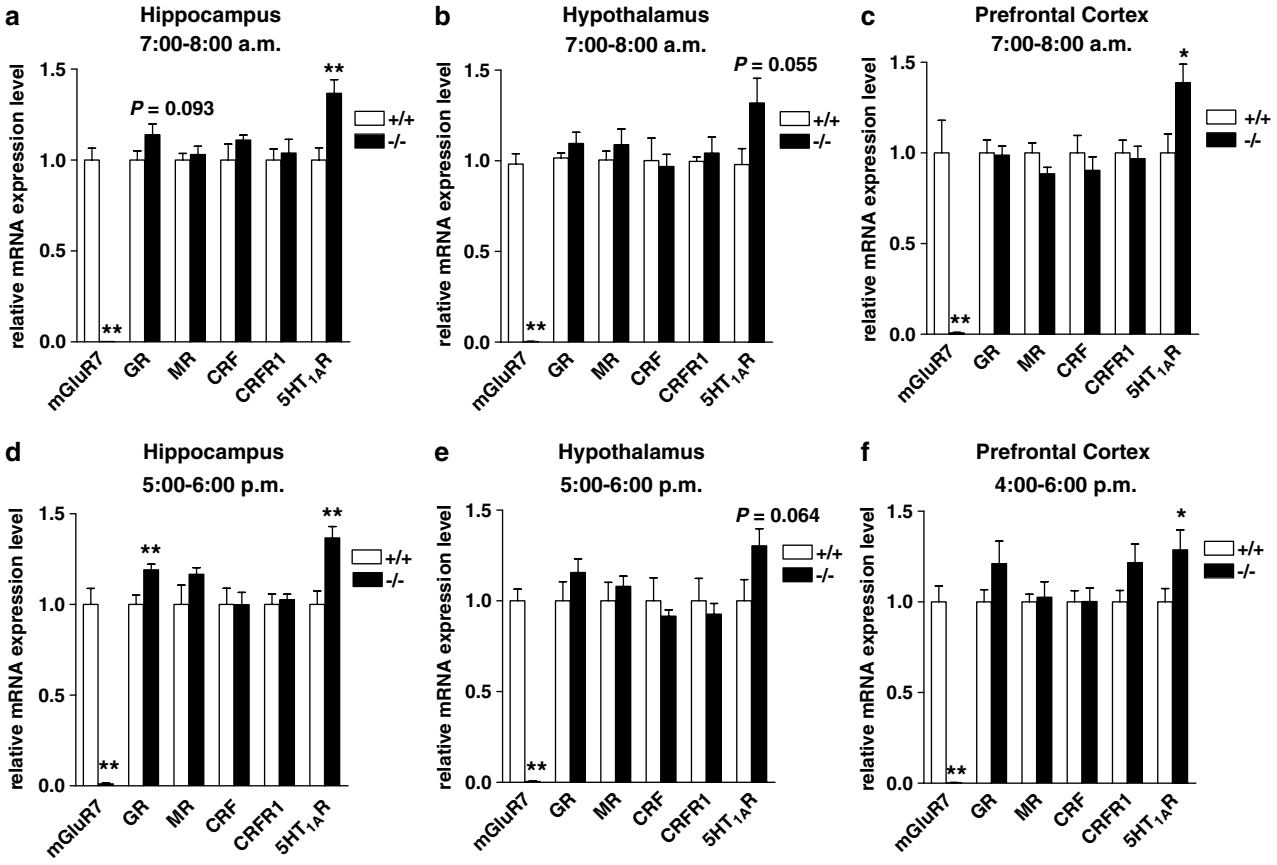

Figure 1 Relative transcript levels of mGluR7 and stress-related genes in the hippocampus (a and d), the hypothalamus (b and e) and the prefrontal cortex (c and f) of male mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice. Sample collection was performed in the morning (0700–0800; a–c) and in the afternoon (1600–1800; d–f). All values are normalized to the expression level of the reference gene 18S rRNA. The mRNA levels of mGluR7$^{+/+}$ mice are set arbitrarily to the expression level of 1.0. Expression levels in mGluR7$^{-/-}$ mice (filled columns) are shown relative to mGluR7$^{+/+}$ animals (opened columns). The data, displayed as means ± SEM, are the average values of two independent determinations (a, b, d, and e) or the average of triplicate determinations (c and f). Each bar represents a group of 7–12 animals. Statistical analysis was carried out by Student's *t*-test. $^{**}p<0.01$, $^{*}p<0.05$; groups that differed significantly from mGluR7$^{+/+}$ mice.

Table 1 Body Weight and Adrenal Gland Weight of Male mGluR7$^{+/+}$ and mGluR7$^{-/-}$ Mice

		Andrenal gland weight		
Genotype	**Body weight (g)**	**mg**	**mg/10 g BW**	***n***
+/+	30.3 ± 0.6	3.2 ± 0.3	1.0 ± 0.1	8
−/−	26.6 ± 1.1	2.7 ± 0.3	1.0 ± 0.1	8

BW, body weight.
Results represent means ± SEM.

glucocorticoid, dexamethasone, to investigate the GR-mediated negative feedback system of the HPA axis. Figure 4 illustrates plasma concentrations of corticosterone and ACTH in mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice injected with saline, 0.03 or 0.1 mg/kg dexamethasone subcutaneously (SC). Plasma corticosterone concentrations of vehicle-treated mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice were not different at afternoon time points (1400–1500; Figure 4a) which is probably due to circadian pattern in the levels of circulating glucocorticoids. Therefore, this further illustrates that caution needs to be taken regarding the time of day that killing of animals is carried out for the assaying of stress hormones.

Administration of 0.03 mg/kg dexamethasone dramatically reduced plasma corticosterone level in mGluR7$^{-/-}$ mice (Figure 4c) and there was a significant difference between these two genotypes ($p = 0.041$). The injection of 0.1 mg/kg dexamethasone showed a remarkable suppression of corticosterone levels both in mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice (Figure 4e) compared to vehicle-treated animals of both genotypes (Figure 4a) and there was no significant difference anymore between the two genotypes. On the other hand, at the same time points (1400–1500), the plasma ACTH level of vehicle-treated mGluR7$^{-/-}$ mice was significantly less than that of mGluR7$^{+/+}$ mice (Figure 4b; $p = 0.022$). Difference between ACTH levels and corticosterone levels may reflect the different temporal patterns of these stress hormones. Full online analysis of these hormones maybe required to discriminate these differences in detail. Administration of 0.03 mg/kg dexamethasone resulted in reduced plasma ACTH levels in mGluR7$^{-/-}$ and mGluR7$^{+/+}$ mice (Figure 4d); ACTH was lower in mGluR7$^{-/-}$ mice than in mGluR7$^{+/+}$ mice, although this was not quite statistically significant ($p = 0.061$). With the injection of 0.1 mg/kg dexamethasone, both mGluR7$^{+/+}$

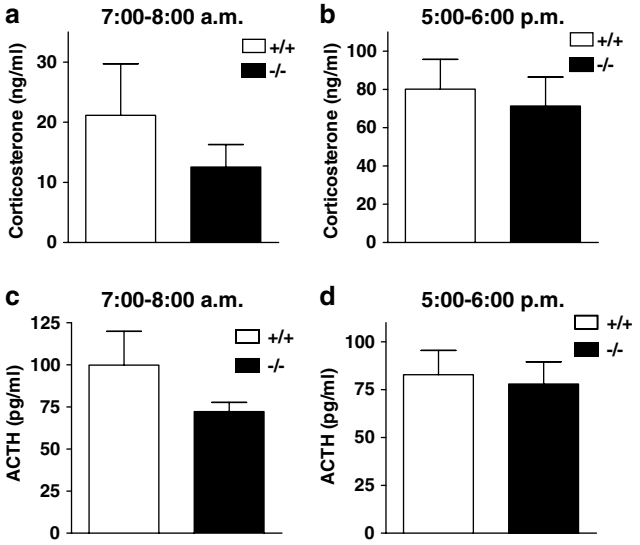

Figure 2 Basal plasma corticosterone (a and b) and ACTH (c and d) concentrations of male mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice ($n=8$–10 per group). Sample collection was performed in the morning (0700–0800; a and c) and in the afternoon (1700–1800; b and d). All bars represent means ± SEM. Statistical analysis was done by Student's *t*-test. No mGluR7$^{-/-}$ group showed significant difference when compared with the mGluR7$^{+/+}$ group ($p>0.05$).

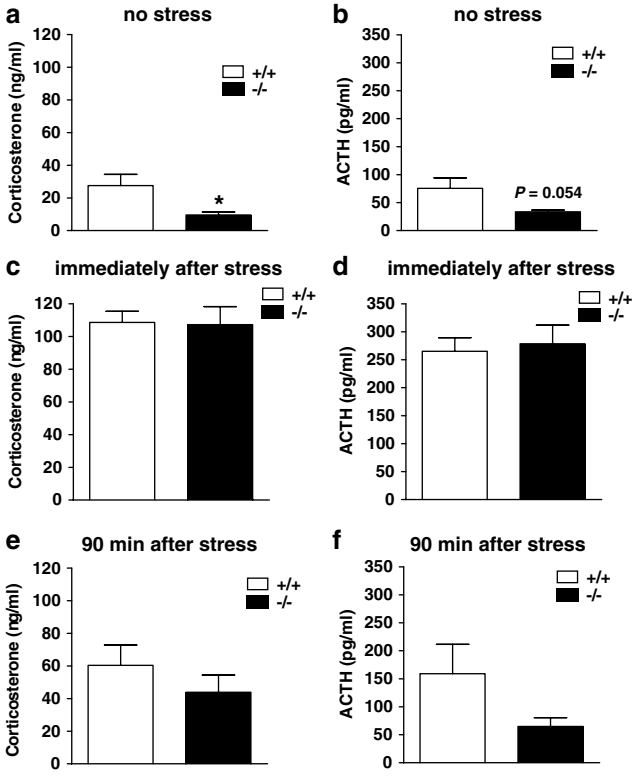

Figure 3 Baseline and swim-stress-modified levels of plasma corticosterone (a, c, and e) and ACTH (b, d, and f) concentrations of male mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice ($n=9$–10 per group). The duration of swim stress was 6 min. Sample collection was performed between 0800 and 1100. All bars represent means ± SEM. Statistical analysis was carried out by Student's *t*-test. *$p<0.05$; groups that differed significantly from mGluR7$^{+/+}$ mice.

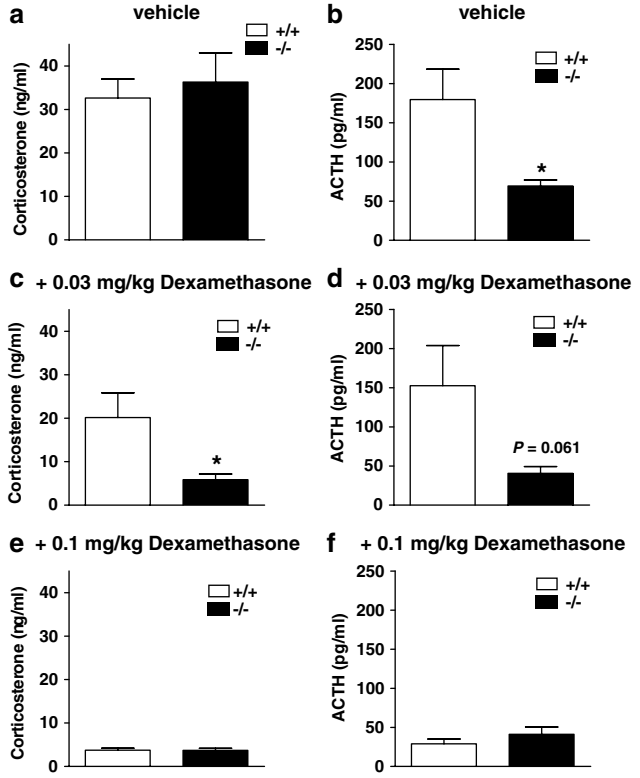

Figure 4 Baseline and dexamethasone-inhibited corticosterone (a, c, and e) and ACTH (b, d, and f) concentrations of male mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice ($n=8$–10 per group). Sample collection was performed between 1400 and 1500, 6 h after subcutaneous (SC) injection of dexamethasone. All bars represent means ± SEM. Statistical analysis was carried out by Student's *t*-test. *$p<0.05$; groups that differed significantly from mGluR7$^{+/+}$ mice.

and mGluR7$^{-/-}$ mice showed suppressed ACTH levels (Figure 4f) and there was no significant difference between the genotypes.

Expression Levels of BDNF Transcript and Protein

Next, we investigated whether BDNF levels are changed in mGluR7$^{-/-}$ mice by using RT-PCR and ELISA assays for mRNA and protein level determination, respectively. Sample collection was in the afternoon (1600–1800). There was no significant difference in BDNF mRNA either in the hippocampus, in the hypothalamus, or in the prefrontal cortex between mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice (Figure 5a). However, mGluR7$^{-/-}$ mice showed a significantly increased level of BDNF protein in the hippocampus as compared to mGluR7$^{+/+}$ mice ($p=0.022$), although the prefrontal cortex showed no difference (Figure 5b) which demonstrates that there is a level of regional specificity to the increases in BDNF. We confirmed this result in the hippocampus by repeating the experiment independently with 9–12 mice per group in tissue that was freshly dissected (Figure 5c). We obtained the same differences between the two genotypes ($p=0.001$), which demonstrates that mGluR7$^{-/-}$ mice show a 20–25% increase of BDNF protein level in the hippocampus as compared to mGluR7$^{+/+}$ animals.

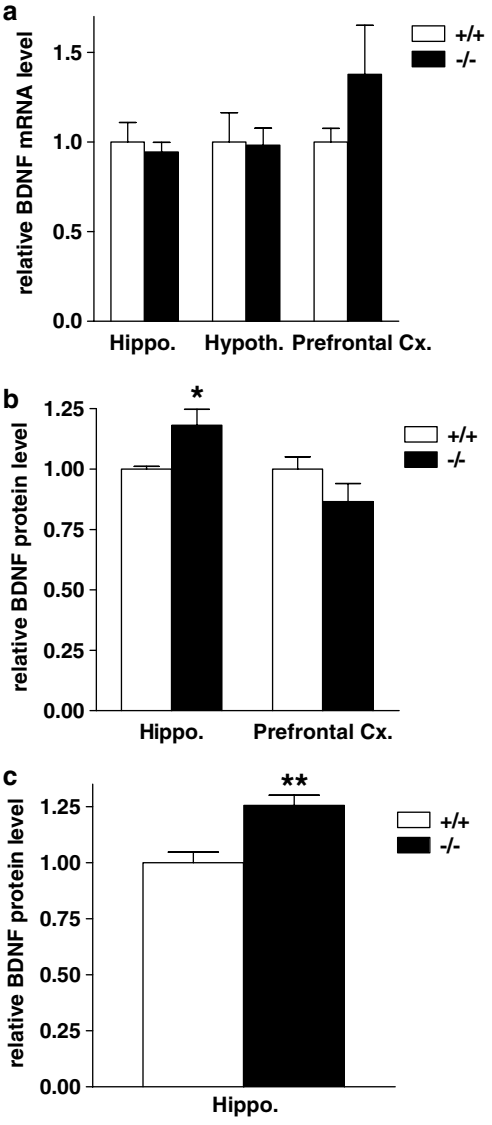

Figure 5 Relative BDNF transcript levels in the hippocampus, the hypothalamus and the prefrontal cortex (a) and protein levels in the hippocampus (b and c) and the prefrontal cortex (b) of male mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice ($n=8$–12 per group). Sample collection was performed in the afternoon (1600–1800) and various regions were dissected either from frozen/freshly thawed brains (a and b) or fresh brains (c). The relative transcript levels are normalized to the expression level of the reference gene 18S rRNA. The mRNA data, displayed as means ± SEM, are the average value of two independent determinations in the hippocampus and the hypothalamus, or the average value of triplicate determinations in the prefrontal cortex ($n=8$–11 animals per group). Expression levels in mGluR7$^{-/-}$ mice (filled columns) are shown relative to those in mGluR7$^{+/+}$ mice (opened columns) that are set arbitrarily to the expression level of 1.0. Statistical analysis was carried out by Student's *t*-test. $^{**}p<0.01$, $^{*}p<0.05$; groups that differed significantly from mGluR7$^{+/+}$ mice. Hippo., hippocampus; Hypoth., hypothalamus; Prefrontal Cx., prefrontal cortex.

DISCUSSION

The present studies demonstrate that selective ablation of the group-III mGluR subtype 7 is associated with several, but selective, changes in molecular targets that participate in the stress response and in psychopathological states. Thus, our findings suggest that drugs acting at mGluR7 may provide innovative and novel approaches to modify stress-related psychiatric disorders such as depression and anxiety.

mGluR7 ablation leads to increased levels of GR and the 5-HT$_{1A}$R in the hippocampus without altering MR, CRF, or CRFR1 mRNA levels. 5-HT$_{1A}$R mRNA was also upregulated in the prefrontal cortex. These transcripts were chosen for analysis because of their key regulatory role in the mammalian stress system (De Kloet *et al*, 2005), which we wanted to better understand in the context of mGluR7 ablation. To our knowledge there are no obvious similarities in the transcriptional control region of the chosen genes, which does not exclude the possibility that they might be regulated in a concerted fashion. Interestingly, we find that only GR and 5-HT$_{1A}$R transcript levels are altered in mGluR7-deficient mice, which implies a selective effect on the feedback system in the HPA axis. The expression levels of both of these genes have previously been shown to be altered by stress, glucocorticoid and antidepressants (Seckl and Fink, 1992; Lopez *et al*, 1998; Okugawa *et al*, 1999; Karandrea *et al*, 2002; Hugin-Flores *et al*, 2004). Further, mice with functional downregulation/ablation of these two receptors have altered emotional behaviors relevant to anxiety and depression (Toth, 2003; Urani and Gass, 2003; Boyle *et al*, 2005). The HPA-axis activity is controlled by a feedback mechanism triggered mainly through the stimulation of GRs located in the cerebral cortex, the hippocampus, and the hypothalamus (Magarinos *et al*, 1987; Diorio *et al*, 1993; Feldman and Weidenfeld, 1999; Mizoguchi *et al*, 2003), and a reduced GR expression and function has been proposed to contribute to HPA-axis alterations in depression (Holsboer and Barden, 1996; Pariante and Miller, 2001; Webster *et al*, 2002). Further, it is clear that the expression and function of the 5-HT$_{1A}$R is regulated by glucocorticoids and GRs (Chalmers *et al*, 1994; Laaris *et al*, 1995; Hery *et al*, 2000; Ou *et al*, 2001; Andrews *et al*, 2004; Froger *et al*, 2004; De Kloet *et al*, 2005). Taken together these data suggest that there is a selective dysregulation of the integration of the stress response in mGluR7$^{-/-}$ mice which is opposite to that seen in some human psychopathological states.

Glutamatergic neurotransmission has long been implicated as a critical regulator of the stress response (van den Pol *et al*, 1990; Brann, 1995). mGluRs in particular appear to play a crucial role in mediating neuroendocrine responses. Specifically, the nonselective mGluR agonist (1S,3R)-1-aminocyclopentane-1,3-dicarboxylate (ACPD), when administered i.c.v., induced a significant increase in plasma corticosterone (Lang and Ajmal, 1995). Whereas, an involvement of group-I and possibly group-II mGluRs has been reported (Johnson *et al*, 2001; Bradbury *et al*, 2003; Scaccianoce *et al*, 2003), it has also been demonstrated that i.c.v. administration of the nonselective group-III mGluR agonists L-AP4 and L-SOP activates the HPA axis (Johnson *et al*, 2001). Our current data showing both altered corticosterone levels and feedback regulation in mGluR7$^{-/-}$ mice suggest that mGluR7, at least in part, may modulates the increase in stress hormones induced by group-III mGluR agonists. The mechanism underlying this increase is currently unclear. However, Johnson *et al* (2001), based on an earlier model of Tasker *et al* (1998), suggested that as group-III mGluRs regulate the activity of GABA

interneurones in the hypothalamus (Schrader and Tasker, 1997), agonists, such as L-AP4 and L-SOP, might act by decreasing L-glutamate release from hippocampal–hypothalamic tracts. Thus, there would be a decreased tone driving GABAergic interneurones and a disinhibition on corticotrophin release factor-containing PVN neurons of the hypothalamus. Alternatively, it has been speculated that group-III agonists might be acting directly on presynaptic terminals of the GABAergic interneurones and activate hetereo-autoreceptors and thus group-III mGluR stimulation could directly limit the degree of inhibitory tone that is on the PVN cells (Johnson *et al* 2001; Tasker *et al*, 1998). Both scenarios might explain how the absence of mGluR7 leads to a state of neuroendocrine dysfunction as has been seen in the present studies which may subsequently induce an altered response in stress-related behaviors.

It is important to note that there are many brain regions other than the hippocampus and hypothalamus, which contribute to stress-related pathology and future studies should focus on whether mGluR7 plays a role in regulating stress-relevant genes in structures such as the amygdala, lateral septum, cortex, dorsal raphe nucleus, and locus coeruleus. Furthermore, the analysis of the expression of mRNA transcripts in different regions within the hippocampus (eg dentate gyrus, CA1 etc), in addition to the examination of the cellular localization of the observed changes in various brain regions will aid in the understanding, at a circuit level, of the role of mGluR7 in stress-related pathologies.

To further examine the potential functional consequences of the changes in stress-related genes we assessed baseline and stress-induced concentrations of corticosterone and ACTH in mGluR7$^{-/-}$ mice. There was a strong trend toward a lower basal corticosterone and ACTH level which suggests that mice lacking mGluR7 function at abnormal homeostatic concentrations of circulating glucocorticoids. However, both genotypes responded equally, in terms of neuroendocrine activation, to swim stress insults which resulted in a marked increase in corticosterone and ACTH levels. It is possible that a ceiling response was observed and that a differential effect could be observed if a less severe stress was employed. However, the rationale for choosing this stressor was based on the fact that mGluR7$^{-/-}$ mice have an altered behavioral response to this stressor (Cryan *et al*, 2003). It is interesting to note that in the swim-stress studies the lower basal levels of stress hormones, which we previously observed in a separate cohort of mice, was also detected and at a level that was statistically significant (see Figure 3), thus giving further credence to the initial findings.

To assess whether there is an altered GR-mediated negative feedback we assessed the effects of the synthetic glucocorticoid dexamethasone which at low doses, such as that used here, acts as a GR agonist (Meijer *et al*, 1998; Cole *et al*, 2000; Groenink *et al*, 2002). Mice lacking mGluR7 show a clear hypersensitivity to dexamethasone at the lower dose tested (0.03 mg/kg). This suggests that there is indeed an altered functional negative feedback of the HPA axis. This enhanced suppression is opposite to that seen in many depressed patients who have a blunted hormonal response to dexamethasone (Holsboer *et al*, 1980; Carroll, 1982). This correlates well with the antidepressant-like response observed in behavioral studies with mGluR7$^{-/-}$ mice (Cryan *et al*, 2003). Despite its historical widespread use in clinical settings there are surprisingly few studies employing the dexamethasone suppression test in genetically modified mice (Cryan and Mombereau, 2004). Here we demonstrate that it can be a sensitive marker of HPA-axis dysregulation. Interestingly, although mGluR7$^{-/-}$ mice have a dysregulated HPA axis, these alterations occur independent of any gross abnormality in adrenal weight with both genotypes having the same relative adrenal weight (Table 1). Increases in relative adrenal weight have been observed following chronic stress (Watanabe *et al*, 1992) and in major depression (Nemeroff *et al*, 1992).

We observed an increase in BDNF protein levels in the hippocampus of mGluR7$^{-/-}$ mice in a reproducible fashion, which also correlates well with the antidepressant-like phenotype observed behaviorally. Intracerebral ventricular or intrahippocampal administration of BDNF has been shown to have an antidepressant-like effect in various animal tests of antidepressant activity (Siuciak *et al*, 1997; Shirayama *et al*, 2002; Hoshaw *et al*, 2005). Furthermore, antidepressant medications have been shown to increase BDNF levels in the hippocampus (Nibuya *et al*, 1995; De Foubert *et al*, 2004; Russo-Neustadt *et al*, 2004; but see Altar *et al*, 2003; Coppell *et al*, 2003; Jacobsen and Mork, 2004). Further evidence for a role of BDNF in antidepressant action comes from studies using genetically modified mice with impaired levels of BDNF or its receptor TRK-B, which are resistant to the behavioral effects of antidepressants (Saarelainen *et al*, 2003). Emerging evidence further suggests that BDNF may play a key role in the pathophysiology of stress-related disorders. Lower hippocampal BDNF levels have been found subsequent to various stressors such as prenatal stress (Fumagalli *et al*, 2004), footshock stress (Rasmusson *et al*, 2002), postnatal stress (MacQueen *et al*, 2003; Roceri *et al*, 2004) and restraint stress (Smith *et al*, 1995; Vaidya *et al*, 1997; Xu *et al*, 2004). Also of interest is the fact that intrahippocampal administration of BDNF protects against stress-induced impairments in spatial learning and memory and LTP (Radecki *et al*, 2005). Mechanisms underlying how stress alters BDNF levels and how BDNF modifies behavior are not currently clear (see Tapia-Arancibia *et al*, 2004 for review). Interestingly, recent studies have shown that BDNF can have direct stimulating effects on the HPA axis suggesting that BDNF could be a stress-responsive intercellular messenger since when it is exogenously administered it acts as an essential factor in the activation and recruitment of hypothalamic CRF neurons (Givalois *et al*, 2004). However, the effects of chronic BDNF administration are unknown and may confer differential influence on the HPA axis. It is important to note, that the changes in protein levels of BDNF observed in mGluR7$^{-/-}$ mice occur independent of detectable changes at the mRNA levels. Reasons for this may in some way be due to the fact that whole tissue homogenate may dilute potential effects at the mRNA levels that occur only in specific subregions of the hippocampus, however this reasoning is unsatisfactory as it should also apply to regulation at the protein level. Moreover, in agreement with our findings, recent studies have also shown very disparate results between BDNF mRNA and protein levels in the same tissue following various antidepressant manipulations

(De Foubert *et al*, 2004; Jacobsen and Mork, 2004). The reasons for such differential responses at mRNA and protein level are currently unclear and warrant further investigation and possibly reflect regulation of BDNF at the translational level.

There has been an upsurge in the use of genetically modified mice to assess depression- and anxiety-related phenotypes (Cryan *et al*, 2002; Cryan and Mombereau, 2004). However, the majority of phenotypic analysis has focused specifically on behavioral readouts, such as immobility in the forced swim test, in isolation of other physiological and molecular biomarkers. Our current data demonstrate the utility of adapting such an approach and exemplify how such analyses can complement behavioral studies. This is perhaps even more important for mice which have genetically modified levels of target proteins, such as in the case of mGluR7, where there are no complimentary pharmacological tools available. However, we cannot rule out that the changes observed here maybe secondary to alterations in other circuits, not yet identified, which are directly influenced by the null mutation of mGluR7. In conclusion, our current data demonstrate that ablation of mGluR7 results in specific changes in key regulators of the HPA axis, namely $5HT_{1A}$ receptors, GR, corticosterone, and in altered BDNF levels, in a direction opposite to that found in human depression or following chronic stress. These changes correlate with the previously identified antistress and antidepressant-like behavioral effects observed in $mGluR7^{-/-}$ mice. Furthermore, the current data suggest that development of selective mGluR7 ligands may provide a novel strategy for modifying stress-related disorders.

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