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Adrenal Insufficiency and Colonic Inflammation after a Novel Chronic Psycho-Social Stress Paradigm in Mice: Implications and Mechanisms

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We investigated chronic psycho-social stress effects on stress-related parameters and on pathohistological changes in the murine colon. Moreover, we aimed to reveal the involvement of adrenal glucocorticoids in chronic stress effects. Chronic subordinate colony housing (CSC, 19 d) resulted in reduced body weight gain, thymus atrophy, adrenal hypertrophy, increased plasma norepinephrine, and increased anxiety. With respect to the time course of CSC effects, CRH mRNA in the hypothalamic paraventricular nucleus, light phase corticosterone and tyrosine hydroxylase expression in colonic tissue were found to be increased, whereas tyrosine hydroxylase expression in the locus coeruleus was found to be decreased on d 2 of CSC; these parameters returned to control levels thereafter. Nevertheless, after 19 d of CSC exposure, the adrenal corticosterone responses *in vivo* and *in vitro*, and glucocorticoid sensitivity of isolated splenic cells were found to be decreased. Importantly, in CSC mice a significant histological damage of the colon was found beginning on d 14 of CSC exposure. Additionally, pro- and antiinflammatory cytokine secretion by mesenteric lymph node cells was increased after CSC exposure. Adrenalectomy before CSC at least partially prevented these chronic stress effects as reflected by less increase in proinflammatory cytokine secretion and an equal histological damage score in adrenalectomized compared with sham-operated CSC mice. In conclusion, chronic exposure to CSC alters relevant neuronal, neuroendocrine and immune functions that could be directly or indirectly involved in the damage of the histological integrity of the colon comparable with that seen during the development of colitis. (*Endocrinology* 148: 670–682, 2007)

CHRONIC STRESS IS a potential risk factor for the development of gastrointestinal disorders, such as irritable bowel syndrome or inflammatory bowel diseases, including ulcerative colitis (1–3). Both animal and human studies demonstrate that exposure to various stressors affects the functional integrity of the gastrointestinal tract leading to altered production and release of mucin and impaired colonic mucosal barrier functions, which may result in increased antigen infiltration (4–11). Thus, acute exposure to 30-min immobilization stress immediately increased mucin release from colonic mucosal tissue (4), which may provide a short-term protection against luminal antigens. In contrast, after an initial increase, colonic mucin production was significantly reduced during repeated cold-restraint stress exposure performed over 5 d consecutively (1 h/d) in Sprague Dawley rats (5). Similarly, reduced colonic mucin production has also been reported in both human patients, suffering from ulcerative colitis (6), and in cotton-top tamarins that have developed a spontaneous, active or quiescent colitis after capture and readjustment to a novel social environment (7). In addition, reduced mean net water absorption, and changed mean net sodium and chloride absorption (8) were reported under conditions of experimental stress in human jejunum (9, 10) and rat ileum and colon (11), possibly contributing to stress-induced diarrhea (12).

Moreover, exposure to relatively acute stressors impaired the mucosal barrier function in different intestinal parts of Swiss 3T3 mice (13) and Wistar Kyoto rats (14, 15). Furthermore, acute forced swimming stress (20 min) increased luminal permeability in all segments of the gastrointestinal tract within 24 h after stress exposure in mice (16), which was thought to be caused by the stress-induced rise in plasma corticosteroids.

Although these data provide evidence for a link between stressor exposure and impaired intestinal barrier functions, pathological alterations of the colonic architectural structure have not been described. This could be due to the fact that the duration of the stressors applied was too short and/or their severity was not efficient to induce histological alterations comparable to those seen, for example, in individuals with inflammatory bowel diseases.

Therefore, in the present study, we aimed to establish chronic subordinate colony housing (CSC) in male mice as a clinically relevant chronic psycho-social stressor measuring relevant behavioral and physiological markers (17–27), and to investigate the influence of exposure to 19 d of CSC on established histological parameters of the colon (28, 29) (experiment 1).

Furthermore, chronic stress effects on gastrointestinal functions are likely to be mediated by the two main stress

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Abbreviations: ADX, Adrenalectomized; CSC, chronic subordinate colony housing; GC, glucocorticoid; HPA, hypothalamo-pituitary-adrenal; HSD, honestly significant difference; IFN, interferon; KRBB, Krebs-Ringer bicarbonate-glucose buffer; LC, locus coeruleus; LPS, lipopolysaccharide; NE, norepinephrine; PVN, paraventricular nucleus of the hypothalamus; SD/OC, social defeat/overcrowding; SHAM, sham-operated; TH, tyrosine hydroxylase.

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systems of the organism, namely the sympathetic nervous system and the hypothalamo-pituitary-adrenal (HPA) axis (30–36). Therefore, we also monitored tyrosine hydroxylase (TH) immunoreactivity in the brain locus coeruleus (LC) and in the colon as parameters of the sympathetic nervous system activity/function, and hypothalamic CRH mRNA expression, adrenal weight, and plasma corticosterone as relevant parameters of the HPA axis on d 2, 3, 7, 14, and 20 of CSC. In parallel, the time course of thymus weight and the histological damage score of the colon were assessed throughout the 19 d of stressor exposure (experiment 2)

To further investigate the recently shown adrenal insufficiency in chronically stressed mice (54), adrenal corticosterone secretion was estimated under stimulated conditions *in vivo* and *in vitro*, *i.e.* during the diurnal rise in plasma corticosterone at the beginning of the dark phase (38, 39) and in response to corticotropin (ACTH), respectively (experiment 3). Additionally, glucocorticoid (GC) sensitivity of isolated splenic cells was determined from the same chronically stressed and control mice, because prolonged GC exposure was shown to reduce corticosterone sensitivity of splenocytes (40–42) (experiment 3). Finally, cytokine secretion by mesenteric lymph node cells in chronically stressed and control mice was assessed after termination of the CSC procedure as an additional parameter describing the ongoing inflammatory processes in colonic tissue. To investigate whether the stress-induced inflammatory processes in colonic tissue were mediated via adrenal mechanisms, we performed adrenalectomy (ADX) 1 wk before the CSC procedure started (experiment 4).

Overall, the present study was designed to determine whether 1) chronic psycho-social stress leads to colonic inflammation in parallel with alterations of the two main stress systems; and 2) adrenal glucocorticoids are involved.

We hypothesized that the initial rise in plasma corticosterone during CSC exposure triggers colonic inflammation. Therefore, stress-induced changes in inflammatory parameters were compared between ADX and sham-operated (SHAM) mice.

Materials and Methods

Animals

Male C57BL/6 mice (Charles River, Sulzfeld, Germany) weighing 19–22 g (experimental mice) or 30–35 g (dominant mice) were individually housed in standard polycarbonate mouse cages (16 × 22 × 14 cm) at least for 1 wk before the experimental procedure started. All mice were kept under standard laboratory conditions (12-h light, 12-h dark cycle, lights on at 0600 h, 22 C, 60% humidity) and had free access to tap water and standard mouse diet. 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 the number of animals used and their suffering.

CSC

To induce a chronic stressful situation, four CSC mice were housed together with a larger dominant male in a polycarbonate observation cage (38 × 22 × 35 cm) for 19 d consecutively. Before the CSC procedure all potential male dominant mice used for CSC procedure were tested for their aggressive behavior. Males that started to injure their opponents by harmful bites were not used for the CSC procedure. To avoid habituation, each dominant male was replaced by a novel dominant male on d 8 and 15. During the first 30 min after colony formation on d 1, 8, and 15, the mice were videotaped for behavioral analyses. In all colonies, the larger male mouse established a dominant status while it was chasing and attacking all four experimental mice. All four experimental mice were considered as subordinates based on their defensive behavior, including flight, retreat, and submissive upright, according to a recent report in rats (43).

Experimental procedure

Experiment 1. Mice were weighed and then randomly assigned to the control or the CSC group. Control mice were singly housed and remained undisturbed in their home cages except for change of bedding once a week. Groups of four CSC mice were housed together with one dominant male over a period of 19 d (see above). On d 19, all mice were weighed again between 1700 and 1800 h, and CSC mice were singly housed. On the next day (d 20), mice were killed between 0800 and 1000 h, and trunk blood was collected for quantification of plasma norepinephrine (NE) and corticosterone concentrations, during light phase. Moreover, relative thymus and adrenal weight, and the histological damage score of the colon were estimated (see below). For assessment of CSC effects on anxiety-related behavior, an additional set of control and CSC mice was tested on the elevated plus-maze (see below) between 0800 and 1100 h on d 20.

Experiment 2. To study the time-course of CSC effects on TH expression in the LC and in colonic tissue, on CRH mRNA expression in the paraventricular nucleus of the hypothalamus (PVN), on adrenal weights, light phase corticosterone concentrations, thymus weights, and the histological damage score of the colon, groups of control and CSC mice were killed before (basal) and on d 2, 3, 7, 14, and 20 of CSC exposure between 0800 and 1000 h.

Experiment 3. To study the effects of CSC on plasma corticosterone concentrations during the dark phase, on adrenal corticosterone secretory responses *in vitro*, and on GC sensitivity of splenic cells, mice were weighed and randomly assigned to the control and CSC group. Control mice were singly housed and remained undisturbed in their home cages except for change of bedding once a week. Groups of four CSC mice were housed together with one dominant male over 19 d (see above). On d 19, CSC mice were singly housed. On the next day (d 20) between 0800 and 1100 h, mice were tested on the elevated plus-maze to confirm the CSC-induced increase in anxiety-related behavior (experiment 1). For assessment of dark-phase corticosterone concentrations, *i.e.* during the diurnal rise in HPA axis activity (38, 39), of adrenal corticosterone secretory responses *in vitro*, and of glucocorticoid sensitivity of splenic cells, the mice were killed between 2000 and 2200 h on d 20.

Experiment 4. To study an additional marker of inflammation in the colonic tissue at the end of the chronic stress procedure and to investigate adrenal mechanisms mediating CSC-induced effects on colonic inflammation, a new set of animals was either ADX or SHAM 1 wk before CSC exposure (see above). After 19 d of CSC or single housing (stress controls), the histological score and the pro- and antiinflammatory cytokine secretion by mesenteric lymph node cells was measured on d 20 of CSC exposure in both SHAM and ADX mice.

Blood sampling and RIA for NE and corticosterone

To determine the effect of CSC exposure on NE and corticosterone concentrations in plasma, all mice of one cage were rapidly killed by decapitation under CO_2 anesthesia within 3 min after entering the animal room. Approximately 200 μl of trunk blood were collected in EDTA-coated tubes on ice (Sarstedt, Nümbrecht, Germany) containing 10 μl aprotinin (Trasylol, Bayer Corp. AG, Leverkusen, Germany) and centrifuged at 4 C (5000 rpm, 10 min). Plasma samples were stored at −80 C until assayed using a commercially available RIA for NE (IBL Immuno Biological Laboratories, Hamburg, Germany; detection limit, 9 pg/ml) and corticosterone (MP Biomedicals GmbH, Eschwege, Germany; detection limit, 10 ng/ml).

Determination of thymus and adrenal weights

To assess chronic stress effects on thymus and adrenal weight, thymus and both adrenal glands were removed after decapitation, pruned

of fat tissue, and weighed. Both the left and right adrenal glands were weighed together. Values were expressed in relation to body weight (milligrams per gram).

Determination of the histological damage score of the colon

To assess the effect of CSC on histological markers of the distal part of the intestinal tract, the colon was removed and mechanically cleaned. Then, 1 cm of the distal third of the colon was cut longitudinally, laid on a filter paper, and fixed in 10% formalin overnight. The next day, the fixed tissue was embedded in paraffin and cut longitudinally. Three 3-μm hematoxylin-eosin stained sections taken at 100 μm distance were evaluated by histological scoring performed by an investigator blind to treatment. For statistics, each individual score represented the mean of the three sections. Histology was scored as follows (28, 29):

Epithelium. 0, Normal morphology; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts; and 4, loss of crypts in large areas.

Infiltration. 0, No infiltration; 1, infiltrate around crypt basis; 2, infiltrate reaching to lamina muscularis mucosae; 3, extensive infiltration reaching the L. muscularis mucosae and thickening of the mucosa with abundant edema; and 4, infiltration of the L. submucosa.

The total histological score of each mouse represents the sum of the epithelium and infiltration score and ranges from 0 to 8.

Elevated plus-maze test

To assess the effect of 19 d of CSC on anxiety-related behavior, CSC mice were housed singly and both controls and CSC mice were transported to the plus-maze test room on d 19. The next day, they were tested on the elevated plus-maze between 0800 and 1100 h for 5 min (44, 45). The elevated plus-maze consisted of two open (6 × 30 cm) and two closed (6 × 30 × 17 cm) arms radiating from a central platform (6 × 6 cm) to form a plus-shaped figure elevated 130 cm above the floor. The open arm edges were 0.3 cm in height to avoid falling. Each mouse was placed on the central platform facing a closed arm. The maze was cleaned thoroughly before each test. The latency to the first open arm entry, the number of entries into the open and closed arms, and the time spent on the respective arms were recorded by means of a video/computer setup to allow calculation of the percentage of time spent on, and the percentage of entries performed into open arms of the maze.

Immunohistochemistry of TH in the LC and colonic tissue

The staining protocol was adopted from Miller *et al.* (2000) (46). Briefly, the separated brainstem and three colon pieces of each mouse were immediately fixed in 3.75% paraformaldehyde in PBS for 24 h. Tissue was then incubated in 20% sucrose in PBS for another 24 h. Thereafter, brainstem and colonic tissue were embedded in protective freezing medium (Tissue-Tek, Sakura Finetek Europe, Zoeterwoude, The Netherlands) and quick-frozen floating on liquid nitrogen for TH staining.

For assessment of LC TH expression, 5-μm cryosections from the LC were placed on precoated slides, air dried (1 h, room temperature), rehydrated in 0.05 M TBS, and unspecific binding was blocked for 1 h with a solution of 10% whole serum (goat and donkey serum; Dako, Glostrup, Denmark) from the host species of the respective secondary antibody in 0.05 M TBS containing 1% BSA and 0.01% NaN_3 (Sigma, Deisenhofen, Germany). The slides were then washed (10 min, 0.05 M TBS) and immunostained in a humid chamber (24 h at room temperature) with primary antibodies against TH (Chemicon, Temecula, CA), the key enzyme for neuronal NE production. The primary antibody solution was diluted 1:500 with 0.05 M TBS containing 1% BSA and 0.01% NaN_3. After three 5-min washes with 0.05 M TBS, an Alexa 546 conjugated secondary goat antirabbit antibody (Molecular Probes, Leiden, The Netherlands) was used to achieve immunofluorescent staining (concentration 1:600, incubation for 90 min, followed by three 5-min washes in the dark). Under control conditions, the respective isotype was used in the above mentioned protocol. TH expression was analyzed as specific fluorescence (pixels with higher fluorescence than a chosen background-threshold were counted as positive pixels; results are presented as the percentage of positive pixels of all pixels of each slice) in 10 slices before and 10 slices after the slice showing the highest fluorescence using a computerized image program (MetaVue, version 5.0r1; Universal Imaging Corp., Downingtown, PA).

For determination of TH expression in colonic tissue, three cryosections (5 μm, 100 μm distance between the slices, each slice contains all three colonic tissues of one mouse) per animal were placed on a precoated slide (SuperFrost Plus; Menzel-Gläser, Braunschweig, Germany). For assessment of colonic TH expression, 17 randomly selected fields of view from each animal were analyzed as specific fluorescence (pixels with higher fluorescence than a chosen background-threshold were counted as positive pixels; results are presented as the percentage of positive pixels of all pixels of each field of view).

In situ hybridization of hypothalamic CRH mRNA

After decapitation, brains were rapidly removed, snap frozen in iso-pentane cooled on dry ice and stored at −80 C for subsequent *in situ* hybridization. A series of six 16-μm cryocut PVN sections per mouse, was thaw-mounted onto slides in a cryostat at −20 C and then was used to assess hypothalamic CRH mRNA expression. The hybridization protocol was adapted from Bosch *et al.* (47). Briefly, hybridization to CRH mRNA was performed by using a 48-base ^{35}S-labeled oligonucleotide probe complementary to bases 64–111 (probe sequence: 5′ ggc ccg cgg cgc tcc aga gac gga tcc cct gct cag cag ggc cct gca). Using the National Center for Biotechnology Information basic local alignment and search tool, the sequence was demonstrated to be specific for the mouse CRH transcript. Hybridized slices were exposed to BioMax MR film under safe red light conditions for 21 d (Eastman Kodak, Rochester, NY). Expression of CRH mRNA was measured as OD on a Macintosh computer with a computerized image program (ImageJ 1.31; National Institutes of Health; http://rsb.info.nih.gov/ij/). Bilateral measures were taken from two to four PVN sections for each mouse, which were pooled to provide individual means per mouse. For tissue background, the OD of a nonhybridized region outside the PVN was measured.

ACTH stimulation of isolated adrenal cells in vitro

To reveal consequences of chronic stress on the capability of adrenal cells to respond to an ACTH challenge *in vitro*, the stimulation protocol was adopted from Bazhan *et al.* (2004) (48). After decapitation, adrenal glands of four mice of the same experimental group (control *vs.* CSC; experiment 3) were pooled and stored in ice-cold Krebs-Ringer bicarbonate-glucose buffer (KRBB, pH 7.4; 0.5% BSA) until they were cleared, cut into small pieces, and incubated in 1.5 ml KRBB (4% BSA, 4% collagenase) for 60 min at 37 C in an atmosphere of 95% O_2 and 5% CO_2. Adrenal cells were then dispersed by gentle homogenization (repeated pipetting) and filtered through five layers of gauze bandage. Then, suspensions were centrifuged (4 C, 10 min at 300 rpm; 10 min at 800 rpm) and washed three times with 2.5 ml cold KRBB (0.5% BSA). Finally, cells were counted (tryphan-blue), cell viability was assessed (tryphan-blue exclusion method), and adjusted to a cell concentration of 4×10^5 cells/ml in cold KRBB (0.5% BSA). Two aliquots (200 μl) of each group were incubated for 2 h at 37 C (95% O_2, 5% CO_2) in the presence of ACTH at different doses (0, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} μM). Then, cells were sedimented by brief centrifugation (5 min at 2000 × *g*) and supernatants were stored at −20 C until radioimmunological quantification of corticosterone. To exclude differences in the adrenal cell preparation between CSC and control groups, experiments were performed by the same experimenter according to the standardized isolation protocol.

Characterization of GC sensitivity of isolated splenocytes

To determine the effects of chronic stress on the GC-dependent cell viability of splenic cells, a protocol was adopted from Engler *et al.* (2005) (42). Briefly, after decapitation, spleens of two mice of the same group (control *vs.* CSC) were pooled, and then splenocytes were isolated and adjusted to a final cell concentration of 5×10^6 cells/ml.

Cell suspensions were then stimulated with lipopolysaccharide (LPS; final concentration was 1 μg/ml) from *Escherichia coli* (serotype O111:B4, Sigma-Aldrich, St. Louis, MO) or remained untreated to assess background activity. To determine the GC sensitivity of unstimulated and LPS-stimulated cells, aliquots of each sample were treated with various

concentrations of corticosterone (Sigma-Aldrich; final concentrations were 0.005, 0.05, 0.1, 0.5, and 5 μM, respectively) including both physiological and pharmacological doses of the hormone. Triplicates of each treatment were transferred to 96-well flat-bottom microtiter plates and incubated for 48 h in a humidified incubator (37 C, 5% CO_2) followed by assessment of cell viability.

The cell viability in the splenocyte cultures was determined with a commercially available colorimetric assay (CellTiter 96 AQueous One Solution Assay; G3580; Promega, Madison, WI). To account for differences in background activity, the mean absorbance of the triplicate set of wells with unstimulated cells for a given treatment was subtracted from the mean absorbance of the three corresponding LPS-stimulated wells.

Surgical procedure/ADX

Surgery was performed under isoflurane anesthesia. After a 2-cm skin incision was performed on the back of the mice at the level of the kidneys (midline), adrenals were removed bilaterally through two peritoneal incisions, performed on the left and right side of the abdomen of the mouse. SHAM mice underwent the same procedure as the ADX mice, but without removal of the adrenals. After surgery, both ADX and SHAM-operated mice received 0.9% saline in drinking water (until they were killed) and were housed singly for 1 wk until the CSC procedure started. Saline helped ADX mice to compensate for loss of mineralocorticoids.

Isolation and incubation of mesenteric lymph node cells

Mesenteric lymph nodes (pooled from each experimental group) were harvested under sterile conditions and collected on ice in cell culture medium [RPMI-1640 supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO-BRL, Eggenstein, Germany), and 3×10^{-5} M β-mercaptoethanol (Sigma, Deisenhofen, Germany)]. Lymph nodes were mechanically disrupted and filtered through a cell strainer (70-μm Nylon, Falcon; Becton Dickinson, Heidelberg, Germany). Then cells were washed three times in cell culture medium and adjusted to a concentration of 10^6 cells/ml. Then 2×10^5 (200 μl) lymph node cells were transferred to wells of a 96-well plate and stimulated by precoating wells with 200 μl of 2.5 μg/ml anti-CD3 antibody in the presence of IL-2 (final concentration 100 U/ml). Eight wells were transferred with the respective number of cells of each experimental group. After incubation for 24 h (37 C, 5% CO_2), cytokine concentrations were measured in the supernatants by ELISA (all from Endogen, Woburn, MA) using four wells per experimental group.

Statistics

For statistical comparisons, the software package SPSS (version 12) was used. Data of the two groups in experiment 1 were compared using the Mann-Whitney *U* test. All comparisons of experiment 2 were performed using a one-way ANOVA (factor time) followed by a *post hoc* Tukey honestly significant different (HSD) test when appropriate. In experiment 3, plasma dark phase corticosterone concentrations as well as δ-cell viability of splenocytes of CSC and control mice were compared using a Mann-Whitney *U* test. The *in vitro* adrenal corticosterone secretory responses as well as the GC sensitivity data were analyzed by repeated measures ANOVA with CSC as between-subject factor and dose as repeated measures within-subject factor followed by a *post hoc* Tukey-HSD test when appropriate. All comparisons of experiment 4 were done using a two-way ANOVA (factor ADX; factor CSC) and followed by a *post hoc* Tukey-HSD test when appropriate. Data are presented as means + SEM. Significance was taken at $P < 0.05$.

Results

Experiment 1

Effects of CSC on body, thymus, and adrenal weight. Compared with singly housed controls, CSC mice gained significantly less body weight during the 19 d of CSC (Fig. 1A). CSC mice also showed a significant decrease in relative thymus weight (Fig. 1B) and a significant increase in relative adrenal weight (Fig. 1C) compared with unstressed control mice after 19 d of CSC exposure.

Effects of CSC on plasma NE and corticosterone concentrations. NE concentrations estimated during the light phase between 0800 and 1000 h were found to be significantly increased in CSC compared with control mice after 19 d of CSC exposure (Fig. 1D). In contrast, exposure to CSC did not alter plasma corticosterone concentrations in the light phase, *i.e.* under basal, unstimulated conditions (Fig. 1E).

Effects of CSC on the histological damage score of the colon. Exposure to the 19-d CSC procedure resulted in histological damage of the colon as reflected by a significantly elevated histological score in CSC compared with control mice (Fig. 1F).

Effects of CSC on anxiety-related behavior. Chronic exposure to CSC significantly increased anxiety-related behavior as reflected by a decrease in the percentage of time spent on the open arms of the maze, and in the percentage of open arm entries compared with control mice (Fig. 1G). The number of entries into the closed arms (control, 9.3 ± 0.8 *vs.* CSC, 9.4 ± 0.6; $P = 0.873$), indicative of locomotor activity, was not altered by CSC exposure.

Experiment 2

Time course of CSC effects on TH expression in the LC and in the colonic tissue. TH immunoreactivity in the LC was found to be significantly affected by CSC exposure ($F_{5, 116} = 4.55$; $P = 0.001$; Fig. 2A). *Post hoc* analysis revealed a significant decrease in TH expression on d 2 of CSC exposure (Fig. 2A; see also Fig. 2D), but on subsequent days of CSC exposure, the level of TH immunoreactivity returned to baseline (Fig. 2A; see also Fig. 2C). Furthermore, a significant main effect of CSC exposure on TH expression in colonic tissue ($F_{5, 611} = 3.59$; $P = 0.003$; Fig. 2B), with a significant increase on d 2 of CSC exposure (Fig. 2B; see also Fig. 2F) compared with basal values (Fig. 2B; see also Fig. 2E) was determined.

Time course of CSC effects on CRH mRNA in the PVN. Exposure to CSC significantly altered CRH mRNA expression in the PVN ($F_{5, 45} = 3.57$; $P = 0.009$; Fig. 3A). *Post hoc* analysis showed a tendency ($P = 0.055$) to elevated CRH mRNA expression on d 2 of CSC exposure (Fig. 3A). This effect reached statistical significance when performing a Mann-Whitney *U* comparison between basal (Fig. 3B) and d 2 values ($P = 0.021$; Fig. 3C). At all other days measured, no differences in CRH mRNA between stressed and basal mice were found.

Time course of CSC effects on adrenal weight and plasma corticosterone concentrations. Relative adrenal weight was found to be significantly increased by CSC exposure ($F_{5, 44} = 18.8$; $P < 0.001$; Fig. 4A). Compared with basal values, the increase in adrenal weight was significantly decreased at all days measured. Plasma corticosterone concentrations during the light phase were also found to be altered by CSC exposure ($F_{5, 45}$

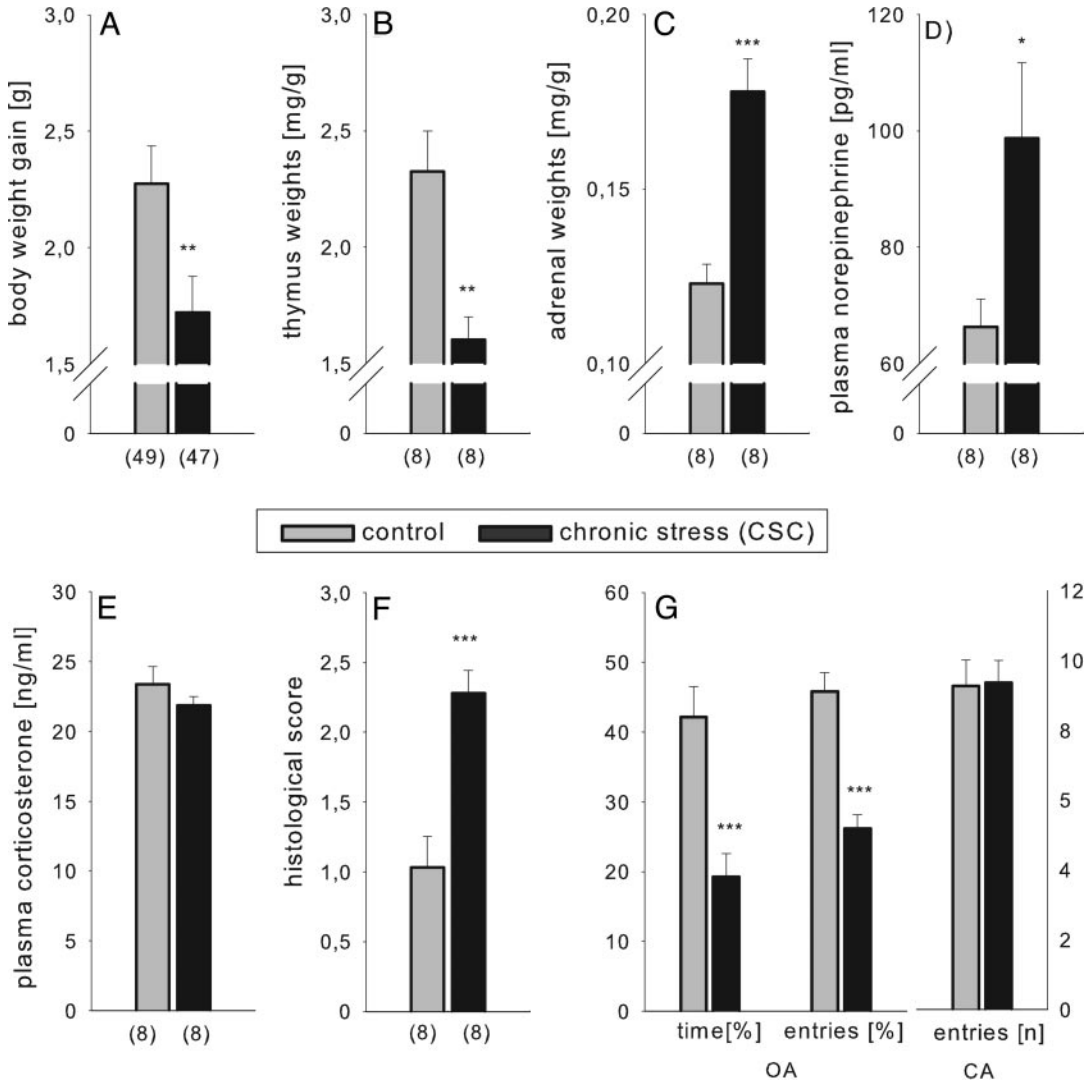

FIG. 1. Exposure to 19 d of CSC leads to a decreased body weight gain (A), decreased relative thymus weight (B), increased relative adrenal weight (C), increased plasma NE concentrations (D), and an increased histological damage score of the colon (F) in CSC compared with unstressed control mice. Plasma light corticosterone concentrations were found to be unchanged (E) by CSC exposure. Additionally, CSC (n = 39) leads to an increased anxiety-related behavior on the elevated plus-maze compared with unstressed control mice (n = 41; G). This is reflected by a reduced percentage of time spent on the open arms (OA) of the plus-maze (time [%]), and percentage of entries onto the open arms (entries [%]). The number of entries onto closed (CA) was equal in both groups, indicating that locomotor activity was not affected by CSC exposure. Data represent means ± SEM; *, $P < 0.05$; **, < 0.01; ***, $P < 0.001$ *vs.* singly housed controls.

= 3.41; $P = 0.012$; Fig. 4B), but significantly elevated hormone levels were only found on d 2, *i.e.* during the initial phase of CSC.

Time course of CSC effects on thymus weight. Relative thymus weight was significantly reduced during 19 d of CSC exposure ($F_{5, 44} = 15.6$; $P < 0.001$; Fig. 5A). In comparison to basal values this reduction in thymus weight was significant at all days measured.

Time course of CSC effects on the histological damage score. The histological damage score of the colon was also found to be dependent on CSC exposure ($F_{5, 47} = 14.6$; $P < 0.001$; Fig. 5B). *Post hoc* analysis revealed that the histological damage score was significantly higher both on d 14 and 20 (Fig. 5D) of CSC exposure compared with values from unstressed control mice (Fig. 5C) reflecting more severe inflammatory infiltration and increased epithelial damage, *i.e.* focal disappearance of mucosal crypts.

Experiment 3

CSC effects on plasma dark phase corticosterone concentrations and adrenal corticosterone secretory responses in vitro (d 20). Plasma corticosterone concentrations determined at the beginning of the dark phase were found to be significantly lower in CSC compared with control mice (Fig. 6A).

No effects of CSC were found on baseline corticosterone secretion from adrenal cells *in vitro* (ACTH dose 0 M; Fig. 6B). Exposure of adrenal cells to ACTH had a stimulating effect on the release of corticosterone in both groups,

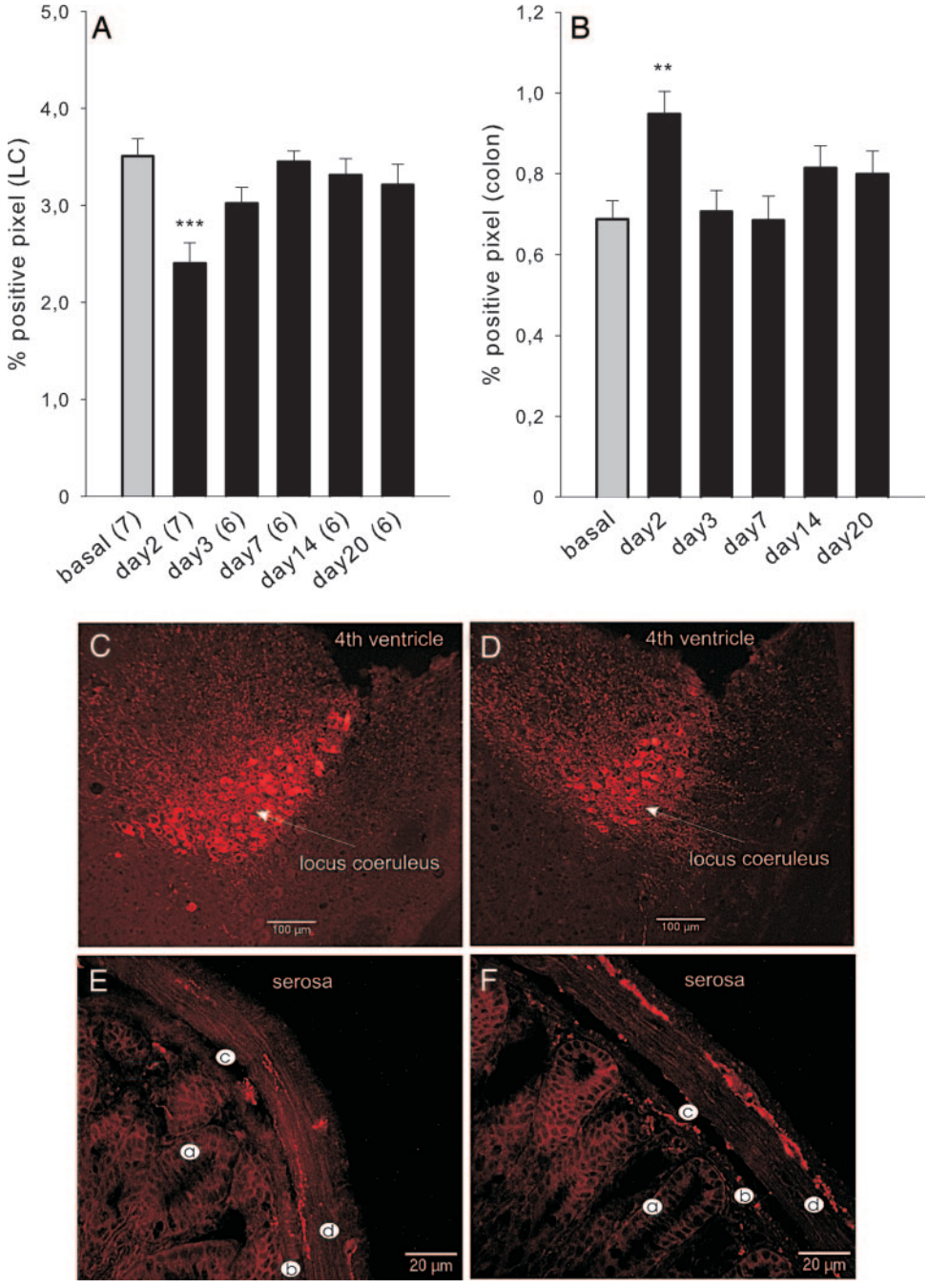

FIG. 2. Effects of 19-d exposure to CSC on TH expression in the LC (A) and in colonic tissue (B) at various time points (d 2, 3, 7, 14, and 20). In A, group sizes are indicated by the *numbers in parentheses*, in B, group size was 6 animals each. Representative images of immunofluorescent TH-staining in the LC and colon [a, lamina mucosa; b, lamina muscularis mucosae; c, lamina submucosae; d, lamina muscularis (circular and longitudinal muscle)], respectively, are shown in C/E (basal values) and D/F (d 2 values). Data represent means + SEM; **, $P < 0.01$; ***, $P < 0.001$ *vs.* basal values.

which was, however, dependent on prior CSC exposure (factor CSC × dose: $F_{4, 8} = 6.0$; $P = 0.016$; Fig. 6B). *Post hoc* Tukey-HSD tests showed a significantly attenuated corticosterone response of adrenal cells from CSC mice at 10^{-12} M and 10^{-11} M ACTH compared with unstressed controls (Fig. 6B).

CSC effects on GC sensitivity of LPS-stimulated splenocytes in vitro *(d 20).* Corticosterone treatment exerted a significant dose-dependent effect on the viability of LPS-stimulated splenocytes ($F_{5, 30} = 83.0$; $P < 0.001$; Fig. 7A), which was, however, dependent on prior CSC exposure (CSC × dose: $F_{5, 30} = 9.85$; $P = 0.001$; Fig. 7A).

In unstressed control mice, a significant increase in splenocyte viability was found in cultures treated with 0.005 µM corticosterone compared with untreated cells. This stimulatory effect of corticosterone at the lowest dose tested was absent in splenocytes from CSC mice indicating reduced corticosterone sensitivity after chronic stress (Fig. 7A).

At the highest corticosterone doses tested (0.5 and 5.0 µM), cell viability of splenocyte cultures of both CSC and control mice was significantly diminished compared with respective untreated splenic cells. This effect tended to be attenuated in CSC mice at 5 µM (Mann-Whitney *U* comparison, CSC *vs.* unstressed control; $P = 0.053$).

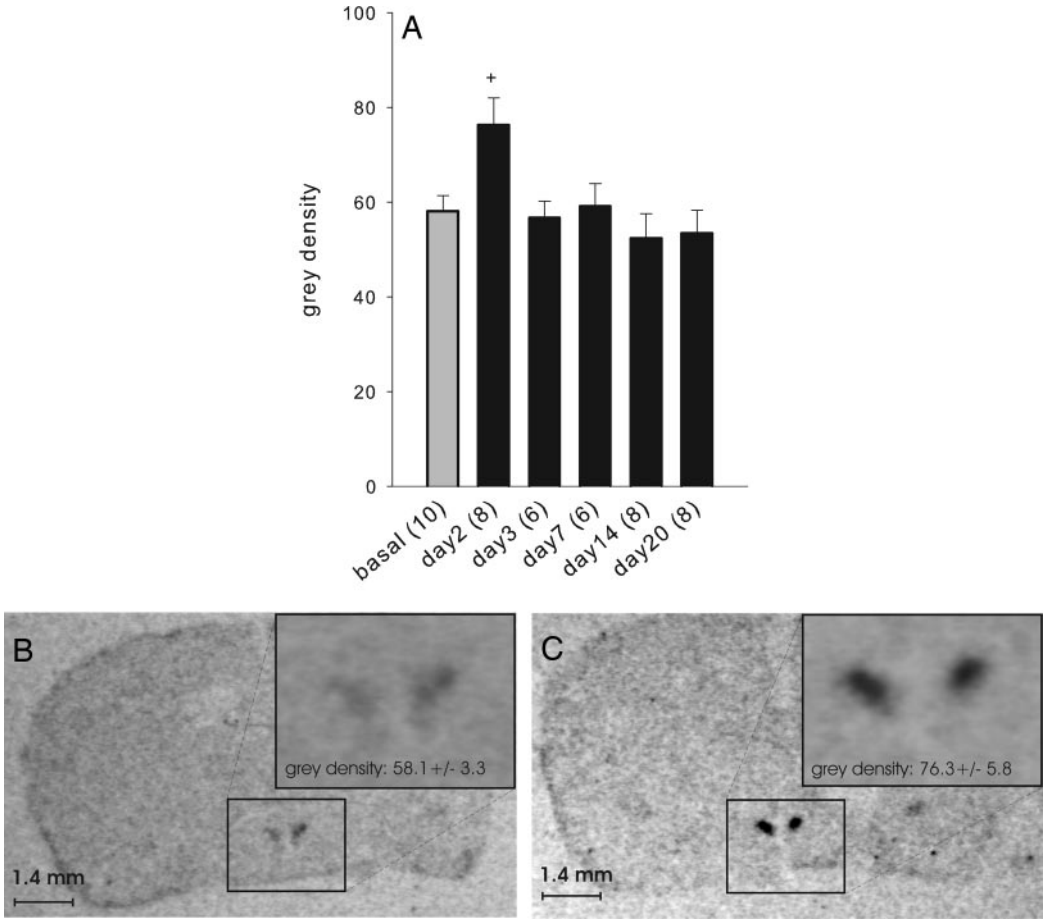

FIG. 3. Effects of 19-d exposure to CSC on CRH mRNA expression (grey density) in the PVN (A) at various time points (d 2, 3, 7, 14, and 20). Furthermore, two representative images of CRH mRNA expression in the PVN of a basal (B) and a CSC mouse killed on d 2 of CSC (C) are shown. *Numbers in parentheses* indicate group sizes. Data represent means + SEM; + $P = 0.055$ *vs.* basal values.

The reduced GC sensitivity of splenic cells of CSC mice is further described by their significantly decreased δ-cell viability (cell viability at 0.005 μM corticosterone − cell viability at 5 μM corticosterone; Fig. 7B) compared with splenic cells of control mice.

Experiment 4

Effects of prior ADX on CSC-induced changes in thymus weight, histological damage score, and cytokine secretion by mesenteric lymph node cells (d 20). Thymus weight was dependent on

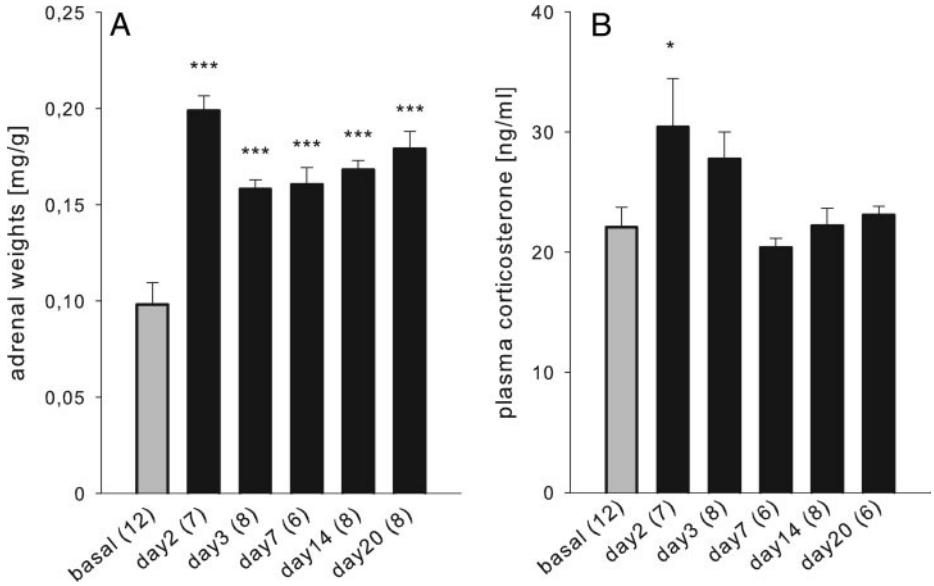

FIG. 4. Effects of 19-d exposure to CSC on relative adrenal weight (A; adrenal weight [mg]/body weight [g]) and light phase plasma corticosterone concentrations [ng/ml] at various time points (d 2, 3, 7, 14, and 20). *Numbers in parentheses* indicate group sizes. Data represent means + SEM; *, $P < 0.05$; ***, $P < 0.001$ *vs.* basal values.

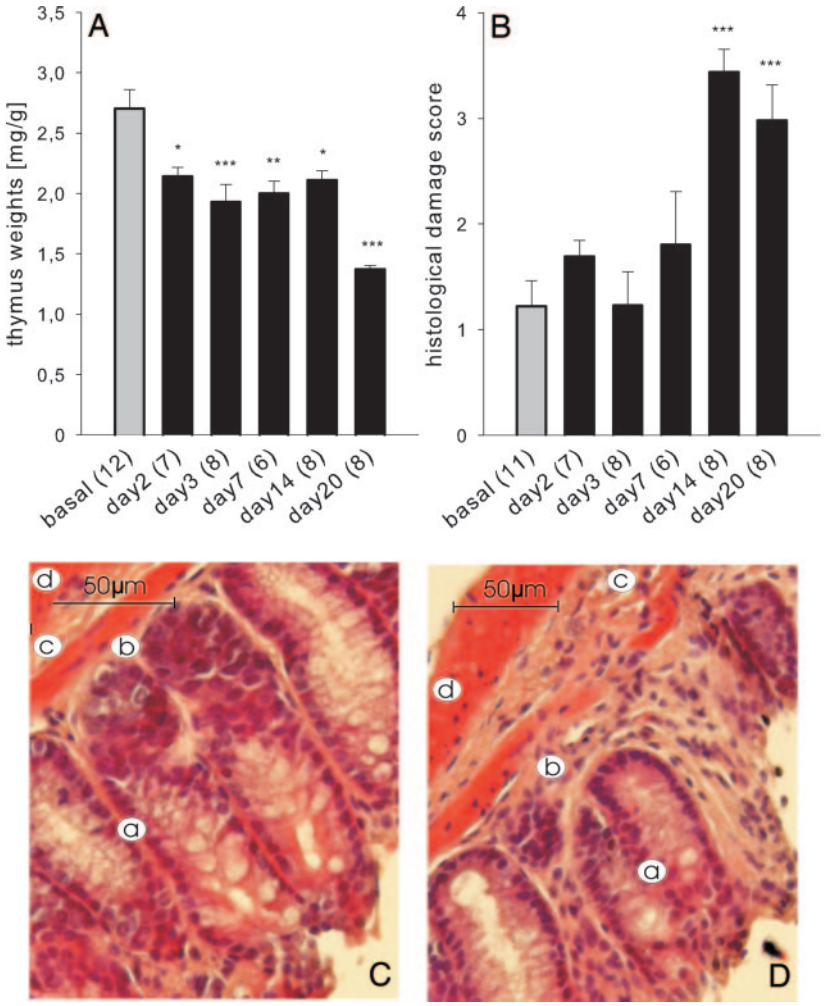

FIG. 5. Effects of 19-d exposure to CSC on relative thymus weight (A, thymus weight [mg]/body weight [g]) and on the histological damage score (B) at various time points (d 2, 3, 7, 14, and 20). *Numbers in parentheses* indicate group sizes. Data represent means + SEM; * $P <$ 0.05; **, $P < 0.01$; ***, $P < 0.001$ *vs.* basal values. Furthermore, two representative colonic hematoxylin and eosin sections are shown [a, lamina mucosa; b, lamina muscularis mucosae; c, lamina submucosae; d: lamina muscularis (circular and longitudinal muscle)] from nonstressed controls (basal; Fig. 4C; normal colon histology) and from chronically stressed mice (d 20; Fig. 4D; goblet cell loss and crypt loss in locally restricted areas; infiltration reaching the Lamina muscularis mucosae; thickening of submucosal areas).

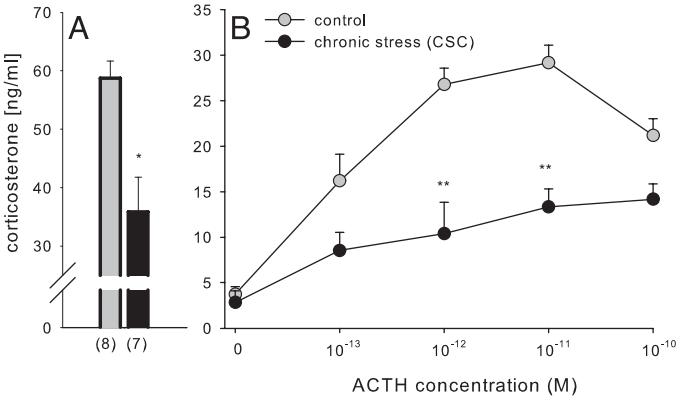

FIG. 6. Effects of 19-d exposure to CSC on plasma dark phase corticosterone concentrations (A; *numbers in parentheses* indicate group sizes) and on the *in vitro* release of corticosterone from isolated adrenal cells after stimulation with ACTH at varying doses (B). Adrenals of four control and four CSC mice, respectively, were pooled (n = 8 pooled samples), and two aliquots (200 μl) of each group were stimulated with ACTH at concentrations between 10^{-13} and 10^{-10} M. Supernatants were analyzed by a corticosterone RIA in triplicates. Adrenal cells of CSC mice showed a reduced response to ACTH at all doses tested. Data represent means ± SEM; **, $P < 0.01$ *vs.* respective unstressed controls.

prior ADX ($F_{1,41} = 9.17$; $P < 0.004$; Table 1) and on CSC exposure ($F_{1,41} = 26.2$; $P < 0.001$; Table 1). CSC mice of both SHAM and ADX groups showed a significantly decreased thymus weight compared with respective control mice (Table 1). Within the nonstressed controls, ADX caused a significant thymus hypertrophy compared with SHAM mice (Table 1).

Statistical analysis revealed a main interaction of ADX and CSC exposure (factor ADX × CSC: $F_{1,41} = 4.78$; $P = 0.035$; Table 1) on the histological damage score. More specifically, histological damage score was not affected by ADX in nonstressed control mice, but the CSC-induced increase determined within the SHAM group was not determined in ADX mice, with the histological damage score of CSC-exposed ADX mice being equal to nonstressed mice of both ADX and SHAM group (Table 1).

Both interferon (IFN)-γ and TNF-α secretion were dependent on prior ADX and CSC exposure (IFN-γ: factor ADX × CSC: $F_{1,12} = 9.15$; $P = 0.011$; TNF-α: factor ADX × CSC: $F_{1,12} = 33.3$; $P < 0.001$; Table 1). Proinflammatory cytokine concentrations of SHAM or ADX mice, which were not exposed to CSC, were not statistically different (Table 1). However, CSC exposure significantly increased the secretion of IFN-γ and TNF-α in SHAM and ADX mice, with the effect being more severe in the SHAM group (Table 1).

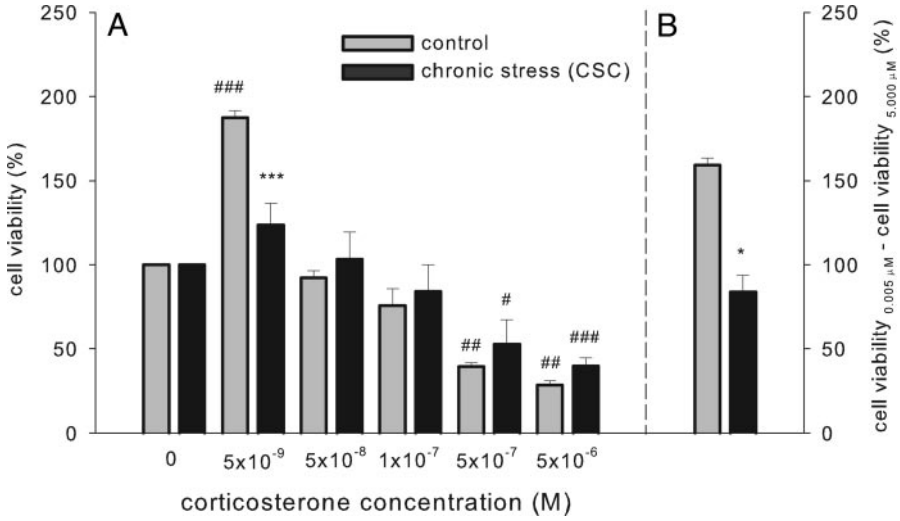

FIG. 7. Effects of 19-d exposure to CSC on the *in vitro* GC sensitivity of splenocytes. Experimental mice were subjected to 19 d of CSC (n = 8; *black bars*). One day before (d 19) being tested on the elevated plus-maze (d 20; from 0800–1000 h) to confirm the chronic stress procedure they were single housed. Twelve hours after the elevated plus-maze testing was performed (from 2000–2200 h), cells from CSC and control mice (n = 10; *gray bars*) were cultured with LPS (1 μg/ml) in presence of various concentrations of corticosterone. After 48 h of incubation, the cell viability was determined using a colorimetric assay. Data are presented as the percentage of cell viability in the absence of corticosterone (A) and δ-cell viability (cell viability at 0.005 μM corticosterone − cell viability at 5.000 μM corticosterone; B). Data represent means ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* single-housed controls.

Secretion of the antiinflammatory cytokine IL-10 was dependent on CSC exposure ($F_{1,41} = 26.2$; $P < 0.001$; Table 1) with CSC mice of both, SHAM and ADX group showing increased secretion compared with controls.

Discussion

The present study reveals that exposure to CSC leads to macroscopic damages of the mucosal layers of the colon and increased secretion of pro- and antiinflammatory cytokines by mesenteric lymph node cells in male mice. In confirmation of studies performed in human (3) and nonhuman primates (49, 50), this is the first report of chronic stress effects on the development of a spontaneous colonic inflammation in a relevant chronic psycho-social stress model in rodents. Furthermore, temporal alterations across the duration of the chronic stress manipulation were shown in the two main stress response systems, the HPA axis, and sympathetic nervous system. Interestingly, CSC-induced colonic inflammation could at least be partially blocked by ADX before CSC exposure. Therefore, the mechanisms underlying the colonic damage are likely to involve the initial activation of the HPA axis during CSC exposure.

Establishment of CSC as model of chronic psycho-social stress in male mice

In agreement with previous studies in the rat (43), we have established the CSC procedure as an effective model of chronic psycho-social stress for male mice. All subordinate mice displayed typical signs of chronic stress, including a reduction in body weight gain (18–21). Interestingly, it has been shown in rats that the extent of body weight gain is related to the rank of the animal within a social group (51). Another indicator of acute or chronic stress is an elevated level of state anxiety (22, 23), which has been described both in rats (21, 24, 25) and in mice (26, 27). In the present study, mice exposed to CSC displayed increased all measured anxiety-related behavior on the elevated plus-maze compared with unstressed, single-housed control mice further validating the CSC model. Importantly, the increase in anxiety-related behavior of CSC mice is not due to single housing for 15 h before plus-maze testing because CSC mice directly transferred from colony housing to the plus-maze were even more anxious (our unpublished observations). As additional indicators of stress, CSC-exposed mice showed robust thymus atrophy and adrenal hypertrophy (17, 21, 52, 53). With the notable exception of the strong influence of CSC on

TABLE 1. Effects of ADX before 19-d exposure to CSC on thymus weight, histological damage score, and pro- and antiinflammatory cytokine secretion by mesenteric lymph node cells in male mice

	SHAM/control	ADX/control	SHAM/CSC	ADX/CSC
Thymus weight (mg/g)	2.2 ± 0.1 (n = 14)	2.8 ± 0.2^{a} (n = 7)	1.7 ± 0.1^{d} (n = 14)	1.9 ± 0.1^{f} (n = 10)
Histological score	1.0 ± 0.1 (n = 14)	1.0 ± 0.2 (n = 7)	1.6 ± 0.2^{d} (n = 14)	0.9 ± 0.2^{a} (n = 10)
IFN-γ (pg/ml)	526.2 ± 69.6 (n = 14)	352.7 ± 45.7 (n = 7)	8649.0 ± 962.3^{f} (n = 14)	4350.0 ± 962.9^{b,e} (n = 10)
TNF-α (pg/ml)	8.9 ± 0.5 (n = 14)	8.4 ± 0.6 (n = 7)	44.7 ± 4.3^{f} (n = 14)	18.8 ± 0.5^{c,d} (n = 10)
IL-10 (pg/ml)	1.0 ± 0.0 (n = 14)	1.6 ± 0.6 (n = 7)	19.2 ± 2.5^{f} (n = 14)	15.2 ± 1.8^{f} (n = 10)

ADX and SHAM mice were housed singly for 1 wk after surgery. A subpopulation of mice from each group (chosen randomly) were exposed to either CSC or further housed singly (control) for a consecutive 19 d. Data represent mean ± SEM. ^{a} $P < 0.05$, ^{b} $P < 0.01$, ^{c} $P < 0.001$ *vs.* respective SHAM-operated mice; ^{d} $P < 0.05$, ^{e} $P < 0.01$, ^{f} $P < 0.001$ *vs.* respective nonstressed control group.

anxiety-related behavior, this model of chronic psycho-social stress is comparable to our previously described SD/OC (social defeat/overcrowding) paradigm (54). However, further testing with the SD/OC model may reveal an anxiety-like phenotype that was not observable on the elevated plus-maze. Additionally, the experimental procedure of the CSC paradigm is simple compared with SD/OC and has the further benefit of avoiding daily manipulation of the animals (54).

On account of these stress-induced differences found between CSC mice and control mice that have been single housed for the same time span we decided to abstain from killing single housed controls at d 2, 4, 7, 14, and 20 of experiment 2. The comparable organ weights, corticosterone levels, and histological scores between the above mentioned control mice of experiment 1 and the basal mice of experiment 2 provide evidence that single housing itself poses no stressor for male mice in our set-up. Remarkably, 24 h after colony formation (d 2), adrenal weight had doubled in comparison to basal values and remained significantly enlarged on d 3, 7, 14, and 20 of stressor exposure. Thymus involution was also seen at all time points measured during CSC procedure. This is in agreement with thymus atrophy seen in defeated rats after 24 h of resident-intruder confrontations (17, 55). The reduction in thymus mass is likely related to a decrease in the absolute cell numbers of all thymocyte subpopulations, most substantially within immature $CD4^+CD8^+$ cells (17). The thymus exhibits a high density of GC type-II receptors, and the immature $CD4^+/CD8^+$-cell population is particularly sensitive to GC (56, 57). Therefore, the elevation of GC observed during the CSC procedure may induce apoptosis and inhibit cell proliferation of immune cells (58, 59). Additionally, the increased thymus weight determined in nonstressed control mice after ADX indicates a basal role of endogenous corticosterone in regulating thymus size. However, in the current study, increased concentrations of plasma corticosterone were only found on d 2 of CSC exposure, which returned to baseline thereafter despite the maintenance of elevated adrenal weight. Therefore, and because of the decreased thymus weight found in ADX mice after 19-d exposure of CSC, an additional factor must be taken into account, contributing to stress-induced loss in thymus mass. In this respect, it is important to note that the medullary part of the thymus expresses high densities of β-adrenergic receptors (60), which are involved in a cAMP-mediated thymocyte apoptosis and consequent decrease of thymocyte numbers (61–63). Thus, the stress-induced activation of the sympathetic-adrenomedullary system, as shown previously (64), and reflected in the present studies by increased plasma NE concentrations even 15 h after the termination of CSC, could be causally involved in the loss of thymus mass of CSC mice. Recent observations in socially defeated rats, treated with the β-adrenergic antagonist propranolol, confirm the hypothesis that thymic atrophy as a result of social stress might, at least partially, be mediated by catecholamines (17). With respect to CSC-induced alterations of the sympathetic nervous system, TH expression in the LC was found to be significantly decreased on d 2 of CSC. This might be due to an acute rise, within the first hours of colony formation, in LC neuronal activity and a rapid decrease thereafter by negative feedback regulation via NE and epinephrine binding to presynaptic a_2-adrenoceptors (65, 66). However, it is known that stressor exposure acutely triggers elevated TH mRNA (67–69) and protein (70) levels in the brainstem. Therefore, it is probable that a combination of these mechanisms is involved in the decreased TH expression in the LC observed 24 h after colony formation in the present study.

Plasma corticosterone concentrations were significantly increased, but only on d 2 of CSC exposure, and returned to baseline afterward. Various adaptive processes concerning the HPA axis are likely to occur during the long-lasting stress exposure, to protect the body from an immunosuppressive, and therefore deleterious, chronic exposure to increased corticosterone concentrations (71, 72). Thus, at the level of hypothalamic CRH neurons, CRH mRNA expression was significantly increased in the same temporal fashion observed for increased plasma corticosterone, *i.e.* only on d 2 of CSC exposure. This initial increase in CRH mRNA expression may reflect the acute activation of the HPA axis described in rats and mice after various acute stressors (73–77). Alterations in CRH mRNA levels measured in the PVN after repeated stressor exposure are controversial and depend upon the type and duration of the stressor (for review see Ref. 78). Therefore, CRH mRNA content of the PVN might not be a reliable indicator of chronic stress in the present study. Severe stress-related changes of HPA axis reactivity are also likely to occur at the level of the adrenal glands. Despite elevated adrenal weights during the CSC procedure, plasma corticosterone concentrations measured at the beginning of the light phase were found to be similar between CSC and control animals (except for the rise seen on d 2 of CSC). Furthermore, moderate stimulation of GC secretion during its diurnal rise (38, 39) seen in control mice was found to be attenuated in chronically stressed mice after 19 d of CSC. As a result, dark phase plasma corticosterone concentrations were significantly lower in CSC compared with control mice. Finally, in addition to the abolished *in vivo* adrenal response upon stimulation, adrenal cells of CSC mice were also found to insufficiently respond to an ACTH challenge *in vitro*. At all doses tested, ACTH-induced corticosterone secretion from isolated adrenal cells was significantly lower in CSC mice compared with controls. Thus, adrenal cells seem to become insufficient to synthesize and/or secrete appropriate amounts of GC as a result of prolonged stressor exposure.

Effects of CSC exposure on colonic inflammation

As outlined in the introduction, exposure to an acute stressor, and the consequently elevated corticosterone levels, has been shown to impair various parameters of intestinal barrier functions (13–16), resulting in increased colonic permeability. The degree of permeability was positively correlated with the severity of the stressor exposure (16, 79). Consequently, the presentation of luminal antigens to the mucosal and nonmucosal immune system is enhanced (80). Although impaired barrier functions have directly been linked to the development of intestinal inflammation (81–83), pathological changes in colon histology have, to our knowledge, never been described immediately after stressor exposure. Thus, in

our study, we present the first evidence in rodents that chronic exposure to a psycho-social stressor induces an increase in colonic inflammation. Because histological damage was not found during the first 2 wk of CSC exposure, it is likely that in other studies the duration of stress was too short to reveal similar histological effects. Additionally, secretion of pro- and antiinflammatory cytokines by mesenteric lymph node cells was found to be increased after exposure to CSC, also reflecting the increased inflammatory state in colonic tissue after chronic stress. Interestingly, CSC-induced effects on colonic histology and proinflammatory cytokine secretion by mesenteric lymph node cells were diminished or even completely abolished by ADX before stressor exposure. Therefore, the initial increase in basal plasma corticosterone concentrations and the increased sympathetic activity as measured by TH expression, described after social defeat (42, 84, 85), and found on d 2 of CSC are likely to increase intestinal permeability and bacterial translocation to mesenteric lymph nodes, liver, and spleen (80). Further studies will be performed to confirm this hypothesis. The commencement of an immune response requires acute activation of the HPA axis as well as the sympathetic nervous system for mobilization of immune cells and their redistribution to relevant body compartments (37). Indeed, with respect to the activation of the sympathetic nervous system, we demonstrate an elevated TH expression directly at the site of bacterial invasion in colonic tissue on d 2 of CSC. Thus, in addition to the hypothesized increased exposure to luminal antigens, CSC mice might also exhibit an acutely improved immune function resulting from the initial activation of these systems.

The initial rise in plasma corticosterone levels and inability of the HPA axis to adequately respond thereafter could partly contribute to the development of spontaneous colonic inflammation after prolonged CSC exposure. With the progression of chronic stress exposure, the HPA axis becomes unable to respond to stress, leading to insufficient production and secretion of antiinflammatory GC. This may contribute to the increased epithelial damage score and the increased cytokine secretion found after prolonged CSC exposure. Indeed, a blunted activation of the HPA axis during stressor exposure makes the animals more vulnerable for chemically induced inflammations (32–35). Moreover, the diminished GC sensitivity found in LPS-stimulated immune cells of CSC mice may additionally contribute to the increased colonic inflammation found on d 14 and 20 of CSC. The development of GC resistance has been suggested as one of the mechanisms by which a hyperinflammatory state may be induced under stressful conditions. Additionally, uncoupling the activity of the HPA axis and sympathetic nervous system during the CSC procedure may promote proinflammatory processes because the synergism of steroid hormones and neurotransmitters of the sympathetic nervous system will be dissipated (31).

In conclusion, this study shows that a subordinate status within a group of male mice over 3 wk is a potent chronic psycho-social stressor that induces a spontaneous colonic inflammation. The inflammatory process is, at least partially, mediated by the initial activation of the HPA axis and rise in plasma corticosterone followed by the inefficiency of the adrenals to secrete corticosterone.

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References

1. **Mitchell CM, Drossman DA** 1987 Survey of the AGA membership relating to patients with functional gastrointestinal disorders. Gastroenterology 92:1282–1284
2. **Robertson DA, Ray J, Diamond I, Edwards JG** 1989 Personality profile and affective state of patients with inflammatory bowel disease. Gut 30:623–626
3. **Salem SN, Shubair KS** 1967 Non-specific ulcerative colitis in Bedouin Arabs. Lancet 1:473–475
4. **Castagliuolo I, Lamont JT, Qiu B, Fleming SM, Bhaskar KR, Nikulasson ST, Kornetsky C, Pothoulakis C** 1996 Acute stress causes mucin release from rat colon: role of corticotropin releasing factor and mast cells. Am J Physiol Gastrointest Liver Physiol 271:G884–G892
5. **Pfeiffer CJ, Qiu B, Lam SK** 2001 Reduction of colonic mucus by repeated short-term stress enhances experimental colitis in rats. J Physiol Paris 95:81–87
6. **Podolsky DK, Isselbacher KJ** 1985 Glycoprotein composition of colonic mucosa: specific alterations in ulcerative colitis. Gastroenterology 87:991–998
7. **Podolsky DK, Madara JL, King N, Sehgal P, Moore R, Winter HS** 1985 Colonic mucin composition in primates. Selective alterations associated with spontaneous colitis in the cotton-top tamarin. Gastroenterology 88:20–25
8. **Barclay G, Turnberg L** 1987 Effect of psychological stress on salt and water transport in the human jejunum. Gastroenterology 93:91–97
9. **Santos J, Saperas E, Nogueiras([Dagger]) C, Mourelle M, Antolin M, Cadahia([Dagger]) A, Malagelada J-R** 1998 Release of mast cell mediators into the jejunum by cold pain stress in humans. Gastroenterology 114:640–648
10. **Barclay G, Turnberg L** 1988 Effect of cold-induced pain on salt and water transport in the human jejunum. Gastroenterology 94:994–998
11. **Empey LR, Fedorak RN** 1989 Effect of misoprostol in preventing stress-induced intestinal fluid secretion in rats. Prostaglandins Leukot Essent Fatty Acids 38:43–48
12. **Markossian S, Kreydiyyeh SI** 2005 TNF-[alpha] down-regulates the Na+-K+ ATPase and the Na+-K+-2Cl-cotransporter in the rat colon via PGE2. Cytokine 30:319–327
13. **Ferrier L, Mazelin L, Cenac N, Desreumaux P, Janin A, Emilie D, Colombel J, Garcia-Villar R, Fioramonti J, Bueno L** 2003 Stress-induced disruption of colonic epithelial barrier: role of interferon-γ and myosin light chain kinase in mice. Gastroenterology 125:795–804
14. **Santos J, Saunders PR, Hanssen NPM, Yang P-C, Yates D, Groot JA, Perdue MH** 1999 Corticotropin-releasing hormone mimics stress-induced colonic epithelial pathophysiology in the rat. Am J Physiol Gastrointest Liver Physiol 277:G391–G399
15. **Saunders PR, Kosecka U, McKay DM, Perdue MH** 1994 Acute stressors stimulate ion secretion and increase epithelial permeability in rat intestine. Am J Physiol Gastrointest Liver Physiol 267:G794–G799
16. **Meddings JB, Swain MG** 2000 Environmental stress-induced gastrointestinal permeability is mediated by endogenous glucocorticoids in the rat. Gastroenterology 119:1019–1028
17. **Engler H, Stefanski V** 2003 Social stress and T cell maturation in male rats: transient and persistent alterations in thymic function. Psychoneuroendocrinology 28:951–969
18. **Von Holst D** 1986 Psychosocial stress and its pathophysiological effects in tree shrews (*Tupaia belangeri*). In: Schmidt TH, Dembrowski TM, Blümchen G, eds. Biological and psychological factors in cardiovascular diseases. Heidelberg: Springer-Verlag 476–490
19. **Sachser N, Lick C** 1989 Social stress in guinea pigs. Physiol Behav 46:137–144
20. **Stefanski V, Engler H** 1999 Social stress, dominance and blood cellular immunity. J Neuroimmunol 94:144–152

21. **Berton O, Aguerre S, Sarrieau A, Mormede P, Chaouloff F** 1998 Differential effects of social stress on central serotonergic activity and emotional reactivity in Lewis and spontaneously hypertensive rats. Neuroscience 82:147–159
22. **McBlane JW, Handley SL** 1994 Effects of two stressors on behaviour in the elevated X-maze: preliminary investigation of their interaction with 8-OH-DPAT. Psychopharmacology (Berl) 116:173–182
23. **McNaughton N, Panickar KS, Logan B** 1996 The pituitary-adrenal axis and the different behavioral effects of buspirone and chlordiazepoxide. Pharmacol Biochem Behav 54:51–56
24. **Heinrichs SC, Pich EM, Miczek KA, Britton KT, Koob GF** 1992 Corticotropin-releasing factor antagonist reduces emotionality in socially defeated rats via direct neurotropic action. Brain Res 581:190–197
25. **Merlot E, Moze E, Dantzer R, Neveu PJ** 2003 Importance of fighting in the immune effects of social defeat. Physiol Behav 80:351–357
26. **Rodgers RJ, Cole JC** 1993 Anxiety enhancement in the murine elevated plus maze by immediate prior exposure to social stressors. Physiol Behav 53:383–388
27. **Hata T, Nishikawa H, Itoh E, Funakami Y** 2001 Anxiety-like behavior in elevated plus-maze tests in repeatedly cold-stressed mice. Jpn J Pharmacol 85:189–196
28. **Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E** 2000 Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. Science 289:1352–1355
29. **Obermeier F, Dunger N, Strauch UG, Grunwald N, Herfarth H, Scholmerich J, Falk W** 2003 Contrasting activity of cytosin-guanosin dinucleotide oligonucleotides in mice with experimental colitis. Clin Exp Immunol 134:217–224
30. **Straub RH, Stebner K, Harle P, Kees F, Falk W, Scholmerich J** 2005 Key role of the sympathetic microenvironment for the interplay of tumour necrosis factor and interleukin 6 in normal but not in inflamed mouse colon mucosa. Gut 54:1098–1106
31. **Straub RH, Herfarth H, Falk W, Andus T, Scholmerich J** 2002 Uncoupling of the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis in inflammatory bowel disease? J Neuroimmunol 126:116–125
32. **Gue M, Bonbonne C, Fioramonti J, More J, Del Rio-Lacheze C, Comera C, Bueno L** 1997 Stress-induced enhancement of colitis in rats: CRF and arginine vasopressin are not involved. Am J Physiol 272:G84–G91
33. **Gulpinar MA, Ozbeyli D, Arbak S, Yegen BC** 2004 Anti-inflammatory effect of acute stress on experimental colitis is mediated by cholecystokinin-B receptors. Life Sci 75:77–91
34. **Cakir B, Bozkurt A, Ercan F, Yegen BC** 2004 The anti-inflammatory effect of leptin on experimental colitis: involvement of endogenous glucocorticoids. Peptides 25:95–104
35. **Million M, Tache Y, Anton P** 1999 Susceptibility of Lewis and Fischer rats to stress-induced worsening of TNB-colitis: protective role of brain CRF. Am J Physiol Gastrointest Liver Physiol 276:G1027–G1036
36. **Härle P, Möbius D, D.J.J C, Schölmerich J, Straub RH** 2005 An opposing time-dependent immune-modulating effect of the sympathetic nervous system conferred by altering the cytokine profile in the local lymph nodes and spleen of mice with type II collagen-induced arthritis. Arthritis Rheum 52:1305–1313
37. **Dhabhar FS, Miller AH, McEwen BS, Spencer RL** 1995 Effects of stress on immune cell distribution. Dynamics and hormonal mechanisms. J Immunol 154:5511–5527
38. **Miller AH, Spencer RL, Pearce BD, Pisell TL, Tanapat P, Leung JJ, Dhabhar FS, McEwen BS, Biron CA** 1997 Effects of viral infection on corticosterone secretion and glucocorticoid receptor binding in immune tissues. Psychoneuroendocrinology 22:455–474
39. **Lechner O, Dietrich H, Oliveira dos Santos A, Wiegers GJ, Schwarz S, Harbutz M, Herold M, Wick G** 2000 Altered circadian rhythms of the stress hormone and melatonin response in lupus-prone MRL/MP-fasIpr mice. J Autoimmun 14:325–333
40. **Avitsur R, Stark JL, Sheridan JF** 2001 Social stress induces glucocorticoid resistance in subordinate animals. Horm Behav 39:247–257
41. **Stark JL, Avitsur R, Hunzeker J, Padgett DA, Sheridan JF** 2002 Interleukin-6 and the development of social disruption-induced glucocorticoid resistance. J Neuroimmunol 124:9–15
42. **Engler H, Engler A, Bailey MT, Sheridan JF** 2005 Tissue-specific alterations in the glucocorticoid sensitivity of immune cells following repeated social defeat in mice. J Neuroimmunol 163:110–119
43. **Stefanski V, Knopf G, Schulz S** 2001 Long-term colony housing in Long Evans rats: immunological, hormonal, and behavioral consequences. J Neuroimmunol 114:122–130
44. **Pellow S, Chopin P, File SE, Briley M** 1985 Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14:149–167
45. **Lister RG** 1987 The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) 92:180–185
46. **Miller LE, Justern H, Scholmerich J, Straub RH** 2000 The loss of sympathetic nerve fibers in the synovial tissue of patients with rheumatoid arthritis is accompanied by increased norepinephrine release from synovial macrophages. FASEB J 14:2097–2107
47. **Bosch OJ, Kromer SA, Neumann ID** 2006 Prenatal stress: opposite effects on anxiety and hypothalamic expression of vasopressin and corticotropin-releasing hormone in rats selectively bred for high and low anxiety. Eur J Neurosci 23:541–551
48. **Bazhan N, Shevchenko A, Karkaeva N, Yakovleva T, Makarova E** 2004 Agouti yellow mutation increases adrenal response to ACTH in mice. Eur J Endocrinol 151:265–270
49. **Drossman DA** 1985 Is the cotton-topped tamarin a model for behavioral research? Dig Dis Sci 30:24S–27S
50. **Gozalo A, Montoya E** 1992 Mortality causes of the moustached tamarin (*Saguinus mystax*) in captivity. J Med Primatol 21:35–38
51. **Stefanski V** 2001 Social stress in laboratory rats: behavior, immune function, and tumor metastasis. Physiol Behav 73:385–391
52. **Haller J, Fuchs E, Halasz J, Makara GB** 1998 Defeat is a major stressor in males while social instability is stressful mainly in females: towards the development of a social stress model in female rats. Brain Res Bull 50:33–39
53. **Zelena D, Mergl Z, Foldes A, Kovacs KJ, Toth Z, Makara GB** 2003 Role of hypothalamic inputs in maintaining pituitary-adrenal responsiveness in repeated restraint. Am J Physiol Endocrinol Metab 285:E1110–E1117
54. **Reber SO, Obermeier F, Straub RH, Falk W, Neumann ID** 2006 Chronic intermittent psychosocial stress (social defeat/overcrowding) in mice increases the severity of an acute DSS-induced colitis and additionally impairs regeneration. Endocrinology 147:4968–4976
55. **Stefanski V, Peschel A, Reber S** 2003 Social stress affects migration of blood T cells into lymphoid organs. J Neuroimmunol 138:17–24
56. **Ashwell JD, Lu FWM, Vacchio MS** 2000 Glucocorticoids in T cell development and function. Annu Rev Immunol 18:309–345
57. **Lowy MT** 1989 Quantification of type I and II adrenal steroid receptors in neuronal, lymphoid and pituitary tissues. Brain Res 503:191–197
58. **Schwartzman RA, Cidlowski JA** 1994 Glucocorticoid-induced apoptosis of lymphoid cells. Int Arch Allergy Immunol 105:347–354
59. **McEwen BS, Biron CA, Brunson KW, Bulloch K, Chambers WH, Dhabhar FS, Goldfarb RH, Kitson RP, Miller AH, Spencer RL, Weiss JM** 1997 The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions. Brain Res Rev 23:79–133
60. **Kendall MD, Al-Shawaf AA** 1991 Innervation of the rat thymus gland. Brain Behav Immun 5:9–28
61. **Durant S** 1986 In vivo effects of catecholamines and glucocorticoids on mouse thymic cAMP content and thymolysis. Cell Immunol 102:136–143
62. **Suzuki K, Tadakuma T, Kizakii H** 1991 Modulation of thymocyte apoptosis by isoproterenol and prostaglandin E2. Cell Immunol 134:235–240
63. **Anderson K, Anderson G, Michell R, Jenkinson E, Owen J** 1996 Intracellular signaling pathways involved in the induction of apoptosis in immature thymic T lymphocytes. J Immunol 156:4083–4091
64. **Koolhaas JM, Meerlo P, De Boer SF, Strubbe JH, Bohus B** 1997 The temporal dynamics of the stress response. Neurosci Biobehav Rev 21:775–782
65. **Starke K** 1987 Presynaptic α-autoreceptors. Rev Physiol Biochem Pharmacol 107:73–146
66. **Aghajanian GK, Cedarbaum JM, Wang RY** 1977 Evidence for norepinephrine-mediated collateral inhibition of locus coeruleus neurons. Brain Res 136:570–577
67. **Nankova B, Kvetnansky R, Hiremagalur B, Sabban B, Rusnak M, Sabban E** 1996 Immobilization stress elevates gene expression for catecholamine biosynthetic enzymes and some neuropeptides in rat sympathetic ganglia: effects of adrenocorticotropin and glucocorticoids. Endocrinology 137:5597–5604
68. **Smith MA, Brady LS, Glowa J, Gold PW, Herkenham M** 1991 Effects of stress and adrenalectomy on tyrosine hydroxylase mRNA levels in the locus ceruleus by in situ hybridization. Brain Res 544:26–32
69. **Zigmond RE, Schon F, Iversen LL** 1974 Increased tyrosine hydroxylase activity in the locus coeruleus of rat brain stem after reserpine treatment and cold stress. Brain Res 70:547–552
70. **Richard F, Faucon-Biguet N, Labatut R, Rollet D, Mallet J, Buda M** 1988 Modulation of tyrosine hydroxylase gene expression in rat brain and adrenals by exposure to cold. J Neurosci Res 20:32–37
71. **Dhabhar FS, McEwen BS** 1999 Enhancing versus suppressive effects of stress hormones on skin immune function. Proc Natl Acad Sci USA 96:1059–1064
72. **Dhabhar FS, McEwen BS** 1997 Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. Brain Behav Immun 11:286–306
73. **Lightman SL, Harbuz MS** 1993 Expression of corticotropin-releasing factor mRNA in response to stress. Ciba Found Symp 172:173–187; discussion 187–98
74. **Imaki T, Nahan J, Rivier C, Sawchenko P, Vale W** 1991 Differential regulation of corticotropin-releasing factor mRNA in rat brain regions by glucocorticoids and stress. J Neurosci 11:585–599
75. **Lightman S, Young 3rd W** 1988 Corticotrophin-releasing factor, vasopressin and pro-opiomelanocortin mRNA responses to stress and opiates in the rat. J Physiol (Lond) 403:511–523
76. **Herman JP, Schafer MK-H, Sladek CD, Day R, Young EA, Akil H, Watson SJ** 1989 Chronic electroconvulsive shock treatment elicits up-regulation of CRF and AVP mRNA in select populations of neuroendocrine neurons. Brain Res 501:235–246
77. **Harbuz M, Lightman S** 1989 Responses of hypothalamic and pituitary mRNA to physical and psychological stress in the rat. J Endocrinol 122:705–711

78. **Aguilera G** 1994 Regulation of pituitary ACTH secretion during chronic stress. Front Neuroendocrinol 15:321–350
79. **Glavin GB, Pare WP, Sandbak T, Bakke H-K, Murison R** 1994 Restraint stress in biomedical research: an update. Neurosci Biobehav Rev 18:223–249
80. **Bailey MT, Engler H, Sheridan JF** 2006 Stress induces the translocation of cutaneous and gastrointestinal microflora to secondary lymphoid organs of C57BL/6 mice. J Neuroimmunol 171:29–37
81. **Yamada T, Sartor R, Marshall S, Specian R, Grisham M** 1993 Mucosal injury and inflammation in a model of chronic granulomatous colitis in rats. Gastroenterology 104:759–771
82. **Hermiston ML, Gordon JI** 1995 Inflammatory bowel disease and adenomas in mice expressing a dominant negative N-Cadherin. Science 270:1203–1207
83. **Rath HC, Herfarth HH, Ikeda JS, Grenther WB, Hamm Jr TE, Balish E, Taurog JD, Hammer RE, Wilson KH, Sartor RB** 1996 Normal luminal bacteria, especially bacteroides species, mediate chronic colitis, gastritis, and arthritis in HLA-B27/human β2 microglobulin transgenic rats. J Clin Invest 98:945–953
84. **Marini F, Pozzato C, Andreetta V, Jansson B, Arban R, Domenici E, Carboni L** 2006 Single exposure to social defeat increases corticotropin-releasing factor and glucocorticoid receptor mRNA expression in rat hippocampus. Brain Res 1067:25–35
85. **Bhatnagar S, Vining C, Iyer V, Kinni V** 2006 Changes in hypothalamic-pituitary-adrenal function, body temperature, body weight and food intake with repeated social stress exposure in rats. J Neuroendocrinol 18:13–24

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