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Full-length Article

Blocking metabotropic glutamate receptor subtype 5 relieves maladaptive chronic stress consequences

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ABSTRACT

Etiology and pharmacotherapy of stress-related psychiatric conditions and somatoform disorders are areas of high unmet medical need. Stressors holding chronic plus psychosocial components thereby bear the highest health risk. Although the metabotropic glutamate receptor subtype 5 (mGlu5) is well studied in the context of acute stress-induced behaviors and physiology, virtually nothing is known about its potential involvement in chronic psychosocial stress. Using the mGlu5 negative allosteric modulator CTEP (2-chloro-4-[2-[2,5-dimethyl-1-[4-(trifluoromethoxy)phenyl]imidazol-4yl]ethynyl]pyridine), a close analogue of the clinically active drug basimglurant – but optimized for rodent studies, as well as mGlu5-deficient mice in combination with a mouse model of male subordination (termed CSC, chronic subordinate colony housing), we demonstrate that mGlu5 mediates multiple physiological, immunological, and behavioral consequences of chronic psychosocial stressor exposure. For instance, CTEP dose-dependently relieved hypothalamo-pituitary-adrenal axis dysfunctions, colonic inflammation as well as the CSC-induced increase in innate anxiety; genetic ablation of mGlu5 in mice largely reproduced the stress-protective effects of CTEP and additionally ameliorated CSC-induced physiological anxiety. Interestingly, CSC also induced an upregulation of mGlu5 in the hippocampus, a stress-regulating brain area. Taken together, our findings provide evidence that mGlu5 is an important mediator for a wide range of chronic psychosocial stress-induced alterations and a potentially valuable drug target for the treatment of chronic stress-related pathologies in man.

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1. Introduction

Chronic psychosocial stress events are a constant burden within modern societies and strong risk factors for the development of multiple medical conditions (de Kloet et al., 2005). These comprise psychiatric disorders, such as anxiety and depression (de Kloet et al., 2005; McEwen, 2004), which are often accompanied by numerous 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). To date, the detailed behavioral, physiological, neural, and immunological mechanisms linking chronic stress with such disorders are not well understood and therapeutic options are still limited. In this context, the chronic subordinate colony housing (CSC) paradigm serves as a valuable animal model, which mimics the type of health compromising stressors of human daily life with strong face and construct validity, as it combines chronic, psychological, and social aspects of stress. It further causes both somatic and affective symptoms, including colonic inflammation, stress axes dysfunctions and increased anxiety-states. Thus, it represents a powerful tool to

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study the mechanisms underlying various chronic stress-induced pathologies (Langgartner et al., 2015; Reber et al., 2007; Uschold-Schmidt et al., 2012).

Glutamatergic neurotransmission is mediated by ionotropic and metabotropic glutamate receptors (mGlus), and many recent studies suggest that glutamatergic imbalance is key in the molecular pathophysiology of stress-related psychiatric and comorbid somatic disorders (Kendell et al., 2005; Mathews et al., 2012; Peterlik et al., 2016; Sanacora et al., 2012; Yim et al., 2012). Concurrently, glutamatergic approaches for the pharmacotherapy of psychiatric disorders have increasingly received attention. Exploratory clinical trials revealed that a single infusion of a subanesthetic dose of the (2*R*)-2-(methylamino)butanedioic acid (NMDA) receptor antagonist ketamine induced rapid and sustained antidepressant efficacy in severely depressed and treatment-resistant patients (Berman et al., 2000; Zarate et al., 2006). However, the abuse potential and other safety liabilities of ketamine, as well as its cognition-altering and dissociative effects limit its widespread application to the off-label use as emergency intervention in controlled clinical settings. In contrast, therapeutic strategies targeting mGlus may represent a more subtle mode, as they are rather modulatory in nature with the promise to have fewer side-effects than ligand-gated ion channel modulators (Cartmell and Schoepp, 2000; Niswender and Conn, 2010). Particularly the mGlu5 subtype, which is expressed in many brain regions associated with emotional processing and stress-related disorders such as the amygdala, striatum, hippocampus, frontal cortex, and thalamus (Ferraguti and Shigemoto, 2006; Romano et al., 1995), and also present in peripheral tissues (Julio-Pieper et al., 2011; Volpi et al., 2012), has become a recent focus for drug discovery efforts.

Of note, there is physical and functional association of mGlu5 with postsynaptic NMDA receptors, suggesting that mGlu5 negative allosteric modulators (NAMs) could negatively modulate NMDA receptor function (O'Leary et al., 2000). Furthermore, mGlu5 NAMs have been reported to have therapeutic potential for numerous conditions including anxiety disorders (Krystal et al., 2010; Palucha and Pilc, 2007) and gastro-esophageal reflux disease (Keywood et al., 2009). For instance, a recent clinical study revealed the mGlu5-selective allosteric antagonist 2-chloro-4-{[1-(4-fluorophenyl)-2,5-dimethyl-1H-imidazol-4-yl]ethynyl}pyridine (basimglurant) as a promising antidepressant drug with the potential to also ameliorate important comorbidities such as anxiety and pain (Jaeschke et al., 2015; Lindemann et al., 2015). Moreover, 2-chloro-4-[2-[2,5-dimethyl-1-[4-(trifluoromethoxy)phenyl]imidazol-4yl]ethynyl]pyridine (CTEP), another selective NAM at mGlu5, chemically similar to the clinically active antidepressant basimglurant and optimized for its pharmacokinetic properties for the use in rodents, showed promising stress-protective effects under both acute and chronic stress conditions. In detail, oral CTEP was shown to be active in reducing the hyperthermic response in the stress-induced hyperthermia (SIH) test (Lindemann et al., 2011) and also able to alleviate some of the chronic social defeat stress (CSDS)-induced depressive-like symptoms while lacking protective effects on selected physiological parameters (Wagner et al., 2014). However, the stress-protective relevance of mGlu5, especially under permanent chronic psychosocial stress conditions that induce an increased anxiety-state, stress axes dysfunctions, and colonic inflammation, as represented by the CSC paradigm, has not been addressed so far.

Here, we investigated the role of mGlu5 in modulating somatic as well as affective consequences of chronic psychosocial stress induced by chronic male subordination during the CSC paradigm. We hypothesized that genetic and pharmacological inhibition of mGlu5 shape the vulnerability to chronic psychosocial stress. To test this hypothesis, we used conventional mGlu5 knockout (KO) mice in comparison with their wildtype (WT) littermates and also WT mice chronically infused with the mGlu5 NAM CTEP, at different doses, and in combination with the CSC paradigm. Furthermore, we also addressed alterations in mGlu5 mRNA expression and receptor binding in stress-relevant brain regions in response to CSC exposure in WT mice.

2. Material and methods

2.1. Animals

Depending on the experiment, male C57BL/6 WT mice (Charles River, Sulzfeld, Germany) or mGlu5 KO mice and their male WT littermates (bred from heterozygous C57BL/6 breeding pairs in the animal facility of the University of Regensburg, Germany), all weighing 19–22 g, were used as experimental mice and individually housed in standard polycarbonate mouse cages ($16 \times 22 \times 14$ cm) for at least one week before starting the CSC procedure. Male CD1 mice weighing 30–35 g from our own breeding were used as dominants. All mice were kept under standard laboratory conditions (12 h light/dark cycle, lights on at 0600 h, 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.

2.2. Experimental design

Experimental mice (n = 6–24 per housing or treatment group, depending on the number of animals used per experiment and the number of experiments performed/pooled) were either chronically stressed by exposure to the CSC paradigm or single-housed for control (SHC) in a genotype-, treatment- and weight-matched setup. The CSC paradigm lasted for 19 consecutive days and was conducted as described elsewhere (Langgartner et al., 2015; Peterlik et al., 2016; Reber et al., 2007) and in detail below.

Experiment 1: To investigate a possible role for mGlu5 in mediating CSC-induced somatic and affective consequences, mGlu5 KO mice and their WT littermates were exposed to CSC and typical CSC-affected physiological, immunological, and behavioral parameters were assessed. Data represent a pool of two independent experiments.

Experiment 2: To extend the findings obtained in the genetic approach using mGlu5 KO mice, we analyzed typical CSC-affected physiological, immunological, and behavioral parameters in more detail following CSC exposure in C57BL/6 WT mice with pharmacological mGlu5 inhibition. Here, the systemically active mGlu5 NAM CTEP (Lindemann et al., 2011) was applied chronically during CSC exposure via micro-osmotic pumps implanted subcutaneously (*s.c.*, for details see below). Depending on the respective parameter assessed, data represent one single experiment or a pool of up to three independent experiments.

Experiment 3: To verify and to confirm the results of CTEP chronically applied *s.c.*, during CSC exposure, CTEP was administered chronically during CSC via micro-osmotic pumps implanted *s.c.* but attached to an intraperitoneal (*i.p.*) catheter. Data represent one single experiment.

Experiment 4: Finally, to assess the impact of chronic stressor exposure on the central mGlu system, C57BL/6 WT mice were exposed to CSC and relative mRNA transcript levels and receptor binding sites of mGlu5 were assessed in different brain regions,

i.e. prefrontal cortex (PFC), hypothalamus (HT) and hippocampus (HC). Data represent one single experiment.

2.3. Chronic subordinate colony housing (CSC) paradigm

The CSC paradigm was conducted as described previously (Langgartner et al., 2015; Peterlik et al., 2016; Reber et al., 2007). Briefly, experimental mice were assigned to the single-housed control (SHC) or chronic subordinate colony housing (CSC) group in a genotype-, treatment- as well as weight-matched manner. Four CSC mice of the same genotype or the same treatment were housed together with a dominant male for 19 consecutive days in order to induce chronic psychosocial stress. To avoid habituation during chronic stressor exposure, each dominant male was replaced by a novel dominant male on days 8 and 15 (see Fig. 1A). As appropriate controls, SHC mice were used (Singewald et al., 2009), being in line with previous studies demonstrating single housing to be less stressful in male mice as compared to group housing (Bartolomucci et al., 2003; Chourbaji et al., 2005; Gasparotto et al., 2005). SHC mice remained undisturbed in their home cage except for change of bedding once a week. On day 14 of CSC, anxiety-related behavior was assessed in the light-dark box (LDB) test (*experiment 3*). On day 18 and 19 of CSC, animals' innate and physiological anxiety was assessed on the elevated plus maze (EPM, day 18; *experiment 2*) and in the stress-induced hyperthermia test (SIH, day 19; *experiment 1* and *2*), respectively. After testing, stressed mice were placed back into their respective CSC colony and SHC mice remained single-housed. On day 20, all mice were rapidly decapitated between 0800 h and 1100 h, trunk blood was collected for quantification of plasma corticosterone (CORT), plasma adrenocorticotropic hormone (ACTH), and the pituitary, adrenal, thymus and spleen weights, as well as the *in vitro* ACTH responsiveness of adrenal explants were assessed. In addition, the histological damage score of the colon, the number of viable mesenterial lymph node cells (mesLNC) and anti-CD3/anti-CD28-stimulated IFN-γ production of mesLNC *in vitro* were determined.

2.4. Drug treatment/surgical procedure

In *experiment 2* and *3*, chronic application of the mGlu5 NAM CTEP (F. Hoffmann-La Roche, Basel, Switzerland) via Alzet® micro-osmotic pumps (pumping rate: 0.11 µl/h, Alzet®, Model 1004, Cupertino, USA) was initiated one week before starting the CSC paradigm (day -6) in order to establish a stable baseline receptor occupancy and was continued until the end of chronic stressor exposure (day 20). CTEP was formulated as a solution in vehicle (VEH, polyethylene glycol 400 (PEG), Sigma Aldrich, Steinheim, Germany) to ensure a continuous substance release of 0.05, 0.5 or 2 mg/kg/day in *experiment 2* and of 2 mg/kg/day in *experiment 3*. As reported by Peters et al. (2014), a micro-osmotic pump was implanted *s.c.* in the abdominal region through a 1 cm long incision at the lower neck of the mouse under isoflurane anesthesia (Baxter, GmbH, Germany). In *experiment 3*, the micro-osmotic pump was attached to a mouse *i.p.* catheter (Alzet®, Cupertino, USA) in accordance to the manufacturer's instructions to ensure proper *i.p.* administration of CTEP. A 1–2 mm long incision into the peritoneal wall was made and the catheter was implanted ending in the peritoneal cavity and fixed with three stitches to ensure proper placement. To avoid post-surgical infections, each mouse received 100 µl of antibiotics (*s.c.*, Baytril® 2.5% Bayer Vitral GmbH, Leverkusen, Germany), followed by wound treatment with betaisodona (Mundipharma GmbH, Limburg, Germany).

2.5. Elevated plus-maze (EPM) and light-dark box (LDB) test

To assess genotype- or treatment-specific effects of CSC on anxiety-related behavior, SHC and CSC mice were tested on the EPM (day 18) (Füchsl et al., 2014; Reber et al., 2007) or in the LDB (day 14) (Peters et al., 2014) between 0800 h and 1100 h. CSC mice were directly taken from the colony cages without single housing prior to EPM or LDB testing. Afterwards, CSC mice were put back in their respective CSC colony and SHC mice were kept single housed.

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 test. Animal movement on the EPM during a 5-min interval was monitored by a camera and subsequently analyzed using the program Plusmaze (DOS program, ©Ernst Fricke, 1993) by an observer blind to the animal's housing and treatment condition. The percentage of time spent on open arms of the EPM was used as a measure of anxiety. In addition, the number of closed-arm entries was taken as a measure of locomotor activity.

The LDB consisted of a bright (27 × 27 × 27 cm; 350 lux) and a dark (18 × 27 × 27 cm; 50 lux) compartment, separated by a partition wall that had a small opening (6 × 6 cm high) at floor level. For habituation, mice were individually placed in the dark compartment, with the opening of the partition wall closed for 30 s. Afterwards, the partition wall was opened and mice were allowed to freely explore the arena for 5 min. The time spent in the bright compartment (as a measure of anxiety) and the number of line crossings (as a measure of locomotor activity) were analyzed by an observer blind to the animal's housing and treatment condition. The LDB was cleaned thoroughly before each test.

2.6. Stress-induced hyperthermia (SIH) test

The SIH test was performed as described elsewhere (Gee et al., 2014; O'Connor et al., 2013; Peterlik et al., 2016) between 0800 h and 1100 h 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*. Rectal temperature was recorded twice at T1 and T2 (15 min later) using a digital thermometer (2.5 mm diameter, Amarell GmbH and Co KG, Kreuzwertheim, Germany). In order to avoid any rectal injuries the thermometer was covered with milking grease before inserting it 1.5 cm into the rectum. Recording of T1 indicated baseline temperature and served as stressor at the same time. Recording of T2 allowed determination of the SIH response defined as the difference between T2 and T1. In *experiment 1* and *2*, SIH measurement was conducted on day 19 in CSC and SHC mice, with all mice being individually housed between the two temperature recordings at T1 and T2.

2.7. Determination of body weight and organ weight

On day 20, mice were weighed immediately before decapitation to assess the effects of CSC on body weight. Afterwards, the pituitary, left and right adrenal glands, thymus and spleen 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% bovine serum albumin (BSA). Values represent absolute measurements (in mg) of the respective organs.

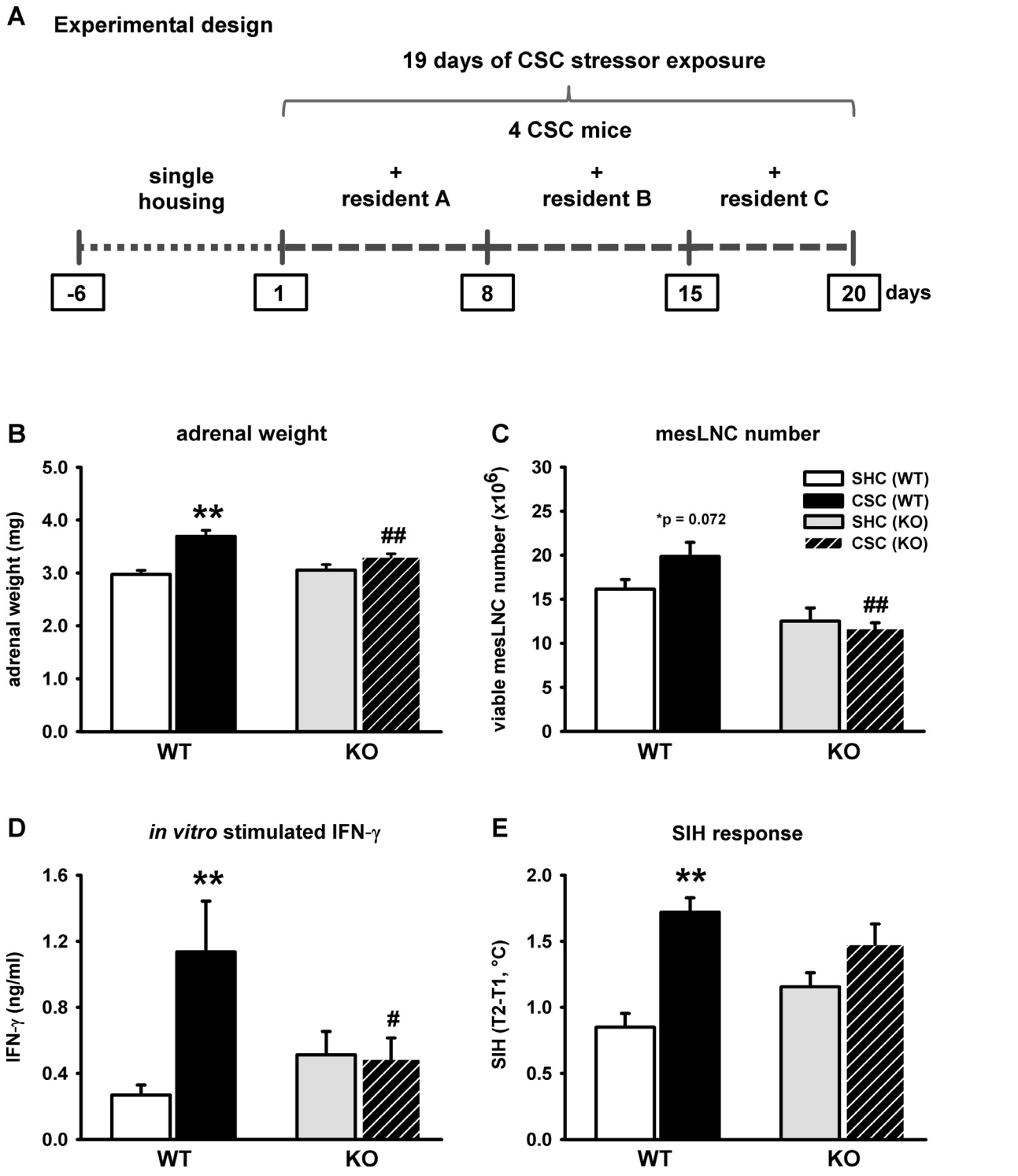

Fig. 1. Experimental design of the chronic subordinate colony housing (CSC, 19 days) paradigm and profile of wildtype (WT) and mGlu5 knockout (KO) mice exposed to CSC. (A) All experimental mice were housed singly for one week before they were assigned in a weight-matched manner to the single-housed control (SHC) or the CSC group. 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 C on day 8 and 15 of CSC, respectively. On day 20 of CSC, (B) absolute adrenal weight (housing: $F_{1,46} = 22.717$, $p \leqslant 0.001$; housing × genotype interaction: $F_{1,46} = 5.516$, $p = 0.023$), (C) the number of viable mesLNC (genotype: $F_{1,47} = 18.228$, $p \leqslant 0.001$), and (D) IFN-γ secretion of stimulated mesLNC *in vitro* (housing × genotype interaction: $F_{1,43} = 4.116$, $p = 0.049$) were assessed in WT and KO mice. (E) SIH response was assessed on day 19 (housing: $F_{1,44} = 23.754$, $p \leqslant 0.001$; housing × genotype interaction: $F_{1,44} = 5.002$, $p = 0.030$). White bar, SHC (WT); black bar, CSC (WT); grey bar, SHC (KO); striped bar, CSC (KO). $n = 9$–16 per genotype and housing group. Data represent mean + SEM. $^{**}p \leqslant 0.01$ *vs.* respective SHC mice; $^{\#}p \leqslant 0.05$, $^{\#\#}p \leqslant 0.01$ *vs.* respective WT genotype; two-way ANOVA followed by Bonferroni *post hoc* analysis or independent Student's *t*-test.

2.8. Trunk blood sampling

To determine the 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 0800 h and 1100 h 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.

2.9. 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; Uschold-Schmidt et al., 2013, 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.

2.10. ELISA for CORT and ACTH

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).

2.11. Determination of the histological damage score of the colon

The histological damage score of colonic tissue was assessed as described previously (Reber et al., 2007). After killing, the colon was removed and mechanically cleaned. 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-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 housing and treatment condition to assess housing- and treatment-specific effects of CSC on the histological damage. Histological damage score ranges from 0 to 8 and represents the sum of the epithelium score (0: normal morphology; 1: loss of goblet cells; 2: loss of goblet cells in large areas; 3: loss of crypts; 4: loss of crypts in large areas) and infiltration score (0: no infiltration; 1: infiltrate around crypt bases; 2: infiltrate reaching to lamina muscularis mucosae; 3: extensive infiltration reaching the lamina muscularis mucosae and thickening of the mucosa with abundant edema; 4: infiltration of the lamina submucosa). The scores (in %) were averaged per animal and group. Scores of respective SHC mice were set to 100%.

2.12. Isolation and incubation of mesLNC

To determine housing-, genotype- and treatment-specific effects of CSC on the IFN-γ secretion of anti-cluster of differentiation 3 (anti-CD3) and anti-CD28-stimulated mesLNC *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). Then, 5×10^5 (100 µl) mesLNC 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) for T cell activation and co-activation, respectively. After incubation for 24 h (*experiment 1*) or 48 h (*experiment 2*) (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.

2.13. RNA processing and quantitative PCR

Total RNA was isolated from prefrontal cortical, hypothalamic and hippocampal tissue using Trizol reagent as described in 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). cDNAs were 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 mGlu5 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA: mGlu5-forward, 5′-TGTGTACCTTCTGCCTCATTGC-3′, mGlu5-reverse, 5′-GGAGAGAGACCGATGCCAATT-3′; GAPDH-forward, 5′-TGTGTCCGTCGTGGATCTGA-3′, GAPDH-reverse, 5′-CCTGCTTCACCACCTTCTTGA-3′. Quantitative PCR 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%).

2.14. Receptor saturation analysis/radioligand binding

Analysis of receptor binding sites was performed using a tritiated version of the mGlu5 PET tracer ABP688 ([^{3}H]ABP688, (Hintermann et al., 2007)) essentially as described elsewhere (Lindemann et al., 2015, 2011). Briefly, membrane preparations isolated from PFC, HT and HC were resuspended in radioligand binding buffer (15 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 1.25 mM $CaCl_2$, and 1.25 mM $MgCl_2$, set to pH 7.4), and the membrane suspension was mixed with the appropriate concentration of radioligand and non-labeled drugs in 96-well plates in a total volume of 200 µl and incubated for 60 min at the appropriate temperature. At the end of the incubation, membranes were filtered onto Whatman (Clifton, NJ, USA) Unifilter (96-well microplate with bonded GF/C filters) preincubated with 0.1% polyethyleneimine in wash buffer (50 mM Tris-HCl, pH 7.4) with a Filtermate 196 harvester (PerkinElmer, Rodgau, Germany) and washed three times with ice-cold wash buffer. Radioactivity captured on the filter was quantified on a Topcount microplate scintillation counter (PerkinElmer, Rodgau, Germany) with quenching correction after the addition of 45 µl of Micro-Scint 40 per well and shaking for 20 min. Protein concentration of membrane suspensions was determined using the Bradford method (Bio-Rad Laboratories,

Reinach, Switzerland) using normalized γ-globulins as standard. Non-specific binding was determined in the presence of 10 μM (unlabeled) MPEP. The receptor binding data are expressed as picomoles per milligram of protein, and nonspecific binding was subtracted from total binding to yield specific binding.

2.15. Statistical analysis

All data represent the mean + or ± S.E.M. and were analyzed using the software IBM SPSS 22.0 (IBM Corporation, Armonk, NY, USA). Parameters depending on one factor (housing or treatment) were analyzed using independent Student's *t-test* or one-way analysis of variance (ANOVA) followed by Bonferroni or least significant difference (LSD) *post hoc* testing. For analyzing parameters depending on two factors (i.e. housing and genotype, housing and treatment, housing and stimulation) two-way ANOVA was employed. Significant main and interaction effects were followed by Bonferroni *post hoc* analysis when appropriate or an independent Student's *t-test*. Data depending on each other were analyzed using repeated measures ANOVA followed by Bonferroni or LSD *post hoc* testing or an independent Student's *t-test*. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Chronic stress-protective phenotype in mice lacking mGlu5

To evaluate a functional role of mGlu5 in CSC-induced somatic and affective alterations, we assessed different physiological, immunological, and anxiety parameters in male mGlu5 KO mice and their WT littermates following CSC exposure. Here, we first examined the effects of CSC on adrenal weight as a highly stress-sensitive hypothalamo-pituitary-adrenal (HPA) axis readout parameter and found a CSC-induced increase in absolute adrenal weight in the WT group ($p \leqslant 0.001$); this stress effect was absent in mGlu5 KO animals. In addition, adrenal weight was lower in CSC KO compared to CSC WT mice ($p = 0.010$; Fig. 1B).

Assessment of the number of mesLNC and stimulated IFN-γ secretion of mesLNC *in vitro* provided information about CSC-induced inflammatory processes. The number of mesLNC was lower in CSC KO mice compared to their CSC WT littermates ($p \leqslant 0.001$) and increased by trend in CSC compared to SHC mice of the WT group (independent Student's *t*-test; $t_{28} = -1.868$ $p = 0.072$; Fig. 1C). Stimulated IFN-γ secretion of mesLNC *in vitro* was increased in CSC compared to SHC mice of the WT group ($p = 0.003$). This stress effect was again absent in KO mice. Moreover, IFN-γ secretion was lower in CSC KO compared to CSC WT mice ($p = 0.043$; Fig. 1D).

To assess the effects of CSC on physiological anxiety, the SIH test was performed on day 19 of CSC. CSC compared to SHC mice of the WT group showed an increased hyperthermic response ($p \leqslant 0.001$); this CSC effect was again absent in KO mice (Fig. 1E). Notably, here we could show for the first time that CSC exposure, in addition to the previously reported increase in innate anxiety, also increased physiological anxiety in WT mice. Moreover, the present results indicate broad stress-protective effects of mGlu5 genetic ablation with respect to the assessed physiological, immunological, and anxiety parameters.

3.2. Chronic pharmacological mGlu5 blockade (s.c. via micro-osmotic pumps) reverses multiple CSC-induced physiological and immunological/inflammatory changes, in a dose-dependent manner, but not the behavioral alterations

To further address the functional involvement of mGlu5 in chronic stress physiology, we assessed whether chronic pharmacological inhibition of mGlu5 interferes with prominent CSC-induced somatic and affective changes. Importantly, testing chronic administration of the vehicle (PEG) and CTEP (2 mg/kg/day) *s.c.* via micro-osmotic pumps in naïve mice for 26 days excluded any undesirable effects on parameters typically assessed after CSC exposure when compared to saline administration (Supplementary Fig. S1A and S1B). This is in line with Peters et al. (2014), revealing no confounding influence of either the surgical procedure or the chronic *i.c.v.* administration of vehicle (in this case Ringer's solution) via micro-osmotic pumps.

In mice implanted with micro-osmotic pumps (*s.c.*) and exposed to CSC, we detected a decreased body weight gain in CSC compared to SHC mice of the vehicle group ($p = 0.028$). This effect was abolished by CTEP at all doses applied. Moreover, CSC mice treated with CTEP at a dose of 0.05 mg/kg/day ($p = 0.011$) and 0.5 mg/kg/day ($p = 0.003$) gained more body weight compared to CSC mice of the vehicle group (Fig. 2A).

A closer look on body weight development of mice treated with vehicle and 2 mg/kg/day of CTEP (exemplary for all CTEP doses) during CSC revealed a positive development of body weight over a period of 19 days in all mice irrespective of treatment and housing. Moreover, independent Student's *t*-tests revealed a decreased body weight in CSC compared to SHC mice of the vehicle group on day 10 ($t_{42} = 2.062$, $p = 0.045$), day 15 ($t_{42} = 2.186$, $p = 0.034$) and day 17 ($t_{42} = 2.116$, $p = 0.040$). In mice treated with 2 mg/kg/day of CTEP, this CSC effect was also present on day 10 ($t_{33} = 2.518$, $p = 0.017$) but absent on day 15 and 17 of CSC (Fig. 2B).

Assessment of different HPA axis-related parameters revealed a CSC-induced increase in pituitary weight (Fig. 2C) concomitant with an increase in basal morning plasma ACTH levels (Fig. 2D) in mice treated with vehicle (pituitary weight: $p = 0.008$; plasma ACTH: independent Student's *t*-test: $t_{42} = -2.297$, $p = 0.027$) and CTEP at a dose of 0.05 mg/kg/day (pituitary weight by trend: $p = 0.057$; plasma ACTH: $p = 0.050$). These CSC-induced effects were not present in mice treated with the two higher doses of CTEP.

CSC exposure also induced an increase in absolute adrenal weight in mice treated with vehicle and CTEP at a dose of 0.05 and 0.5 mg/kg/day ($p \leqslant 0.001$ for each). This CSC effect was completely abolished with CTEP at a dose of 2 mg/kg/day. Consequently, adrenal weight was lower in CSC mice treated with the high dose of CTEP compared to CSC mice of the vehicle group ($p \leqslant 0.001$; Fig. 2E).

Basal morning plasma CORT levels were neither influenced by housing nor by treatment (Fig. 2F).

Next, we analyzed adrenal *in vitro* ACTH responsiveness. In all groups, adrenal explants from both SHC ($p \leqslant 0.001$ for each) and CSC (vehicle: $p = 0.013$; 0.05 CTEP: $p = 0.015$; 0.5 and 2 CTEP: $p \leqslant 0.001$) mice showed an increased CORT secretion in response to ACTH compared to basal (saline) stimulation. However, in mice treated with vehicle and 0.05 mg/kg/day of CTEP, ACTH-induced adrenal CORT secretion was lower in CSC compared to SHC mice ($p \leqslant 0.001$ for each). This CSC-induced attenuation of adrenal *in vitro* ACTH responsiveness was absent in mice treated with 0.5 and 2 mg/kg/day of CTEP (Fig. 2G).

CSC housing has also been shown to induce immunological consequences, e.g. thymus atrophy and splenomegaly and to cause spontaneous colitis (Füchsl et al., 2014; Langgartner et al., 2015; Reber et al., 2007). In the present study, absolute thymus weight was decreased in CSC compared to SHC mice treated with vehicle ($p \leqslant 0.001$) and 0.05 mg/kg/day of CTEP ($p = 0.021$). This CSC-induced thymus atrophy was blocked by treatment with CTEP at doses of 0.5 and 2 mg/kg/day. Furthermore, thymus weight was higher in CSC mice treated with CTEP at a dose of 0.5 ($p = 0.005$) and 2 mg/kg/day ($p = 0.047$) compared to CSC mice of the vehicle group (Fig. 3A).

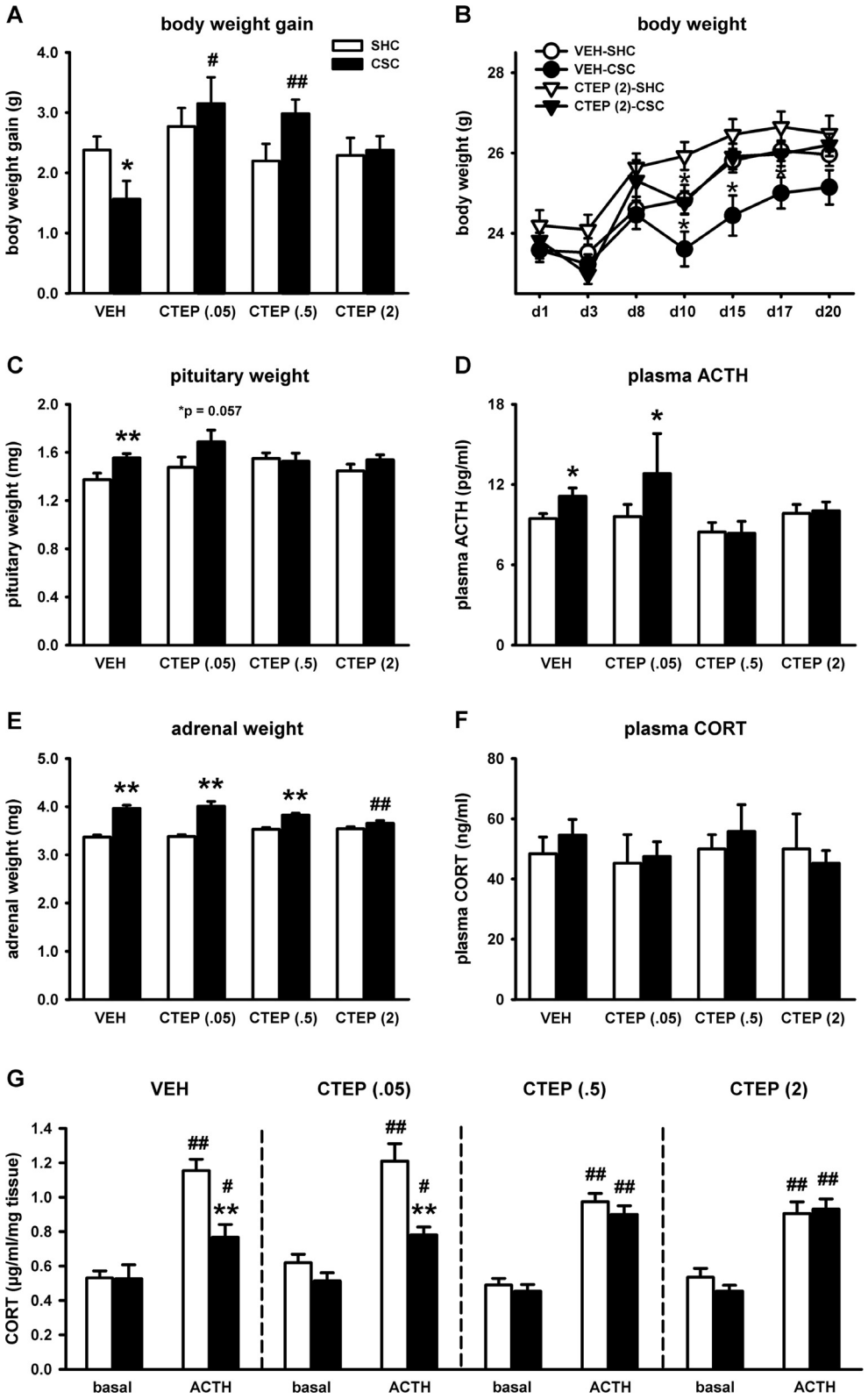

Fig. 2. Stress-protective effects of chronic CTEP treatment (*s.c.*) on CSC-induced physiological alterations. Mice were chronically treated with vehicle or CTEP at different doses during CSC exposure and (A) body weight gain (day 1 to day 20) (treatment: $F_{3,125} = 3.352$; $p = 0.021$; housing × treatment interaction: $F_{3,125} = 3.017$; $p = 0.032$), (B) body weight development during CSC (housing: $F_{3,75} = 3.373$, $p = 0.023$; time: $F_{6,450} = 41.282$, $p \leqslant 0.001$; housing × time interaction: $F_{18,450} = 1.658$, $p = 0.044$), (C) absolute pituitary weight (housing: $F_{1,127} = 7.366$, $p = 0.008$), basal morning plasma ACTH levels (housing: $F_{1,123} = 3.873$, $p = 0.051$; treatment: $F_{3,123} = 3.356$, $p = 0.021$), (E) absolute adrenal weight (housing: $F_{1,128} = 96.834$, $p \leqslant 0.001$; housing × treatment interaction: $F_{3,128} = 9.646$, $p \leqslant 0.001$), (F) basal morning plasma CORT levels, and (G) adrenal *in vitro* ACTH responsiveness (vehicle: housing: $F_{1,86} = 8.660$, $p = 0.004$; stimulation: $F_{1,86} = 42.362$, $p \leqslant 0.001$; housing × stimulation interaction: $F_{1,86} = 8.282$, $p = 0.005$; 0.05 CTEP: housing: $F_{1,32} = 14.705$, $p \leqslant 0.001$; stimulation: $F_{1,32} = 37.689$, $p \leqslant 0.001$; housing × stimulation interaction: $F_{1,32} = 5.313$, $p = 0.028$; 0.5 CTEP: stimulation: $F_{1,64} = 108.316$, $p \leqslant 0.001$; 2 CTEP: stimulation: $F_{1,58} = 60.518$, $p \leqslant 0.001$) were assessed on day 20 of CSC. White bar, SHC; black bar, CSC. $n = 8$–24 per treatment/stimulation and housing group. Data represent mean + SEM. $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$ *vs.* respective SHC mice; $^{\#}p \leqslant 0.05$, $^{\#\#}p \leqslant 0.01$ *vs.* respective vehicle (VEH) group (A, E); $^{\#}p \leqslant 0.05$, $^{\#\#}p \leqslant 0.01$ *vs.* respective basal stimulation (G); two-way (A, C, D, E, F, G) or repeated measures (B) ANOVA followed by Bonferroni *post hoc* analysis or independent Student's *t*-test.

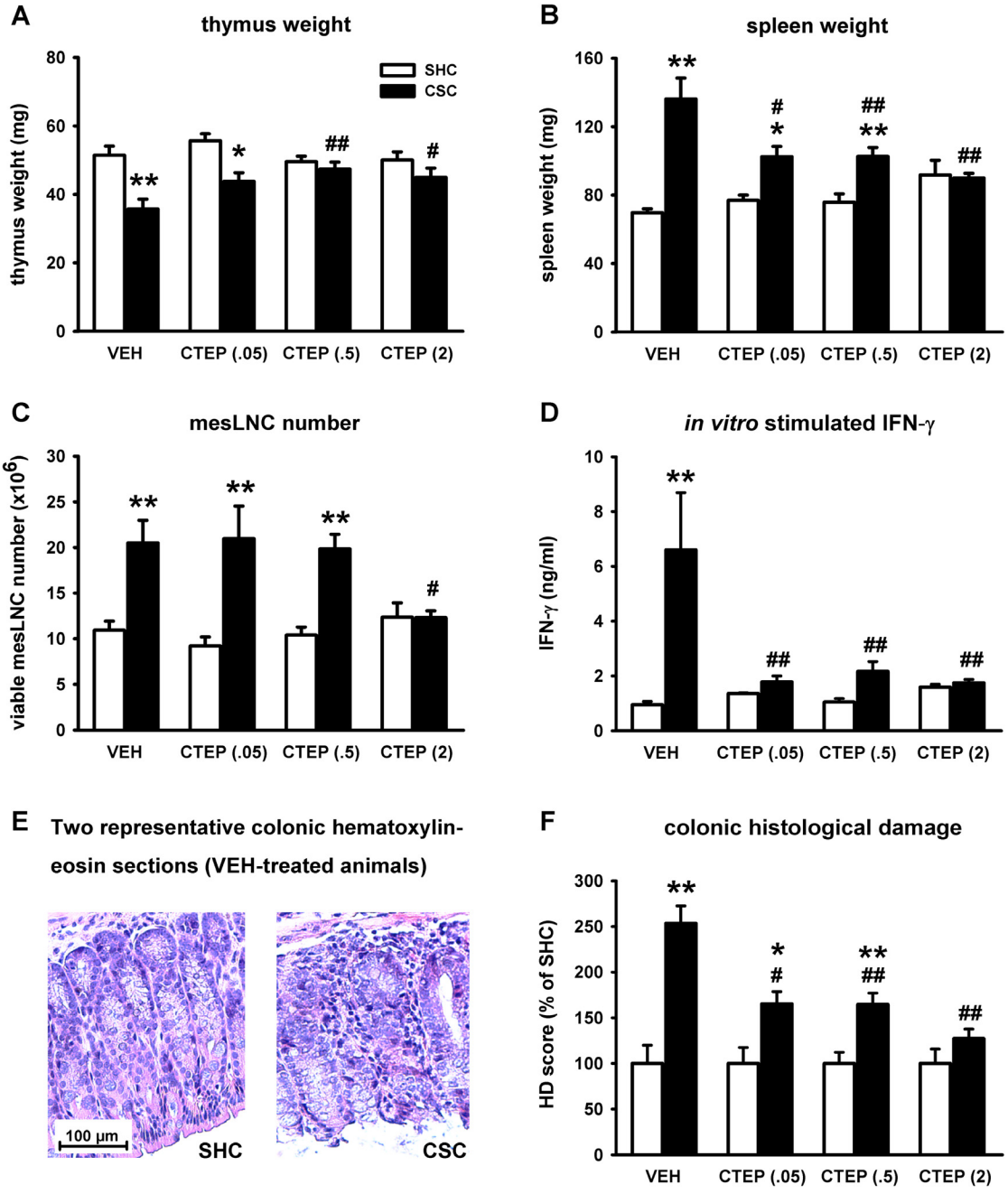

Fig. 3. Stress-protective effects of chronic CTEP (*s.c.*) on CSC-induced immunological alterations. Mice were chronically treated with vehicle or CTEP at different doses during CSC exposure and (A) absolute thymus weight (housing: $F_{1,128} = 19.837$, $p \leqslant 0.001$; housing treatment interaction: $F_{3,128} = 3.296$, $p = 0.023$), (B) absolute spleen weight (housing: $F_{1,95} = 30.239$, $p \leqslant 0.001$; housing × treatment: $F_{3,95} = 7.527$, $p \leqslant 0.001$), (C) the number of viable mesLNC (housing: $F_{1,93} = 36.841$, $p \leqslant 0.001$; housing × treatment interaction: $F_{3,93} = 3.424$, $p = 0.020$), (D) stimulated IFN-γ secretion of mesLNC *in vitro* (housing: $F_{1,89} = 7.708$, $p = 0.007$; treatment: $F_{3,89} = 3.401$, $p = 0.021$; housing × treatment interaction: $F_{3,89} = 4.555$, $p = 0.005$), and (F) the histological damage (HD) score of colonic tissue (housing: $F_{1,82} = 34.776$, $p \leqslant 0.001$; treatment: $F_{3,82} = 5.075$, $p = 0.003$; housing × treatment interaction: $F_{3,82} = 5.075$, $p = 0.003$) were assessed on day 20 of CSC. (E) Representative colonic sections stained with hematoxylin and eosin from SHC (left; normal colon histology) and CSC (right; goblet cell loss and crypt loss in locally restricted areas and infiltration of cells reaching the Lamina muscularis mucosae) mice of the vehicle group. White bar, SHC; black bar, CSC. $n = 8$–24 per treatment and housing group. Data represent mean + SEM. $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$ *vs.* respective SHC mice; $^{\#}p \leqslant 0.05$, $^{\#\#}p \leqslant 0.01$ *vs.* respective vehicle (VEH) group; two-way ANOVA followed by Bonferroni *post hoc* analysis.

CSC-induced splenomegaly, i.e. an increase in absolute spleen weight, was detected in mice treated with vehicle ($p \leqslant 0.001$) and CTEP at doses of 0.05 ($p = 0.037$) and 0.5 mg/kg/day ($p = 0.002$). This CSC effect was blocked by treatment with 2 mg/kg/day of CTEP. Furthermore, spleen weight was also found to be lower in CSC mice of all CTEP groups compared to CSC mice treated with vehicle (0.05 CTEP: $p = 0.017$; 0.5 CTEP: $p = 0.002$; 2 CTEP: $p \leqslant 0.001$; Fig. 3B).

Beneficial effects of chronic mGlu5 blockade were also found with respect to CSC-induced colonic inflammation. The number of viable mesLNC was found to be increased in CSC compared to SHC mice treated with vehicle and CTEP at doses of 0.05 and 0.5 mg/kg/day ($p \leqslant 0.001$ for each). This increase was completely blocked by 2 mg/kg/day of CTEP. Moreover, the number of viable mesLNC was lower in CSC mice treated with CTEP at a dose of 2 mg/kg/day compared to CSC mice of the vehicle group

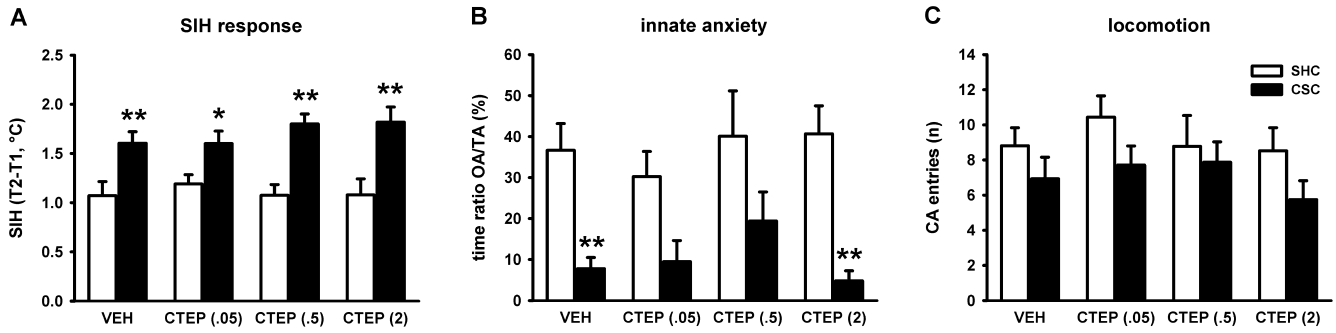

Fig. 4. Behavioral profile of mice with chronic CTEP treatment *(s.c.)* during 19 days of CSC. Mice were chronically treated with vehicle or CTEP at different doses during CSC exposure and (A) the SIH response was assessed on day 19 of CSC (housing: $F_{1,129} = 32.902$, $p \leqslant 0.001$). One day before on day 18 of CSC, (B) the percentage of time spent on the open arms (time ratio OA/TA in %; housing: $F_{1,92} = 31.780$, $p \leqslant 0.001$) and (C) the number of closed arm (CA) entries (housing: $F_{1,92} = 4.501$, $p = 0.037$) on the EPM were assessed. White bar, SHC; black bar, CSC. Data represent mean + SEM. $^{*}p \leq 0.05$, $^{**}p \leqslant 0.01$ *vs.* respective SHC mice. $n = 8$–24 per treatment and housing group; two-way ANOVA followed by Bonferroni *post hoc* analysis or independent Student's *t*-test.

($p = 0.015$; Fig. 3C). IFN-γ secretion of mesLNC *in vitro* was increased in CSC compared to SHC mice treated with vehicle ($p \leqslant 0.001$). This CSC effect was blocked by CTEP-treatment independent of the dose applied. Furthermore, *in vitro* IFN-γ secretion was lower in CSC mice of all CTEP groups compared to CSC mice of the vehicle group (0.05 CTEP: $p = 0.006$; 0.5 CTEP: $p = 0.001$; 2 CTEP: $p = 0.003$; Fig. 3D).

In support of the development of colonic inflammation, we also detected an increased colonic histological damage score in CSC compared to SHC mice treated with vehicle ($p \leqslant 0.001$; Fig. 3F), reflected by increased epithelial damage and more severe inflammatory infiltration (Fig. 3E). This CSC effect was also present in mice treated with CTEP at doses of 0.05 ($p = 0.035$) and 0.5 mg/kg/day ($p = 0.003$), but absent in mice treated with 2 mg/kg/day of CTEP. Moreover, colonic histological damage was higher in CSC mice treated with vehicle compared to CSC mice of all three CTEP groups (each $p \leqslant 0.014$) (Fig. 3F).

As CSC exposure reliably increases anxiety-related behavior in mice (Langgartner et al., 2015; Reber and Neumann, 2008), we evaluated possible beneficial effects of CTEP-treatment on CSC-induced innate as well as physiological anxiety with the EPM and SIH test, respectively. With respect to the latter, ANOVA revealed a CSC-induced increase in the hyperthermic response in mice treated with vehicle ($p = 0.002$) as well as CTEP at doses of 0.5 and 2 mg/kg/day ($p \leqslant 0.001$ for each), indicative for an increase in physiological anxiety. An independent Student's *t*-test additionally revealed an increased hyperthermic response in CSC compared to SHC mice treated with 0.05 mg/kg/day ($t_{16} = -2.645$, $p = 0.018$; Fig. 4A).

The time the mice spent on the open arms of the EPM was decreased in CSC compared to SHC mice of the vehicle ($p \leqslant 0.001$) as well as all CTEP groups (0.05 CTEP: by trend $p = 0.065$; 0.5 CTEP: by trend $p = 0.057$; 2 CTEP: $p \leqslant 0.001$). These results suggest also no beneficial effects of CTEP on the CSC-induced increase in innate anxiety (Fig. 4B). In addition, there were no significant differences between CSC and SHC mice of all groups with respect to the number of closed arm (CA) entries, indicating no adverse effects of CSC or CTEP treatment on locomotor activity (Fig. 4C).

3.3. Chronic pharmacological mGlu5 blockade (i.p. via micro-osmotic pumps) reverses multiple CSC-induced physiological, immunological/inflammatory, and behavioral alterations

To verify and confirm CTEP's chronic stress protective potential obtained via *s.c.* administration, we applied CTEP chronically via an alternative route (*i.p.*) at a dose of 2 mg/kg/day during CSC exposure and assessed selected physiological, immunological, and behavioral parameters. 2 mg/kg/day of CTEP given *i.p.* were able to attenuate the CSC-induced increase in absolute adrenal weight seen in vehicle-treated CSC *versus* SHC mice on day 20 (CTEP: $p \leqslant 0.001$; vehicle: $p = 0.013$). In addition, absolute adrenal weight was slightly higher in CTEP-treated compared to vehicle-treated SHC mice ($p = 0.020$) and lower in CTEP-treated compared to vehicle-treated CSC mice ($p = 0.003$; Table 1).

In vitro stimulation of adrenal explants revealed an increased CORT secretion in response to ACTH compared to basal (saline) stimulation in SHC and CSC mice from both the CTEP and vehicle group ($p \leqslant 0.023$ for each). However, in mice treated with vehicle, ACTH-induced adrenal CORT secretion was lower in CSC compared to SHC mice ($p = 0.006$). This CSC-induced attenuation was absent in mice treated with 2 mg/kg/day of CTEP (Table 1).

Table 1
Stress-protective effects of chronic CTEP treatment (*i.p.*) on CSC-induced physiological, immunological, and behavioral alterations. Mice were chronically treated with vehicle or CTEP at a dose of 2 mg/kg/day during CSC exposure and absolute adrenal weight (housing: $F_{1,27} = 58.132$, $p \leqslant 0.001$; housing × treatment interaction: $F_{1,27} = 16.247$, $p \leqslant 0.001$), *in vitro* adrenal CORT secretion (vehicle: housing: $F_{1,24} = 5.306$, $p = 0.03$; treatment: $F_{1,24} = 25.514$, $p \leqslant 0.001$; CTEP: treatment: $F_{1,30} = 26.711$, $p \leqslant 0.001$), absolute thymus weight (housing: $F_{1,26} = 6.783$, $p = 0.015$), and absolute spleen weight (housing: $F_{1,24} = 8.898$, $p = 0.006$) were assessed on day 20. In addition, on day 14 of CSC the time the mice spent in the bright compartment in the LDB (housing: $F_{1,29} = 7.243$, $p = 0.012$; treatment: $F_{1,29} = 5.527$, $p = 0.026$) and the number of line crossings (housing: $F_{1,29} = 5.311$, $p = 0.029$; treatment: $F_{1,29} = 5.492$, $p = 0.026$) during the LDB test were assessed as a measure of innate anxiety levels and locomotor activity, respectively. $n = 6$–10 per housing and treatment group. Data represent mean ± SEM. $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$ *vs.* respective SHC mice; $^{\#}p \leqslant 0.05$, $^{\#\#}p \leqslant 0.01$ *vs.* respective vehicle (VEH) group (adrenal weight and time in bright compartment) or respective basal values (adrenal CORT secretion); two-way ANOVA followed by Bonferroni *post hoc* analysis.

Parameter	SHC-VEH	CSC-VEH	SHC-CTEP(2)	CSC-CTEP(2)
Absolute adrenal weight (mg)	3.371 ± 0.047	4.371 ± 0.141**	3.667 ± 0.065$^{\#}$	3.975 ± 0.073$^{*,\#\#}$
In vitro adrenal CORT secretion – basal (μg/ml/mg)	0.434 ± 0.033	0.406 ± 0.063	0.430 ± 0.044	0.371 ± 0.052
In vitro adrenal CORT secretion – ACTH (μg/ml/mg)	0.947 ± 0.077$^{\#\#}$	0.649 ± 0.095$^{**,\#}$	0.673 ± 0.056$^{\#\#}$	0.687 ± 0.064$^{\#\#}$
Absolute thymus weight (mg)	47.957 ± 2.169	36.757 ± 4.010*	43.140 ± 1.822	38.817 ± 4.085
Absolute spleen weight (mg)	79.650 ± 3.403	112.457 ± 8.627**	76.089 ± 3.995	88.133 ± 12.405
LDB – time in bright compartment (s)	156.267 ± 14.357	99.101 ± 6.687*	178.074 ± 20.204	150.938 ± 13.252$^{\#}$
LDB – line crossings (n)	83.000 ± 7.345	64.750 ± 6.982	109.500 ± 12.126	83.375 ± 8.004

With respect to immunological parameters, we detected a CSC-induced atrophy of the thymus ($p = 0.015$) and a CSC-induced splenomegaly ($p = 0.006$) in mice treated with vehicle. Both CSC effects were absent in mice treated with 2 mg/kg/day of CTEP.

As mentioned above, CSC exposure reliably increases anxiety-related behavior in mice. This CSC effect is already detectable around day 15 of CSC exposure (Peters et al., 2014). As we did not see any effects of chronic CTEP treatment with respect to anxiety on day 18 (EPM) and day 19 (SIH) of CSC in the *s.c.* approach, maybe due to insufficient receptor saturation in this late phase of CSC exposure, we assessed the effects of chronic CTEP given *i.p.* on anxiety-related behavior already on day 14 of CSC (one day before the final change of the dominant male) in the LDB test. We detected a decreased time spent in the bright compartment of the LDB in CSC compared to SHC mice of the vehicle group ($p = 0.019$), indicative for an increase in anxiety. This CSC effect was absent in mice treated with 2 mg/kg/day of CTEP. In addition, CSC mice treated with CTEP spent more time in the bright compartment than CSC mice of the vehicle group ($p = 0.027$). No significant differences between CSC and SHC mice of both groups were found with respect to the number of line crossings, suggesting no adverse effects of CSC or CTEP treatment on locomotor activity (Table 1).

3.4. Chronic psychosocial stress in mice affects mGlu5 mRNA expression and receptor binding in the CNS

Assessing the molecular changes that occur within the mGlu system in the CNS upon chronic psychosocial stressor exposure in mice, we found that CSC resulted in a trend towards increased mGlu5 mRNA transcript levels in the hypothalamus ($p = 0.071$; see also Peterlik et al., 2016). No CSC-induced changes could be detected in the prefrontal cortex or in the hippocampus, when compared to SHC mice (Fig. 5A). Furthermore, analysis of mGlu5 receptor binding sites in the same brain regions as above, using radioligand binding on membrane preparations from micro-dissected brain regions with [^{3}H]-ABP688, revealed a CSC-induced increase in receptor binding sites in hippocampal membrane preparations ($p = 0.001$). In contrast, no changes were detectable in membrane preparations of the prefrontal cortex and hypothalamus compared to the SHC groups (Fig. 5B). Importantly, the k_d (dissociation equilibrium constant) as a measure for CTEP's affinity to mGlu5 did not differ between the groups (data not shown).

4. Discussion

In the present study we provide first evidence for the involvement of mGlu5 in mediating physiological, immunological, and behavioral consequences of chronic psychosocial stress in mice using a chronic male subordination paradigm, termed CSC. First, we demonstrate that genetic ablation of mGlu5 decreases the vulnerability to multiple alterations induced by 19 days of CSC, including physiological anxiety. Second, we show that chronic treatment of mice with the systemically active mGlu5 NAM CTEP, a close analogue to the clinically active drug basimglurant – but optimized for rodent studies, is protective against the physiological and immunological consequences of 19 days of CSC, and also ameliorates the anxiety-prone phenotype on day 14 of CSC exposure. Third, we demonstrate a CSC-induced upregulation of mGlu5 expression in the hippocampus, indicating region-specific activation of the mGlu5 system during chronic psychosocial stress. Together, these findings suggest for mGlu5 to be a valuable and novel target for the treatment of chronic stress-induced pathologies in man.

4.1. Stress-protective phenotype in mice lacking mGlu5

Assessment of multiple parameters suggests a stress-protective phenotype in mice with genetic ablation of mGlu5. At first, the effect of CSC on the main stress response system, namely the HPA axis, was assessed. We focused therefore on the adrenal glands as the effector organs of the HPA axis, which are highly sensitive indicators for chronic stress. As previously described for the CSC paradigm (Füchsl et al., 2014; Reber et al., 2007; Uschold-Schmidt et al., 2012), WT mice of the present study also showed a CSC-induced adrenal hypertrophy. This chronic stress effect was absent in mice lacking mGlu5. Furthermore, CSC exposure induced a trend towards an increased number of isolated viable mesLNC as well as significantly increased secretion of the pro-inflammatory cytokine IFN-γ from these cells following *in vitro* anti-CD3/CD28 stimulation in the WT group. Both findings – in agreement with previous reports and indicative for the development of spontaneous colonic inflammation (Langgartner et al., 2015; Reber et al., 2011, 2007) – were again not present in mice lacking mGlu5.

CSC exposure also reliably results in an increase in innate anxiety, recorded e.g. in the EPM, in the elevated platform, in the light-dark box, and in the open-field tests (Reber and Neumann, 2008; Reber et al., 2007; Slattery et al., 2012; Uschold-Schmidt

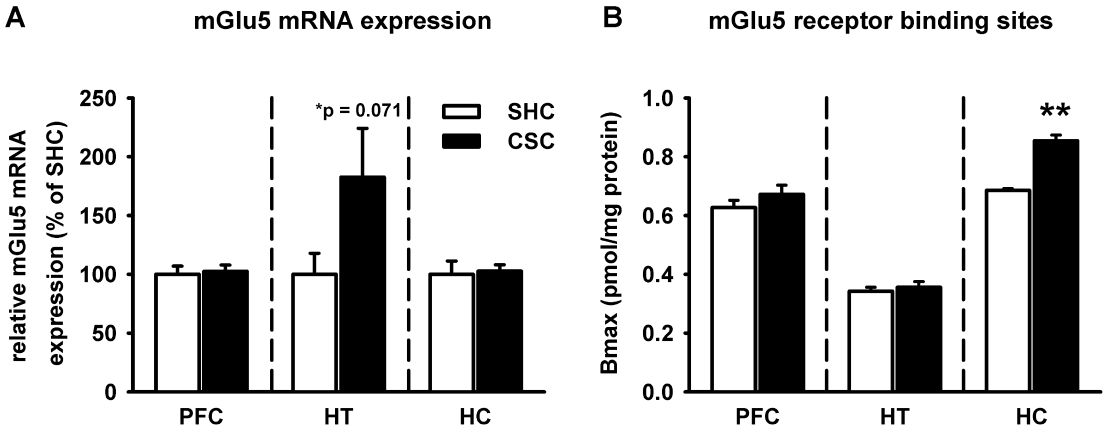

Fig. 5. Effects of CSC exposure on brain mGlu5 mRNA and receptor binding sites. Following 19 days of CSC, (A) relative mGlu5 mRNA expression was assessed in the hypothalamus (HT), prefrontal cortex (PFC) and hippocampus (HC). White bar, SHC ($n = 6$); black bar, CSC ($n = 9$–11). In addition, (B) mGlu5 binding site were assessed in membrane preparations from the same brain regions using the highly selective radioligand [^{3}H]-ABP688. White bar, SHC ($n = 3$, pool of 8 animals for each n); black bar, CSC ($n = 3$, pool of 8 animals for each n). Data represent mean + SEM. $^{**}p \leq 0.01$ *vs.* respective SHC mice, independent Student's *t*-test.

et al., 2012). In the present study, CSC *vs.* SHC mice were exposed to the SIH test on day 19 of CSC, an animal model addressing a physiological component of anxiety that is sensitive to anxiolytic drugs (Adriaan Bouwknecht et al., 2007; Borsini et al., 1989). Interestingly, we could show for the first time that CSC exposure, in addition to innate anxiety, also increases physiological anxiety levels. This was indicated by a CSC-induced increased hyperthermic response in WT mice in the SIH test. Again, this chronic stress effect was absent in mGlu5 KO mice. The previously reported anxiolytic-like effect of mGlu5 depletion (Brodkin et al., 2002) was not reproduced, as we didn't find a lower SIH response in KO compared to WT mice within the SHC group. This might be due to genetic differences between mouse strains (B6;129-*Gprc1e*^{tm1Rod} *vs.* C57/BL6 mice) and differences in the experimental setup (measurement of T2 at 30 min *vs.* 15 min after T1 in the SIH test).

Together, our findings suggest that mice deficient for mGlu5 are protected against important CSC-induced alterations of physiological, immunological, and anxiety parameters, at least with respect to physiological anxiety. Thus, mGlu5 KO mice seem to be resilient to a broad variety of maladaptive consequences of chronic psychosocial stressor exposure.

4.2. Stress-protective effects of chronic CTEP treatment

Given the promising chronic stress-protective phenotype of mGlu5 KO mice, we further analyzed the effects of pharmacological mGlu5 inhibition in mice exposed to CSC. Relevant CSC-affected parameters were analyzed in even more detail after chronic (during CSC) *s.c.* and *i.p.* administration of the mGlu5 NAM CTEP. Different doses of CTEP (0.05, 0.5, and 2 mg/kg/day) were used in the *s.c.* approach to evaluate dose-dependency. In the *i.p.* approach we focused on 2 mg/kg/day of CTEP. The doses used were chosen according to previously published data suggesting sufficient plasma and brain exposure, sustained receptor occupancy as well as activity in animal models of anxiety (SIH, Vogel conflict drinking test) and Fragile X syndrome (Lindemann et al., 2011; Michalon et al., 2012).

Well in line with previous studies using untreated mice, vehicle-treated CSC *vs.* SHC mice of the present study, showed typical reliable CSC-induced physiological and immunological alterations following 19 days of CSC, independent of *s.c.* or *i.p.* administration via micro-osmotic pumps. They showed a reduced body weight gain (Reber et al., 2007; Veenema et al., 2008) and alterations in HPA axis functionality including hypertrophy of the pituitary and of the adrenal glands together with a reduced ACTH reactivity of the latter (Füchsl et al., 2013; Langgartner et al., 2015; Uschold-Schmidt et al., 2012). Furthermore, we detected different immunological changes including thymus atrophy and splenomegaly, as well as an increased number of isolated viable mesLNC accompanied by an increased *in vitro* IFN-γ secretion from these cells. Together with the increased histological damage score of the colon in these mice, these findings indicate CSC-induced colonic inflammation (Füchsl et al., 2014; Reber et al., 2011, 2007). Interestingly, all these CSC-induced alterations found in vehicle-treated mice were attenuated or even abolished in mice chronically treated with CTEP *s.c.* or *i.p.*, with the effects being mostly dose-dependent in the *s.c.* approach. Together, our findings indicate that mice with pharmacological blockade of mGlu5 are less vulnerable to CSC-induced physiological and immunological/inflammatory alterations.

It has to be mentioned that the finding of a stress-protective effect of CTEP with respect to alterations in HPA axis functionality in the present study differs from to the study conducted by Wagner et al. (2014), showing no beneficial effects of oral CTEP (2 mg/kg; every 48 h) on the dysregulated HPA axis activity in mice exposed to chronic social defeat stress (CSDS; 21 days). These discrepancies might be due to the use of different chronic stress paradigms, likely resulting in divergent changes in the mGlu5 system of the brain and/or periphery and, thus, different CTEP-mediated effects. Moreover, it cannot be excluded that the stress induced by repeated oral applications of CTEP interfered with possible beneficial effects of mGlu5 blockade on the CSDS-induced HPA axis dysregulation.

Also in line with previous data (Reber and Neumann, 2008; Reber et al., 2007) and the above mentioned data from WT mice, CSC exposure of vehicle-treated mice in the *s.c.* approach of the present study increased innate and physiological anxiety, measured on the EPM (day 18) and in the SIH test (day 19), respectively. Different than expected, CTEP applied *s.c.* in our study did not normalize the CSC-induced anxiety-prone phenotype, despite the above reported physiological anxiety-preventing effects in mGlu5 KO CSC mice (also assessed on day 19 of CSC) and the recovery of behavioral alterations by chronic oral CTEP administration in mice exposed to CSDS for 21 days (Wagner et al., 2014). However, when anxiety-related behavior was assessed in the LDB on day 14 of CSC in the *i.p.* approach, CTEP treatment was actually able to prevent the CSC-induced anxiety-prone phenotype seen in vehicle-treated mice. This discrepancy with the *s.c.* approach might be explained by sufficient mGlu5 receptor saturation on day 14 (*i.p.* approach), but sub-maximal saturation with CTEP on day 18/19 of CSC (*s.c.* approach), possibly due to loss of pump functioning. In addition, receptor saturation on day 18/19 in the *s.c.* approach might also have been lower than the level of saturation reached by Wagner et al. (2014) with repeated oral administration and therefore insufficient to correct behavioral effects in this late stage of CSC exposure. Moreover, the stress-protective effect with respect to physiological anxiety on day 19 of CSC in mGlu5 KO mice suggests that a higher receptor saturation in brain regions involved in emotion circuitry on day 18/19 might, in addition to physiological and immunological changes, also correct the CSC-induced anxiety-prone phenotype in this late stage of CSC. This would also be consistent with the robust anxiolytic effect of CTEP applied *i.p.* in naïve mice using doses of 0.5 and 2 mg/kg in an acute (1 h) and 2 mg/kg/day in a sub-chronic (day 16 and 24) experimental design (Supplemental Fig. S2A–S2C), which might be due to a sufficient mGlu5 receptor saturation at the moment of behavioral testing. However, future studies will need to resolve the underpinnings of the discrepancies in the behavioral effects of CTEP, e.g. ensuring high receptor saturation until the end of CSC exposure.

The mechanisms underlying the stress-protective effects of mGlu5 blockade still remain elusive. However, based on the literature, the synergistic reciprocal interaction between mGlu5 and NMDA receptors (O'Leary et al., 2000; Palucha and Pilc, 2007) or the close interaction between the glutamatergic and the serotonergic system (Fukumoto and Chaki, 2015) might play a role. Other studies suggest the involvement of neuropeptides that are crucial for modulating stress responses in the stress-protective action of mGlu5 blockade, such as neuropeptide Y (Wierońska et al., 2004). On the other hand, similar to the proposed mechanisms underlying the effects of the clinically active CTEP analogue basimglurant (Fuxe and Borroto-Escuela, 2015), different types of mGlu5 heteroreceptor complexes of cortical limbic GABA interneurons may be targeted, resulting in disinhibition of glutamate projection neurons of the circuits of stress-related mood and emotion pathways and restoration of activity other stress-relevant brain circuits. In addition, as mGlu5 expression is abundant in peripheral endocrine organs like the adrenal glands (Pokusa et al., 2014), immune cells (Storto et al., 2000), and in the gastrointestinal tract (Julio-Pieper et al., 2011), also peripheral mechanisms might play a role in the receptor's involvement in chronic

psychosocial stress. For example, mGlu5 was shown to be expressed in addition to astrocytes in the CNS also on enteric glial cells (Nasser et al., 2007), which play an important role in gastrointestinal homeostasis (Sharkey, 2015). Interestingly, Nasser et al. (2007) found a dysregulation of mGlu5 distribution and expression on enteric glia in rodent models of chemically induced colitis and spontaneous chronic intestinal inflammation, respectively, suggesting an involvement of mGlu5 in inflammatory processes. Thus, it is quite likely that CSC-induced colonic inflammation is, at least partly, induced by changes in glial mGlu5 expression in the enteric nervous system. Furthermore, as these enteric glial cells also regulate neuronal activity, comparable to astrocytes in the CNS, changes in the expression patterns of glial mGlu5 may also influence gut-brain communication mediated e.g. by the vagal nerve (Carabotti et al., 2015; Savidge et al., 2007); the resulting physiological alterations may be blocked by CTEP treatment. To finally proof this, further studies are required.

4.3. CSC-induced changes in brain mGlu5 expression

In addition to the peripheral expression, the effects of CSC on the brain glutamatergic system are also little investigated. The latter was addressed in the present study. We describe a trend towards a CSC-induced increase of mGlu5 mRNA levels specifically in the hypothalamus. This is in accordance with our previous report, where we additionally found no CSC-induced changes of mGlu2 and mGlu3 mRNA levels in the hippocampus, hypothalamus or the prefrontal cortex (Peterlik et al., 2016). These are interesting differences given that also roles for group II mGlus in modulating stress physiology have been reported (Chaki et al., 2013; Laloux et al., 2012; Lee et al., 2006; Matrisciano et al., 2012). Furthermore, assessing mGlu5 protein expression by [^{3}H]ABP688 radioligand binding in the present study revealed a specific increase in the hippocampus of CSC *vs.* SHC mice. Together with the unchanged mGlu5 transcript levels in this region, this suggests that mGlu5 protein upregulation occurs independent from transcriptional processes. These results are in line with previous reports showing alterations in brain mGlu5 following chronic stressor exposure. Employing the chronic mild stress model, Wierońska et al. (2001) showed changes in hippocampal mGlu5 protein expression with an increase in the CA1 and a decrease in the CA3 region, paralleled by an increase in depressive-like behavior. In contrast, CSC mice typically show an increased anxiety-related behavior without changes in depressive-like behavior (Slattery et al., 2012). Thus, different qualities of chronic stressors (i.e. a continuous psychosocial stressor with only repeated physical interaction *vs.* continuous exposure to a combination of physical, psychological and physiological stress) may have different effects on brain mGlu5 expression and/or signaling. Taken together, our data suggest increased mGlu5 activity in brain regions involved in the regulation of behavior and HPA axis functionality following CSC exposure. This mGlu5 dysregulation may, at least in part, contribute to the consequences of CSC exposure.

In conclusion, our studies employing genetic and pharmacological inhibition of mGlu5 activity suggest a chronic stress-protective effect of mGlu5 functional blockade. Moreover, our results suggest that the stress-protective effects are due to acute mGlu5 inhibition as opposed to potential neurodevelopmental effects of mGlu5 ablation in the KO mice. Furthermore, we show that CSC exposure upregulates mGlu5 expression in brain regions regulating behavioral and physiological stress responses. Together, these findings strongly indicate a role for mGlu5 in mediating a broad variety of consequences induced by chronic psychosocial stressor exposure and, thus, suggest for mGlu5 to be valuable as a novel target for the treatment of chronic stress-induced pathologies in man.

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Conflict of interest

Lothar Lindemann and Georg Jaeschke are full-time employees of F. Hoffman-La Roche AG, Basel, Switzerland. The other authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2016.08.007.

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