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Relief from detrimental consequences of chronic psychosocial stress in mice deficient for the metabotropic glutamate receptor subtype 7

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ABSTRACT

Chronic stress-related psychiatric conditions and comorbid somatic pathologies are an enormous public health concern in modern society. The etiology of these disorders is complex, with stressors holding a chronic and psychosocial component representing the most acknowledged risk factor. During the last decades, research on the metabotropic glutamate receptor (mGlu) system advanced dramatically and much attention was given to the role of the metabotropic glutamate receptor subtype 7 (mGlu7) in acute stress-related behavior and physiology. However, virtually nothing is known about the potential involvement of mGlu7 in chronic psychosocial stress-related conditions. Using the chronic subordinate colony housing (CSC, 19 days) in male mice, we addressed whether central mGlu7 is altered upon chronic psychosocial stressor exposure and whether genetic ablation of mGlu7 interferes with the multitude of chronic stress-induced alterations. CSC exposure resulted in a downregulation of mGlu7 mRNA transcript levels in the prefrontal cortex, a brain region relevant for stress-related behaviors and physiology. Interestingly, mGlu7 deficiency relieved multiple chronic stress-induced alterations including the CSC-induced anxiety-prone phenotype; mGlu7 ablation also ameliorated CSC-induced physiological and immunological consequences such as hypothalamo-pituitary-adrenal (HPA) axis dysfunctions and colonic inflammation, respectively. Together, our findings provide first evidence for the involvement of mGlu7 in a wide range of behavioral and physiological alterations in response to chronic psychosocial stressor exposure. Moreover, the stress-protective phenotype of genetic mGlu7 ablation suggests mGlu7 pharmacological blockade to be a relevant option for the treatment of chronic stress-related emotional and somatic dysfunctions.

This article is part of the Special Issue entitled 'Metabotropic Glutamate Receptors, 5 years on'.

1. Introduction

Chronic stress-related medical conditions such as anxiety and depression are an enormous public health concern in modern society (De Kloet et al., 2005). These disorders are often accompanied by somatic comorbidities including cardiovascular diseases (Buckley et al., 2009; Dimsdale, 2008), inflammatory bowel disease (Bernstein et al., 2010; Duffy et al., 1991; Levenstein et al., 2000) as well as chronic pain and infectious diseases (Cohen et al., 1991; Coker et al., 2000; Kiecolt-Glaser et al., 1996). The etiology of these pathologies is complex, with chronic psychosocial stress representing the most acknowledged risk factor (Chrousos, 2009; Heim and Nemeroff, 2001, 1999; Lupien et al., 2009). To date, there is still a dearth of knowledge about the underlying behavioral, physiological, neural, and immunological mechanisms linking chronic stress with such disorders and treatment options are

Abbreviations: ACTH, adrenocorticotropic hormone; ADX71743, 6-(2,4-dimethylphenyl)-2-ethyl-4,5,6,7-tetrahydro-1,3-benzoxazol-4-one; AMN082, *N,N′*-dibenzhydrylethane-1,2-diamine dihydrochloride; ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; CA, closed arm; CNS, central nervous system; CORT, corticosterone; CSC, chronic subordinate colony housing; CV, coefficients of variation; EPM, elevated plus-maze; GR, glucocorticoid receptor; HC, hippocampus; HPA, hypothalamo-pituitary-adrenal; HT, hypothalamus; IFN-γ, Interferon-γ; iGlu, ionotropic glutamate receptor; KO, knockout; mesLNC, mesenteric lymph node cell; mGlu, metabotropic glutamate receptor; mGlu7, metabotropic glutamate receptor subtype 7; MMPIP, 6-(4-methoxyphenyl)-5-methyl-3-(pyridin-4-yl)-4H,5H-[1,2]oxazolo[4,5-*c*]pyridin-4-one; NAM, negative allosteric modulator; OA, open arm; PFC, prefrontal cortex; S.E.M., standard error of the mean; SHC, single-housed control; SIH, stress-induced hyperthermia; TA, total arm; VFTD, Venus flytrap domain; WT, wildtype; XAP044, 7-hydroxy-3-(4-iodophenoxy)-4Hchromen-4-one.

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insufficient. Notably, the chronic subordinate colony housing (CSC) paradigm represents a valuable animal model as it mimics the type of health compromising stressors of human daily life through a combination of chronic, psychological, and social aspects of stress. CSC exposure reliably leads to both somatic and affective consequences, including colonic inflammation, stress axes dysfunctions and increased anxiety-related behavior, and thus, represents a powerful model to study the mechanisms underlying various chronic stress-induced pathologies (Langgartner et al., 2015; Reber et al., 2007; Uschold-Schmidt et al., 2012).

The L-glutamatergic system, the primary excitatory neurotransmitter system in the mammalian brain, consists of a diverse family of receptors broadly divided into ionotropic glutamate receptors (iGlus) and metabotropic glutamate receptors (mGlus). In the last decades, many studies focused on the L-glutamate system in the context of acute stress-related behavior and physiology (Kew and Kemp, 2005; Swanson et al., 2005) thereby indicating a clear link to mental illness. The mGlu7 subtype received particular attention. It shows the highest degree of evolutionary conservation and is the most widely distributed mGlu family member in key limbic brain regions associated with anxiety and other stress-related mental conditions (Kinoshita et al., 1998; Masugi et al., 1999). Mainly localized in the presynaptic active zone of glutamate and GABA neurons (Dalezios et al., 2002; Kinoshita et al., 1998; Kosinski et al., 1999), mGlu7 is thought to act as an auto- and heteroreceptor that becomes activated by excessive glutamate release during very high synaptic activity. Mice with genetic ablation of mGlu7 are characterized by deficits in amygdala-dependent fear learning and aversive responses (Fendt et al., 2008; Masugi et al., 1999). Moreover, they display an antidepressant- and anxiolytic-like phenotype in acute behavioral tests (Cryan et al., 2003) as well as alterations in hypothalamo-pituitary-adrenal (HPA) axis functionality including upregulated glucocorticoid receptor- (GR-) dependent feedback suppression and increased hippocampal brain-derived neurotrophic factor (BDNF) protein levels (Mitsukawa et al., 2006). In line with that, partial siRNA-mediated knockdown of mGlu7 also resulted in behavioral alterations including blockade of extinction of conditioned fear and anxiolytic-like effects (Fendt et al., 2008; O'Connor et al., 2013).

With respect to pharmacological manipulation, only a few systemically active allosteric modulators for mGlu7 were identified so far yielding interesting results in acute behavioral paradigms. For instance, the first mGlu7-selective allosteric agonist *N,N′*-dibenzhydrylethane-1,2-diamine dihydrochloride (AMN082) was shown to elevate plasma adrenocorticotropic hormone (ACTH) and corticosterone (CORT) levels (Mitsukawa et al., 2005), displayed mGlu7-dependent antidepressant-like activity (Bradley et al., 2012; Palucha et al., 2007), and modulated acquisition and extinction of conditioned fear (Fendt et al., 2008; O'Connor et al., 2013). Interestingly, besides activating mGlu7, AMN082 was shown to facilitate rapid and lasting mGlu7 internalization – a form of functional antagonism and a possible mechanism for the drugs anxiolytic and antidepressant activity (Pelkey et al., 2007; Peterlik et al., 2016). The two systemically active mGlu7 negative allosteric modulators (NAMs) 6-(4-methoxyphenyl)-5-methyl-3-(pyridin-4-yl)-4H,5H-[1,2]oxazolo[4,5-*c*]pyridin-4-one (MMPIP) and 6-(2,4-dimethylphenyl)-2-ethyl-4,5,6,7-tetrahydro-1,3-benzoxazol-4-one (ADX71743) induced discrepant effects in behavioral tests. MMPIP showed only low anxiolytic activity and reversed the AMN082-induced antidepressant-like effects (O'Connor and Cryan, 2013; Pałucha-Poniewiera and Pilc, 2013), whereas ADX71743 showed robust anxiolytic-like effects in several tests (Kalinichev et al., 2013). Furthermore, the recently discovered first systemically active and mGlu7-selective orthosteric-like antagonist 7-hydroxy-3-(4-iodophenoxy)-4Hchromen-4-one (XAP044), which binds within the Venus flytrap domain (VFTD) of mGlu7, demonstrated a wide spectrum of antistress-, antidepressant-, as well as anxiolytic-like efficacy in acute tests *in vivo* (Gee et al., 2014). Taken together, there is clear evidence for a prominent role of mGlu7 in the modulation of acute stress-related behavior and physiology.

Less attention was given to the role of the L-glutamate system in chronic psychosocial stress in rodents or humans. However, in the last years some data emerged suggesting the L-glutamatergic system also to be a relevant therapeutic target for chronic stress-related emotional disorders (Kendell et al., 2005; Mathews et al., 2012; Sanacora et al., 2012; Yim et al., 2012). Recently, we have described transcript levels of mGlu5 and mGlu7 were affected in the central nervous system (CNS) by chronic psychosocial stressor exposure using the CSC paradigm, suggesting the involvement of the mGlu7 (and mGlu5) subtype in chronic stress-induced somatic and affective pathologies (Peterlik et al., 2016).

The aim of the present study was therefore to investigate in detail the role of mGlu7 in modulating somatic as well as affective consequences induced by chronic male subordination. Here, we focused on genetic ablation of mGlu7 by using knockout (KO) mice in combination with the CSC paradigm and hypothesized that genetic ablation of mGlu7 shapes the vulnerability to chronic psychosocial stress.

2. Material and methods

2.1. Animals

Depending on the experiment, male wildtype C57BL/6 mice (Charles River, Sulzfeld, Germany) or male mGlu7 receptor knockout mice (mGlu7 KO, bred from heterozygous C57BL/6 breeding pairs in the animal facility of the University of Regensburg, Germany) and their male wildtype (WT) littermates, all weighing 19–22 g, were used as experimental mice and individually housed in standard polycarbonate mouse cages (16 × 22 × 14 cm) for one week before the start of CSC procedure. Male CD1 mice (Charles River, Sulzfeld, Germany), weighing 30–35 g, were used as dominants. All mice were kept under standard laboratory conditions (12 h light/dark cycle, lights on at 06:00 a.m., 22 °C, 60% humidity) with free access to tap water and standard mouse diet (ssniff Spezialdiäten GmbH, Soest, Germany). All experimental protocols were approved by the Committee on Animal Health and Care of the local government and conformed to international guidelines on the ethical use of animals. All efforts were made to minimize animals suffering and to reduce number of animals used.

2.2. Experimental design

Experimental mice were either exposed to 19 days of CSC or single-housed for control (SHC) in a genotype- and weight-matched setup.

Experiment 1. To assess the impact of chronic stressor exposure on the metabotropic glutamate receptor system in the CNS, relative mGlu7 transcript levels were assessed in the prefrontal cortex (PFC), hypothalamus (HT) and hippocampus (HC) of C57BL/6 WT SHC and CSC mice. Data represent a pool of two independent experiments.

Experiment 2. To investigate a possible functional role of the mGlu7 receptor in mediating CSC-induced somatic and affective consequences, behavioral, physiological, and immunological parameters typically affected by 19 days of CSC exposure were assessed in mGlu7 KO mice and their WT littermates. In detail, on day 19 of CSC, mice were tested on the EPM between 08:00 and 11:00 a.m. to verify genotype-specific effects of CSC exposure on

anxiety-related behavior. Afterwards, CSC mice were put back in their respective CSC colony and SHC mice were kept singly. On day 20 of CSC, mice were rapidly killed between 08:00 and 11:00 a.m. for analysis of genotype-specific CSC effects on physiological and immunological parameters. Trunk blood was collected for quantification of plasma ACTH and CORT concentrations. Furthermore, body weight gain, pituitary, adrenal, and spleen weight, *in vitro* adrenal ACTH responsiveness, *in vitro* interferon-γ (IFN-γ) secretion of isolated and stimulated mesenteric lymph node cells (mesLNCs), and the histological damage (HD) score of colonic tissue were assessed in SHC and CSC mice of the WT and the KO group. Data of spleen weight and IFN-γ secretion derive from one experiment. All other data represent a pool of at least three independent experiments.

2.3. Chronic subordinate colony housing (CSC)

The CSC paradigm was conducted as described previously (Langgartner et al., 2015; Peterlik et al., 2016; Reber et al., 2007). Briefly, four experimental CSC mice of the same genotype were housed together with a larger dominant male (resident) for 19 consecutive days, in order to induce chronic psychosocial stress. To avoid habituation, each dominant male was replaced by a novel one on days 8 and 15. As appropriate controls single-housed (SHC) mice were used (Singewald et al., 2009), being in line with previous studies demonstrating single housing to be less stressful in male mice compared to group housing (Bartolomucci et al., 2003; Chourbaji et al., 2005; Gasparotto et al., 2005).

2.4. Elevated plus-maze (EPM) test

To assess genotype-specific effects of CSC on anxiety-related behavior, mice were tested on the EPM on day 19 of CSC between 08:00 and 11:00 a.m. in a room different from where the animals were housed before, in order to avoid any confounding influence of housing on test performance and vice versa. The test was performed as previously described (Peterlik et al., 2016; Reber et al., 2007). The EPM consisted of two open (6 cm × 30 cm) and two closed (6 cm × 30 cm × 17 cm) arms radiating from a central platform (6 cm × 6 cm) to form a plus-shaped figure elevated 30 cm above the floor. The open arm edges were 0.3 cm in height to prevent mice from falling. Each mouse was placed on the central platform facing a closed arm. The maze was cleaned thoroughly before each mouse. Animal movement on the EPM was monitored by a camera and subsequently analyzed using the program Plus-maze (DOS program, ©Ernst Fricke, 1993) by an observer blind to the animal's genotype and housing condition. The calculated percentage of time spent on the open arms and the number of closed arm entries were used as measures of anxiety and locomotor activity, respectively.

2.5. Determination of spleen, pituitary, and adrenal weight

After decapitation on day 20, the spleen, pituitary, and left and right adrenal glands of each animal were removed, pruned from fat, and weighed separately. In addition, the sum of left and right absolute adrenal weights was calculated for each animal. Until all mice were killed and adrenals removed, the latter were stored in ice-cold DMEM (DMEM/F-12, Life Technologies, Darmstadt, Germany) containing 0.1% BSA. Values represent absolute measurements (in mg) of the respective organs.

2.6. Trunk blood sampling

To determine the genotype-specific effects of CSC exposure on basal morning plasma ACTH and CORT concentrations, mice were rapidly killed by decapitation under CO_2 anesthesia within 3 min after entering the animal room between 08:00 and 11:00 a.m. on day 20, and trunk blood was collected in EDTA-coated tubes (Sarstedt, Nuembrecht, Germany) on ice and centrifuged at 4 °C (5000 rpm for 5 min). Plasma samples were stored at −20 °C until assayed.

2.7. ACTH stimulation of adrenal explants in vitro

Stimulation of adrenal explants with ACTH (100 nM) *in vitro* was performed as previously described (Füchsl et al., 2014; Peters et al., 2014; Uschold-Schmidt et al., 2012). Briefly, left and right adrenals were stored in ice-cold DMEM/F-12 (Life Technologies, Darmstadt, Germany) containing 0.1% BSA until all mice were killed and adrenals removed. Afterwards, each left and right adrenal gland was cut into two halves each containing cortical and medullary tissue. The halves were then weighed and pre-incubated in 200 μl DMEM/F-12 for 4 h (37 °C, 5% CO_2) before any further treatment. Culture medium was then replaced, and each half of one adrenal was incubated with medium containing either 0.9% saline (basal) or 0.9% saline plus ACTH (100 nM) for 6 h (37 °C, 5% CO_2). After incubation, supernatants were carefully removed and stored at −20 °C until being analyzed using a commercially available ELISA for CORT (IBL International, Hamburg, Germany). CORT concentrations were calculated in relation to the weight of the respective adrenal explants (*i.e.*, relative CORT secretion). To illustrate the *in vitro* adrenal CORT secretion in relation to the whole organism, relative CORT secretion from the left and right adrenal gland of each mouse was summed up.

2.8. ELISA for ACTH and CORT

Plasma and supernatant samples were analyzed using a commercially available ELISA for CORT (analytical sensitivity < 1.631 nmol/l and intra-assay and inter-assay coefficients of variation (CV) ≤ 6.35 %; IBL International, Hamburg, Germany) and ACTH (plasma samples only; analytical sensitivity 0.22 pg/ml and intra-assay and inter-assay CV ≤ 7.1%; IBL International, Hamburg, Germany) according to the instructions of the manufacturer.

2.9. Determination of the HD score of the colon

The HD score of colonic tissue was assessed as described previously (Reber et al., 2007). The colon was removed and mechanically cleaned to assess genotype-specific effects of CSC on the histological damage. Afterwards, 1 cm of the distal third was cut longitudinally, laid on a filter paper, and fixed in 5% paraformaldehyde overnight. The next day, the fixed tissue was embedded in paraffin and cut longitudinally. For each animal two 3-μm hematoxylin- and eosin-stained sections taken at 100 μm distance were evaluated by histological scoring as reported previously (Obermeier et al., 2003; Reber et al., 2007) by an investigator blind to treatment. The scores were then averaged per animal and group. Respective SHC values were set to 100%.

2.10. Isolation and incubation of mesLNCs

To determine genotype-specific effects of CSC on the IFN-γ secretion of anti-CD3 and anti-CD28-stimulated mesLNCs *in vitro*, mesenteric lymph nodes were isolated from each animal as described previously (Füchsl et al., 2014; Reber et al., 2007). Afterwards, cell number was assessed using a cell viability analyzer (Vi-Cell XR; Beckman Coulter, Krefeld, Germany) and 5 × 10^5 mesLNCs (100 μl) were transferred to 2 wells of a 96-well plate pre-

coated with 2.5 μg/ml anti-CD3 antibody and additionally stimulated with anti-CD28 antibody (0.5 μg/well, 100 μl). After incubation for 48 h (37 °C, 5% CO_2), IFN-γ levels were measured in the supernatants of 2 wells per animal by ELISA (BioLegend, San Diego, USA) and averaged per animal and group.

2.11. RNA processing, reverse transcription and quantitative PCR

After decapitation of the mice, brains were removed and PFC, HT, and HC tissue was stored in Trizol reagent (Peqlab, Erlangen, Germany). Afterwards, total RNA was isolated according to the manufacturer's instructions (Peqlab, Erlangen, Germany). RNA was re-suspended in 20 μl of RNase free water and its concentration and quality were analyzed spectrophotometrically (NanoDrop Spectrophotometer, Peqlab, Erlangen, Germany). Single strand cDNA was prepared from 500 ng of total RNA using SuperScript III (Invitrogen, Karlsruhe, Germany) in a 20 μl final reverse transcription reaction. Quantitative PCR was performed using the Power SYBR® Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) and the 7500 Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). The following primers (Metabion International AG, Martinsried, Germany) were used to quantify mGlu7 receptor mRNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels: mGlu7-forward, 5′-GCAGAAGGAGCCATCACCAT-3′, mGlu7-reverse, 5′-GTCCGGGATGTGAAGTAAGCA-3′; GAPDH-forward, 5′-TGTGTCCGTCGTGGATCTGA-3′, GAPDH-reverse, 5′-CCTGCTTCACCACCTTCTTGA-3′. Thermal cycler conditions included an initial enzyme activation step of 95 °C/20 s, followed by 40 cycles of 95 °C/3 s and 60 °C/30 s. Samples were prepared in triplicates and changes in gene expression were determined according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) by using GAPDH for normalization and SHC as control (set to 100%).

2.12. Statistical analysis

All data presented are shown as mean + S.E.M. and were analyzed by the software IBM SPSS 22.0. Parameters depending on one factor (housing) were analyzed using independent Student's *t*-test. Parameters depending on two factors (i.e. housing and genotype or housing and stimulation) were analyzed using two-way analysis of variance (ANOVA). Significant main and interaction effects were followed by Bonferroni *post hoc* analysis when appropriate. Statistical significance was accepted at $p \leq 0.05$.

3. Results

3.1. Chronic psychosocial stress in mice affects mGlu7 gene expression in the CNS

Using the CSC paradigm, we assessed central mGlu7 transcript levels in response to chronic psychosocial stressor exposure in mice. 19 days of CSC exposure resulted in significantly decreased relative mGlu7 mRNA levels in the PFC ($t_{17} = 2.456$, $p = 0.025$), when compared to SHC mice (Fig. 1b); no effects of CSC were found in the HT ($t_{16} = -1.443$, $p = 0.168$) and in the HC ($t_{16} = 0.339$, $p = 0.741$; see also Peterlik et al., 2016). As previously shown, relative mGlu5 mRNA levels increased by trend in response to 19 days of CSC specifically in the HT, whereas mGlu2 and mGlu3 mRNA levels remained unaffected in each of the three brain regions assessed (Peterlik et al., 2016). Together, these findings suggest a specific regulation of mGlu7 mRNA levels in the PFC in response to chronic psychosocial stressor exposure.

3.2. Chronic stress-protective phenotype in mice lacking mGlu7

To evaluate a functional role of mGlu7 in chronic psychosocial stress-induced affective and somatic consequences, different behavioral, physiological and immunological parameters were assessed in male mGlu7 KO mice and their WT littermates following 19 days of CSC exposure.

3.2.1. mGlu7 genetic ablation protects against the CSC-induced anxiety-prone phenotype

mGlu7 KO mice were repeatedly shown to have a stress-protective phenotype with respect to acute stressor exposure. For example, they show a reduced hyperthermic response in the stress-induced hyperthermia (SIH) test as well as reduced anxiety-related behavior when tested on the EPM compared to their WT littermates (Cryan et al., 2003). In the present study, we assessed whether mGlu7 deletion also influences the behavioral outcome of chronic psychosocial stressor exposure, more precisely the CSC-induced anxiety-prone phenotype (Füchsl et al., 2014; Peters et al., 2014; Reber et al., 2007). Assessment of anxiety-related behavior in the EPM test revealed a main effect of the factors housing ($F_{1,88} = 13.658$, $p \leq 0.001$) and genotype ($F_{1,88} = 5.741$, $p = 0.019$), with a significantly decreased percentage of time spent on the open arms (time ratio open arms (OA)/total arms (TA) in %) in CSC compared to SHC mice of the WT group ($p \leq 0.001$; Fig. 2a). This finding is in accordance with the literature and indicative for a CSC-induced increase in anxiety-related behavior. Interestingly, this CSC-induced decreased percentage of time spent on the OA was absent in the KO group. Bonferroni *post hoc* analysis further revealed a significantly higher percentage of time spent on the OA in CSC KO compared to CSC WT mice ($p = 0.012$; Fig. 2a). Importantly, the number of closed arm (CA) entries, indicative for locomotor activity, was neither affected by CSC exposure nor by genotype (Fig. 2b). Together, the present findings suggest that mice lacking mGlu7 are protected from developing the CSC-induced anxiety-prone phenotype.

3.2.2. mGlu7 genetic ablation protects against CSC-induced physiological and neuroendocrine alterations

Exposure to 19 days of CSC has been shown to result also in profound physiological and neuroendocrine changes (Füchsl et al., 2014; Reber et al., 2007; Uschold-Schmidt et al., 2012; Veenema et al., 2008). In the present study, we assessed in detail different relevant parameters following CSC exposure in mGlu7 KO mice and their WT littermates to evaluate a potential involvement of mGlu7 in CSC-induced HPA axis dysfunction and other physiological parameters. First of all, assessing body weight gain revealed no effects of CSC exposure in WT mice, which is in line with the literature (Füchsl et al., 2014; Slattery et al., 2012; Veenema et al., 2008). In addition, no effects were also found in the mGlu7 KO group (Fig. 3a). Absolute pituitary weight was found to be dependent on both the factor housing ($F_{1,70} = 4.596$, $p = 0.036$) and the factor genotype ($F_{1,70} = 10.613$, $p = 0.002$), with a significant increase in pituitary weight in WT animals exposed to CSC compared to respective SHC mice ($p = 0.003$). Interestingly, this CSC effect was absent in mGlu7 KO animals. Moreover, pituitary weight was significantly lower in CSC KO compared to CSC WT mice ($p \leq 0.001$; Fig. 3b). Analysis of absolute adrenal weight also revealed a main effect of both the factor housing ($F_{1,69} = 56.725$, $p \leq 0.001$) and the factor genotype ($F_{1,69} = 14.080$, $p \leq 0.001$). Bonferroni *post hoc* analysis showed a significant increase in weight in CSC compared to SHC mice of the WT group ($p \leq 0.001$). This CSC effect was also present in the KO group, but less pronounced ($p = 0.002$). Therefore, adrenal weight was also significantly lower in CSC KO compared to CSC WT mice ($p \leq 0.001$; Fig. 3c).

Furthermore, basal morning plasma ACTH levels were dependent on the factor housing ($F_{1,68} = 6.462$, $p = 0.013$), with significantly higher basal morning plasma ACTH levels in CSC compared to SHC mice of the WT group ($p = 0.004$). This stress effect was absent in the KO group (Fig. 4a). Basal morning plasma CORT levels were neither dependent on the factor housing nor on the factor genotype (Fig. 4b). We further analyzed potential genotype-dependent effects of CSC exposure on adrenal ACTH responsiveness *in vitro*. In the WT group, there was a main effect of the factors housing ($F_{1,48} = 15.587$, $p \leq 0.001$) and stimulation ($F_{1,48} = 43.881$, $p \leq 0.001$), as well as a housing × stimulation interaction ($F_{1,48} = 11.160$, $p = 0.002$) on adrenal CORT secretion *in vitro*. In contrast, adrenal CORT secretion from explants of mGlu7 KO mice were only dependent on the factor stimulation ($F_{1,44} = 31.576$, $p \leq 0.001$). Adrenal explants from both SHC ($p \leq 0.001$ for each genotype) and CSC (WT: $p = 0.025$; KO: $p \leq 0.001$) mice showed an increased CORT secretion in response to ACTH compared to basal (saline) stimulation. However, adrenal CORT secretion in response to ACTH was significantly lower in CSC compared to SHC mice of the WT group ($p \leq 0.001$). This CSC-induced attenuation of adrenal *in vitro* ACTH responsiveness was absent in mGlu7 KO mice (Fig. 4c). Together, these findings indicate that mGlu7 genetic ablation protects against developing CSC-induced HPA axis dysfunction.

3.2.3. *mGlu7 genetic ablation protects against CSC-induced immunological alterations*

Reliable immunological alterations induced by CSC exposure include splenomegaly and the development of spontaneous colitis (Füchsl et al., 2014; Langgartner et al., 2015; Reber et al., 2007). In the present study, absolute spleen weight was dependent on both the factor housing ($F_{1,22} = 11.069$, $p = 0.003$) as well as the factor genotype ($F_{1,22} = 12.917$, $p = 0.002$). In line with the literature, Bonferroni *post hoc* analysis revealed a significant increase in spleen weight in CSC compared to SHC mice of the WT group ($p = 0.001$). This CSC effect was absent in the KO group. In addition, spleen weight was significantly lower in CSC KO compared to CSC WT mice ($p \leq 0.001$; Fig. 5a). Beneficial effects of mGlu7 genetic ablation were also found with respect to CSC-induced colonic inflammation. *In vitro* IFN-γ secretion from isolated and anti-CD3/anti-CD28-stimulated mesLNC were found to be dependent on the factors housing ($F_{1,19} = 5.476$, $p = 0.030$) and genotype ($F_{1,19} = 12.358$, $p = 0.002$), as well as a factor housing × genotype interaction ($F_{1,19} = 5.632$, $p = 0.028$). Bonferroni *post hoc* analysis revealed a significantly increased IFN-γ secretion in CSC compared to SHC mice of the WT group ($p = 0.004$). This CSC effect was again absent in mGlu7 KO mice. Moreover, IFN-γ secretion was significantly lower in the CSC KO group compared to CSC WT mice ($p \leq 0.001$; Fig. 5b). In support of colonic inflammation, the HD score was found to be dependent on the factors housing ($F_{1,49} = 6.332$, $p = 0.015$) and genotype ($F_{1,49} = 6.967$, $p = 0.011$), as well as a factor housing × treatment interaction ($F_{1,49} = 6.967$, $p = 0.011$). Bonferroni *post hoc* analysis revealed a significantly increased damage score in CSC compared to SHC mice of the WT group ($p \leq 0.001$; Fig. 5c), reflected by an increased epithelial damage and a more severe inflammatory infiltration (Fig. 5d). This CSC effect was again absent in the mGlu7 KO group. Furthermore, the damage score was significantly lower in CSC KO compared to CSC WT mice ($p \leq 0.001$; Fig. 5c). Together, these findings suggest that mGlu7 genetic ablation also protects against developing CSC-induced immunological alterations including colonic inflammation.

4. Discussion

The data of the present study strongly suggest an involvement of the mGlu7 receptor subtype in mediating behavioral, physiological and immunological consequences of chronic psychosocial stress in rodents using CSC as a chronic subordination paradigm for male mice. We demonstrate a significant CSC-induced downregulation of mGlu7 mRNA expression in the PFC, indicating region specific modulation of the mGlu7 gene in response to chronic psychosocial stress. Beyond that, genetic ablation of mGlu7 in mice relieves multiple chronic stress-induced alterations. In addition to protection against the CSC-induced anxiety-prone phenotype, mGlu7 deficient mice were also less vulnerable to CSC with respect to reliable physiological and immunological consequences such as HPA axis dysfunctions and colonic inflammation. Together, these findings provide evidence for the involvement of the mGlu7 subtype in a wide range of affective and somatic alterations that emerge upon chronic psychosocial stressor exposure. Moreover, the stress-protective phenotype of genetic mGlu7 ablation may suggest that mGlu7 pharmacological blockers could be a relevant

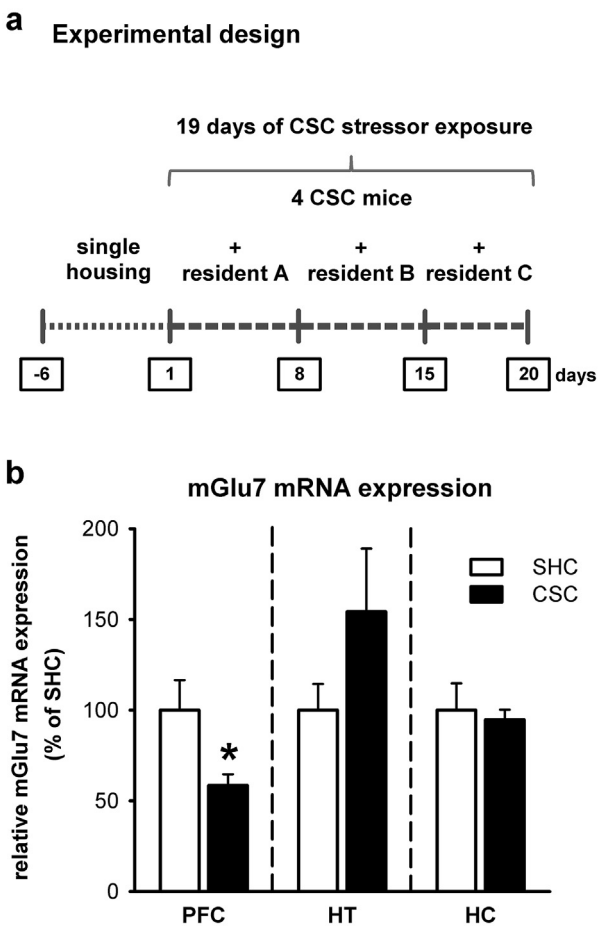

Fig. 1. Schematic illustration of the experimental design of the chronic subordinate colony housing (CSC, 19 days) paradigm and effects of CSC exposure on the brain mGlu7 system. (a) All experimental mice were housed singly for one week before they were assigned in a genotype- and weight-matched manner to the single-housed control (SHC) or the CSC group. In order to induce chronic psychosocial stress, CSC mice were housed together with a larger dominant male for 19 consecutive days. In detail, four experimental CSC mice were put into the homecage of resident A on day 1 of CSC, resulting in immediate subordination of the four intruder CSC mice. In order to avoid habituation, the four experimental CSC mice were transferred into the homecage of resident B and resident C on day 8 and on day 15 of CSC, respectively. (b) 19 days of CSC resulted in a significant decrease of relative mGlu7 receptor transcript levels in the prefrontal cortex (PFC), whereas no significant effects were observed in the hypothalamus (HT) and hippocampus (HC). White bar, SHC ($n = 9$); black bar, CSC ($n = 9–10$). Data represent mean + SEM. $^*p \leq 0.05$ *vs.* respective SHC mice, Student's *t*-test.

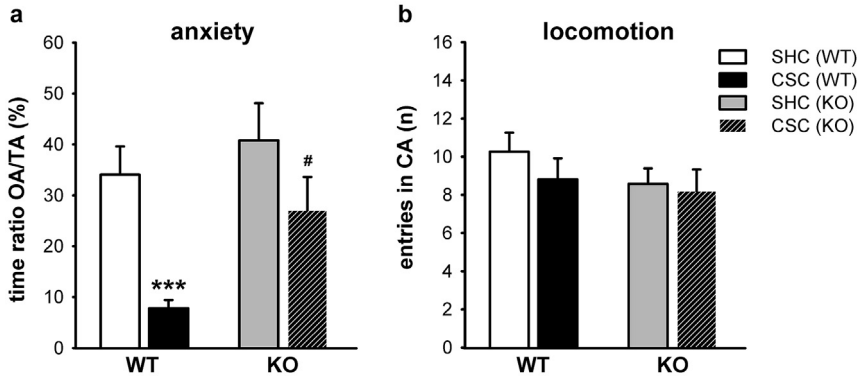

Fig. 2. Stress-protective effects of mGlu7 deficiency on the CSC-induced anxiety-prone phenotype. To assess chronic psychosocial stress effects on anxiety-related behavior, SHC and CSC mice of the wildtype (WT) and knockout (KO) group were exposed to the EPM on day 19 of CSC. (a) The percentage of time spent on the open arms (time ratio OA/TA in %) on the EPM was decreased in CSC compared to SHC mice only of the WT group, indicative for an increase in innate anxiety. Moreover, the percentage of time spent on the open arms was significantly higher in CSC KO compared to CSC WT mice. (b) The number of closed arm (CA) entries was neither affected by CSC nor by genotype, indicating unaffected locomotor activity. White bar, SHC (WT); black bar, CSC (WT); grey bar, SHC (KO); dashed bar, CSC (KO). $n = 19–26$ per genotype and housing group. Data represent mean + SEM. $^{***}p \leq 0.001$ *vs.* respective SHC group; $^{\#}p \leq 0.05$ *vs.* respective WT group; two-way ANOVA followed by Bonferroni *post hoc* analysis.

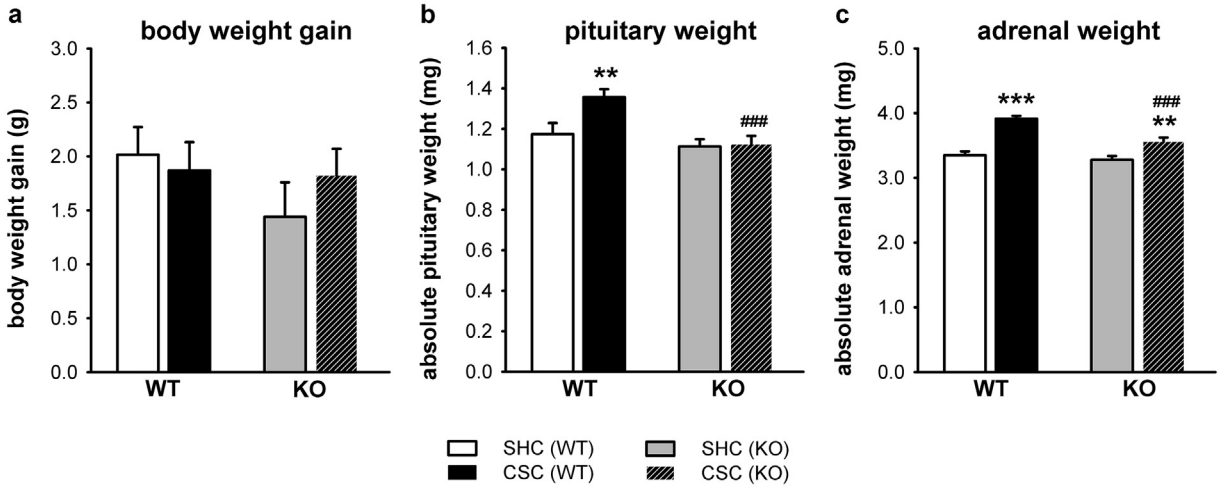

Fig. 3. Stress-protective effects of mGlu7 deficiency on CSC-induced physiological alterations. (a) Body weight gain during 19 days of CSC was neither affected by CSC nor by genotype. (b) 19 days of CSC induced a significant increase in absolute pituitary weight in the wildtype (WT) but not in the knockout (KO) group. In addition, pituitary weight was significantly lower in CSC KO compared to CSC WT mice. (c) CSC exposure also induced a significant increase in absolute adrenal weight in WT mice and a less pronounced increase also in KO mice. Hence, adrenal weight was significantly lower in CSC KO compared to CSC WT mice. White bar, SHC (WT); black bar, CSC (WT); grey bar, SHC (KO); dashed bar, CSC (KO). $n = 14–23$ per genotype and housing group. Data represent mean + SEM. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ *vs.* respective SHC group; $^{\#\#\#}p \leq 0.001$ *vs.* respective WT group; two-way ANOVA followed by Bonferroni *post hoc* analysis.

option for the treatment of chronic stress-related emotional and somatic dysfunctions in man.

4.1. Chronic psychosocial stress in mice affects mGlu7 gene expression in the CNS

Due to the lack of data relating to the impact of CSC on brain mGlu7 expression, we decided to first address the effects of CSC on mGlu7 mRNA levels in brain regions involved in regulating behavioral and physiological stress responses. CSC resulted in a downregulation of mGlu7 transcript levels in the prefrontal cortex but little or no effects were observed in the hypothalamus or in the hippocampus (for comparison see also Peterlik et al., 2016). Notably, we previously showed a trend towards CSC-induced dysregulation of mGlu5 mRNA levels specifically in the hypothalamus, whereas mGlu2 and mGlu3 mRNA levels were not affected in any of the three brain regions assessed (Peterlik et al., 2016). Thus, certain mGlu subtypes respond to chronic psychosocial stressor exposure at the mRNA level, and in a region-specific manner, whereas others do not. Interestingly, in the case of mGlu7, there seems to be a specific CSC-induced downregulation in the PFC.

4.2. Chronic stress-protective phenotype in mice lacking mGlu7

Genetic ablation of mGlu7 has been demonstrated to be associated with several selective changes in molecular targets that participate in the stress response and in psychopathological states (Cryan et al., 2003; Mitsukawa et al., 2006). In particular, increased GR levels in the hippocampus combined with an increased GR-mediated feedback suppression of the HPA axis, as well as elevated hippocampal BDNF and 5-HT_{1A} receptor levels in mGlu7 KO mice suggest selective dysregulation of stress response integration opposite to that found in humans suffering from chronic stress-related pathologies (Holsboer and Barden, 1996; Webster et al., 2002). Moreover, these changes correlate well with the previously identified acute antistress, antidepressant- and anxiolytic-like phenotype in mice with genetic mGlu7 ablation (Callaerts-Vegh et al., 2006; Cryan et al., 2003), with pharmacological

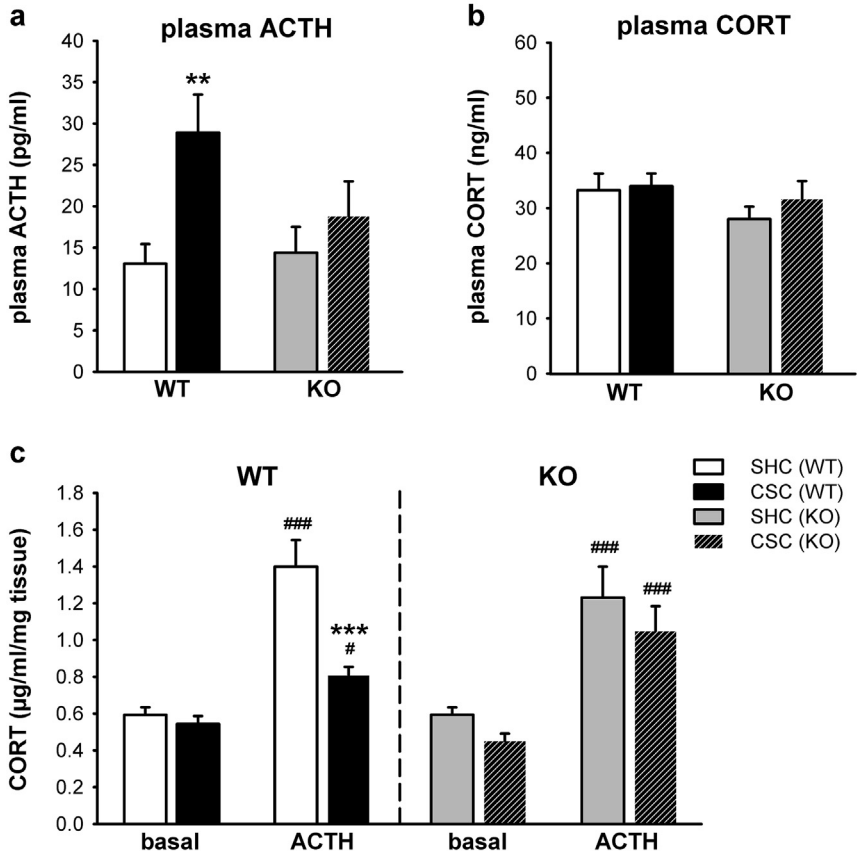

Fig. 4. Stress-protective effects of mGlu7 deficiency on CSC-induced alterations in HPA axis functionality. (a) The CSC-induced increase in basal morning plasma ACTH levels in wildtype (WT) mice was absent in knockout (KO) mice. (b) There was neither an effect of housing nor genotype on basal morning plasma CORT levels. (c) 19 days of CSC resulted in an impaired adrenal *in vitro* ACTH responsiveness in WT mice (left panels), an effect absent in KO mice (right panels). White bar, SHC (WT); black bar, CSC (WT); grey bar, SHC (KO); dashed bar, CSC (KO). $n = 15–23$ (a, b) and $n = 11–13$ (c) per genotype and housing group. Data represent mean + SEM. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ *vs.* respective SHC group; $^{\#}p \leq 0.05$, $^{\#\#\#}p \leq 0.001$ *vs.* respective basal stimulation (c); two-way ANOVA followed by Bonferroni *post hoc* analysis.

mGlu7 blockade (Gee et al., 2014; Kalinichev et al., 2013) as well as with siRNA-mediated knockdown of mGlu7 (Fendt et al., 2008; O'Connor et al., 2013).

In the present study, we demonstrate a stress-protective phenotype in mice with genetic ablation of mGlu7 in the context of chronic psychosocial stress. First of all, in support of previous findings (Füchsl et al., 2014; Peters et al., 2013; Reber and Neumann, 2008; Reber et al., 2007; Slattery et al., 2012; Uschold-Schmidt et al., 2012), CSC exposure resulted in increased anxiety-related behavior in mice of the WT group tested on the EPM. However, this CSC-induced anxiety-prone phenotype was not present in mGlu7 KO mice, suggesting a stress-protective effect of genetic mGlu7 ablation with respect to the chronic psychosocial stress-induced increase in innate anxiety. As mentioned above, we did not reproduce the previously described anxiolytic-like effect of genetic mGlu7 ablation; in the present study innate anxiety levels were comparable between mGlu7 KO and WT mice as seen in the SHC group tested on the EPM. However, our findings are still in line with the study of Cryan et al. (2003) who showed a significant anxiolytic-like effect of genetic ablation of mGlu7 in mice using the light/dark box test, but couldn't easily confirm this effect with the EPM test. Thus, it seems that the detection of the anxiolytic-like phenotype of mGlu7 KO mice depends on the exact behavioral test employed.

A stress-protective effect of genetic mGlu7 ablation was also found regarding HPA axis functionality. In mGlu7 KO mice, we neither detected a CSC-induced increase in pituitary weight and associated basal morning plasma ACTH levels nor a CSC-induced increase in absolute adrenal weight accompanied by a reduction in adrenal ACTH responsiveness *in vitro,* which are reliable indicators for chronic psychosocial stress induced by CSC exposure (Füchsl et al., 2013; Langgartner et al., 2015) (Uschold-Schmidt et al., 2012) that we have confirmed in the WT group of the current study. These findings suggest that the HPA axis of mGlu7-deficient mice is less vulnerable to chronic psychosocial stressor exposure.

Analysis of immunological parameters further supported a stress-protective phenotype in mGlu7 KO mice. The previously described CSC-induced splenomegaly (Füchsl et al., 2014), which was confirmed in the WT group of the present study, appears completely abolished in mGlu7 KO mice. Given the suggested role for mGlu7 in the regulation of gastrointestinal function (Julio-Pieper et al., 2010), it was also of great interest to assess whether genetic ablation of mGlu7 is protective against CSC-induced development of spontaneous colitis. Indeed, an increased IFN-γ secretion from isolated and anti-CD3/anti-CD28-stimulated mesLNCs and an increased histological damage of colonic tissue, indicating development of mild colonic inflammation (Füchsl et al., 2014; Reber et al., 2007), were detected in CSC mice of the WT group but not in mGlu7 KOs.

Together, the present findings indicate that mice with genetic ablation of mGlu7 are less vulnerable to behavioral, physiological as well as immunological consequences of chronic psychosocial stressor exposure. The mechanisms underlying these stress-protective effects of genetic mGlu7 ablation might be explained by the role of mGlu7 in regulating neurotransmitter release. The

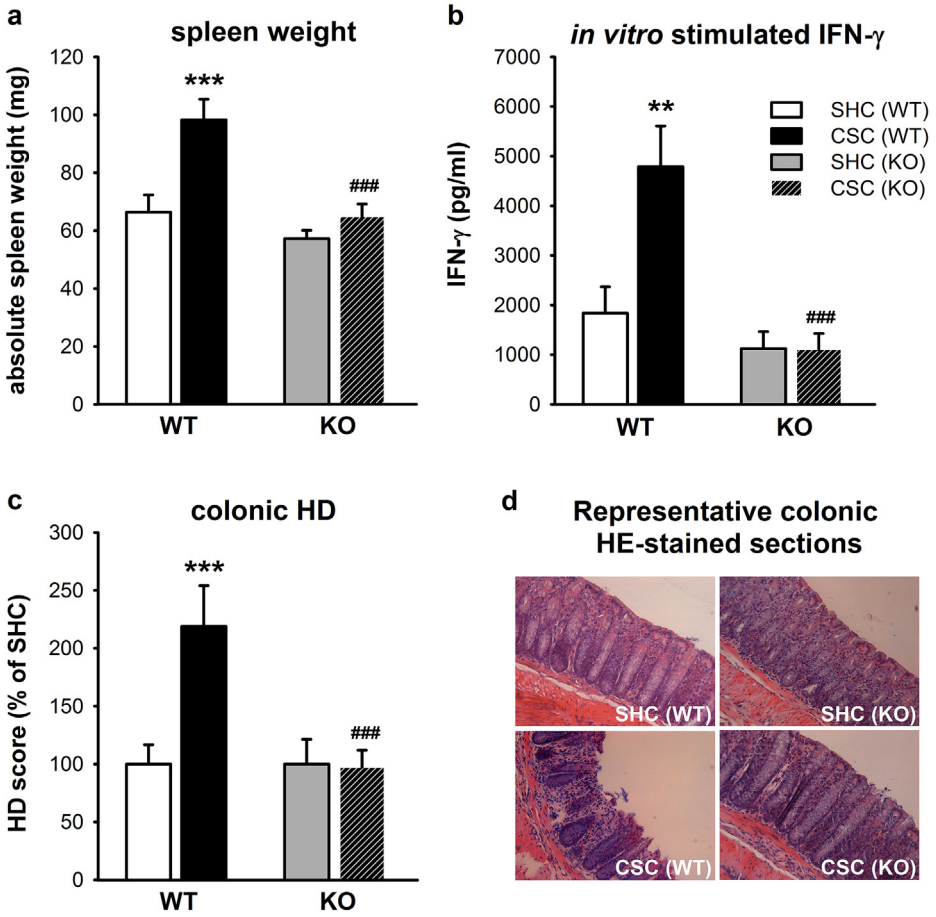

Fig. 5. Stress-protective effects of mGlu7 deficiency on CSC-induced immunological alterations. (a) Absolute spleen weight was significantly increased in CSC compared to SHC mice of the wildtype (WT) but not of the knockout (KO) group. In addition, spleen weight was significantly lower in CSC KO compared to CSC WT mice. (b) 19 days of CSC resulted in a significantly increased secretion of IFN-γ from isolated and anti-CD3/anti-CD28 stimulated mesLNCs. This effect was absent in KO mice. Hence, IFN-γ secretion was also significantly lower in CSC KO compared to CSC WT mice. (c) CSC exposure induced a significant increase in the HD score of colonic tissue in the WT group, whereas this increase was not present in KO mice. Furthermore, HD score was significantly lower in CSC KO compared to CSC WT mice. (d) Representative colonic sections stained with hematoxylin and eosin from SHC WT (left; normal colon histology) and CSC WT (right; goblet cell loss and crypt loss in locally restricted areas and infiltration of cells reaching the Lamina muscularis mucosae) mice. White bar, SHC (WT); black bar, CSC (WT); grey bar, SHC (KO); dashed bar, CSC (KO). $n = 5–8$ (a), $n = 4–8$ (b) and $n = 11–15$ (c) per genotype and housing group. Data represent mean + SEM. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ *vs.* respective SHC group; $^{###}p \leq 0.001$ *vs.* respective WT group; two-way ANOVA followed by Bonferroni *post hoc* analysis.

presynaptically located mGlu7 is thought to act as an auto- and heteroreceptor on glutamatergic and GABAergic neurons in key limbic brain regions involved in the regulation of the stress response. Thus, a complete lack of mGlu7 might induce region-specific changes in excitatory and inhibitory neurotransmitter release through altered negative feedback regulation leading eventually to a stress-resilient phenotype. Moreover, downstream effects on other neurotransmitter systems such as the dopamine and serotonin systems (Müller and Schwarz, 2007) that are known to alter e.g. anxiety-related behavior (Lucki, 1998) and/or altered neurodevelopmental mechanisms due to mGlu7 deficiency may also account for stress resilience observed in mGlu7 KO mice. Our finding that 19 days of CSC led to a decrease of mGlu7 mRNA levels in the PFC in WT mice seems to be consistent with our physiological/behavioral observations: A downregulation of mGlu7 and hence an L-glutamate/GABA imbalance in a specific brain region, namely the PFC, may account for an increase in anxiety-related behavior following CSC. This would be in line with studies suggesting that hyperexcitability in the PFC contributes to an anxiety-prone phenotype (Bi et al., 2013; Bruening et al., 2006; Singewald et al., 2003). Conversely, altered inhibition and/or excitation in several brain regions at the same time, due to the complete lack of mGlu7, may compensate CSC-induced changes in neurotransmitter release via complex circuitry adaptations and thereby account for the stress-protective phenotype of mGlu7 KO mice.

In addition, mGlu7 is known to be expressed in endocrine organs like adrenal glands and also in the gastrointestinal tract (Julio-Pieper et al., 2011). Therefore, peripheral mechanisms may also contribute to the stress-protective phenotype in mice lacking mGlu7, at least with respect to immunological/inflammatory parameters.

In conclusion, 19 days of CSC decrease mGlu7 transcript levels specifically in the PFC, a brain region involved in the regulation of behavioral and physiological stress responses. Furthermore, the present results demonstrate a stress protective phenotype of mice lacking mGlu7 in the context of chronic psychosocial stress, and thus clearly indicate a role for mGlu7 in mediating affective as well as somatic consequences induced by chronic psychosocial stressor exposure. Thus, selective pharmacological blockers of mGlu7 may represent an innovative option for the treatment of chronic stress-induced human pathologies.

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