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Alterations in anxiety-like behavior following knockout of the *uncoupling protein 2* (*ucp2*) gene in mice

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ABSTRACT

Aims: Uncoupling protein 2 (UCP2) is a mitochondrial protein that reduces oxidative stress and has a protective function in chronic inflammatory diseases such as multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus. UCP2 is strongly expressed in areas implicated in the central regulation of stress and anxiety. Therefore, we compared the neuroendocrine regulation of stress responses, immunity and behavior in UCP2-deficient and wildtype C57BL/6J mice under psychological stress.
Main methods: Stress was induced by social disruption (SDR) and anxiety-like behavior was examined using the elevated plus-maze (EPM). Serum corticosterone was determined by radioimmunoassay and brain neurotransmitter concentrations were analyzed by HPLC of whole brain homogenates. T cell activation and tumor necrosis factor (TNF)-α production of mitogen-activated splenocytes were determined in vitro by flow cytometry staining of CD25, CD69 and CD71 on CD4 cells, and ELISA, respectively. The influence of corticosterone on UCP2 expression of splenocytes was analyzed by Western blot.
Key findings: At baseline, UCP2-deficient mice were significantly more anxious in the EPM than wildtype mice, and this phenotype was exacerbated after SDR stress. The corticosterone response after SDR + EPM was reduced in UCP2-deficient mice compared to wildtype mice. Corticosterone in turn downregulates UCP2 expression in splenocyte cultures of wildtype mice at physiological concentrations. Dopaminergic and serotonergic turnovers were increased in UCP2-deficient mice after SDR + EPM. While T-helper cell activation-marker expression was reduced, TNF-α production was increased in UCP2-deficient mice after SDR + EPM.
Significance: Our study shows that UCP2 is involved in anxiety-like behavior and modulates neuroendocrine and immune responses after stress.

Introduction

Uncoupling protein 2 (UCP2) is a member of the mitochondrial transporter superfamily which reduces the production of reactive oxygen species (ROS) (Arsenijevic et al., 2000). UCP2 is expressed in a wide variety of tissues, including the brain (Richard et al., 1998; Richard et al., 2001). Here, UCP2 prevents neuronal death and diminishes brain dysfunction after stroke and brain trauma (Mattiasson et al., 2003; Mehta and Li, 2009). The *ucp2* gene has been identified as a susceptibility gene in multiple sclerosis (MS) (Vogler et al., 2005). Furthermore, a *ucp2* promoter polymorphism has been linked to chronic inflammatory diseases such as rheumatoid arthritis and systemic lupus erythematosus (Yu et al., 2009b). Stressful life events may lead to exacerbation of these diseases (Dobkin et al., 1998; Mohr et al., 2004; Thomason et al., 1992). Remarkably, UCP2 is expressed in neurons of stress-related regions of the hypothalamic–pituitary–adrenal (HPA) axis, the limbic system and neurotransmitter-regulating regions, for example the hypothalamus, the locus coeruleus (LC), the amygdala and the raphe nuclei. Hypothalamus, LC and amygdala are interrelated via neuronal projections, hormones and transmitter substances which play a role in anxiety (Bale et al., 2000; Butler et al., 1990; Reyes et al., 2005). UCP2 is also strongly expressed in the ventral tegmental area and the substantia nigra of the dopaminergic system (Richard et al., 1998; Richard et al., 2001). In the brain, the dopamine (DA) turnover is altered in UCP2-deficient (UCP2−/−) mice and the number of mitochondria in DA neurons of the substantia nigra and the ventral tegmental area is reduced in UCP2−/− mice, whereas ROS production is increased (Andrews et al., 2006; Fuxe et al., 2005).

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It has been shown that UCP2 protein is also expressed in the spleen (Pecqueur et al., 2001) and immune cells, such as macrophages and T cells (Alves-Guerra et al., 2003; Kizaki et al., 2002). In experimental autoimmune encephalomyelitis, a mouse model for MS, UCP2−/− mice develop a more severe disease than wildtype mice and show differences in T cell responses (Vogler et al., 2006). It is known that immune functions are modulated by psychosocial stress, e.g., in mice and rats (Bailey et al., 2009; Merlot et al., 2004; Stefanski, 1998). However, nothing is known about a possible immune modulation by UCP2 deficiency under the influence of stress.

The aim of this study was to investigate whether UCP2-deficient mice show alterations in anxiety, neuroendocrine responses to psychological stress and in their adaptive and innate immune response following stress. We used social defeat as a psychosocial stressor that has been shown to induce anxiety in mice (Kinsey et al., 2007) and combined it with a behavioral test, i.e., the elevated plus maze (EPM), which is anxiogenic (Belzung and Griebel, 2001). We analyzed anxiety-like behavior, serum corticosterone concentrations and the brain neurotransmitters DA, noradrenaline (NA) and 5-hydroxytryptamine (5-HT) and their metabolites in response to stress. We investigated adaptive immunity by flow cytometric staining of early (CD69, CD25) and late (CD71) CD4+ T cell activation markers in response to mitogens. Innate immunity was assessed by studying splenocytic TNF-α production following lipopolysaccharide (LPS) stimulation in UCP2−/− mice compared to wildtype controls under psychological stress conditions.

Materials and methods

Mouse strains

The inbred strains C57BL/6J and NZB/B1n were obtained from Jackson Laboratory (Bar Harbor, MA, U.S.A.). Generation of transgenic animals on a C57BL/6J background deficient for UCP2 has been previously described (Alves-Guerra et al., 2003; Arsenijevic et al., 2000). Briefly, the *ucp2* gene was interrupted by insertion of a (phosphoglycerate kinase)-neor cassette in exon 3 of the gene. The linearized targeting construct was electroporated into ES cell clone RW4, and G418-resistant clones were isolated and analyzed by PCR. Correctly targeted clones were microinjected into C57BL/6J mouse blastocysts and heterozygous mutant mice were generated from two of those lines. Heterogenous mutants were backcrossed for ten generations with C57BL/6J mice. Separate mutant and wildtype homozygote lines were then established and bred in the same facility. The UCP2 −/− line was backcrossed again with C57BL/6J mice every ten generations to avoid the risk of de novo mutations. A total of 108 six-week old male mice were used for the experiments. Animals were allowed to adapt to housing conditions at the animal facility at the University of Rostock for at least 3 weeks prior to experiments. They were housed with their siblings (three males per cage) before and during experiments. Mice were housed in a climate-controlled environment (21 °C) with a 12-h light/dark cycle and fed conventional rodent chow and water ad libitum. All procedures were pre-approved by the local Animal Care Committee and are in accordance with the European Council Directive of 24 November 1986 (86/609/EEC).

Social disruption (SDR) stress

Cages of three mice (brothers) were randomly assigned as either control or SDR groups. Control mice remained undisturbed in their home cage. Stressed mice experienced two SDR events over 2 days: Stress was induced by introducing an aggressive intruder, i.e., a retired old breeder male (NZB/B1n, >1 year of age), into the home cage for 6 h (0700 h to 1300 h) and again from 0630 h to 0930 h the following day. Behavior was observed to ensure that the intruder attacked the residents and that the residents showed submissive postures. All of the attacked mice received small back or tail bite wounds. None of the group-housed control mice were injured. After the second SDR cycle, mice were either immediately sacrificed by cervical dislocation or tested on an EPM for 10 min and then sacrificed immediately afterwards, i.e. within seconds after the EPM test. EPM tests started 20 min past the end of the second SDR cycle with one mouse at a time, i.e. 0950 h to 1100 h when six mice were tested which was the maximum per day.

Sample collection

After cervical dislocation, mice were rapidly decapitated, and their trunk blood was collected. Brains (i.e. the cerebrum after cutting off the olfactory bulb and the cerebellum) were isolated, snap-frozen in liquid nitrogen and stored at −80 °C until neurotransmitter analysis. Blood was allowed to clot for 2 h and was centrifuged at 2,000×*g* for 10 min. Sera were stored at −20 °C until corticosterone analysis. Spleens were isolated for cell cultures.

Behavioral test

The elevated plus maze consists of four arms of 30-cm length and 5-cm width each. Two opposing arms are enclosed in opaque 15-cm high side and end walls. The elevation of the structure was 60 cm. We tested anxiety based on the following parameters: i) entries into open arms; ii) time spent in open arms; and iii) distance in open arms. Locomotor activity was determined as i) total entries into open and closed arms; ii) entries into closed arms; and iii) distance in closed arms. The conflict between approaching and avoiding risk, i.e., to enter the “dangerous” open arms was tested by i) entries into central position, ii) time in central position, and iii) head dips from a closed into an open arm. Mice were put into the central position of the maze and left undisturbed for 10 min. Their behavior was recorded by video camera and later analyzed by a trained observer blind to the treatment. Control and SDR-stressed mice were alternately tested to minimize influences of time progression after SDR stress and time of day. The test apparatus was thoroughly cleaned between sessions. The EPM test was carried out by an investigator who was blind to the treatment (SDR or control) which was applied in another room. To avoid stress influences on the mice, the sacrificing of the mice was performed in a separate laboratory.

Corticosterone analysis

Serum corticosterone concentrations were measured in duplicate using a commercially available double antibody rat corticosterone ^{125}I-RIA kit (DRG Diagnostics, Marburg, Germany) according to the manufacturer's instructions. Cross-reactivities of the antibody with any potentially competing serum steroids were lower than 0.5%. The test sensitivity was 7.2 ng/ml, and intra- and interassay coefficients of variation (CV) were 6.9% and 8.1%, respectively.

Western blot of UCP2 expression

We chose splenocytes as model cells because UCP2 expression has been confirmed in this group of cells (Alves-Guerra et al., 2003; Kizaki et al., 2002). Splenocytes from C57BL/6J mice were cultured in RPMI 1640 medium (PAA) supplemented with 14.3 μM 2-mercaptoethanol and 10% fetal bovine serum. Cells were stimulated with 0, 0.1, 1, 10 and 100 μM of corticosterone (C2505, Sigma Aldrich) for 24 h. Protein content was precipitated by adding 400 μl of 10 mM NaN_3, 2% trichloroacetate (TCA) and an equal volume of 0.5 mm glass beads (Biospec products distributed by VWR International, Fontenay-sous-Bois, France) and then thoroughly mixed. Glass beads were washed twice with TCA solution and all supernatant was centrifuged at 6000×*g* for 20 min. Protein in the resulting pellet was resuspended in 10 μl of

Laemmli buffer per million cells. Twenty-five microliters of each protein sample was loaded on a 12.5% SDS-PAGE. UCP2 protein was immunodetected by chemiluminescence using the hUCP2-605 antibody as previously described (Pecqueur et al., 2001). The anti-porin antibody (clone 20B12; Molecular Probes, Leiden, The Netherlands) was used to estimate the mitochondrial protein content of each sample as a reference because no variation in the amount of porin was observed upon stimulation with LPS, cytokines or glutamine (Emre et al., 2007; Hurtaud et al., 2007; Tagen et al., 2009). UCP2 quantification was performed by densitometry using the volume tools of Quantity One® (BioRad, Hercules, CA, USA). A box was defined that was used on all bands. The quantity of the volume is the sum of the intensities of all the pixels within the volume boundary multiplied by the area of each pixel. Units are intensity × mm^2. Blanks were quantified using the same box on the background of the blots. They were subtracted for graphical presentation.

Neurotransmitter analyses

Whole brain concentrations of NA and its metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG), DA and its metabolite homovanillic acid (HVA) and 5-HT and its metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) were determined in duplicate using high performance liquid chromatography (HPLC) with electrochemical detection. This method was recently described in detail by Otten et al. (2010), and slightly adapted by using 4 ml of 0.2 M perchloric acid for each extraction step. The concentrations of neurotransmitters and metabolites were expressed as ng per g of wet brain tissue. The intraassay CV values for NA, MHPG, DA, HVA, 5-HT and 5-HIAA were 2.4, 7.1, 5.3, 4.8, 3.7 and 2.7%, and the interassay CV values were 6.4, 7.9, 7.0, 5.4, 3.0 and 2.8%, respectively. The limits of quantification were 39, 10, 27, 54, 22 and 37 ng/g for NA, MHPG, DA, HVA, 5-HT and 5-HIAA, respectively.

Analysis of T cell activation and TNF-α production

Spleen cell suspensions were freshly prepared from sacrificed mice as previously described (Gimsa et al., 2009). Cells were stimulated with either 5 ng/ml phorbol myristate acetate (PMA) plus 1 μg/ml ionomycin, 2 μg/ml concanavalin A or 10 μg/ml LPS for 24 h for flow cytometry (all reagents from Sigma-Aldrich). Additionally, LPS-stimulated splenocytes were cultured for 72 h for TNF-α analysis. Expression of CD25, CD69 and CD71 in CD4+ lymphocytes (reagents from BD Biosciences, Heidelberg, Germany; for details see (Gimsa et al., 2009)) were analyzed on a flow cytometer (EPICS XL-MCL, Beckman Coulter, Krefeld, Germany). Samples were gated on live lymphocytes on the basis of forward and side scatter parameters. Supernatants were tested by a murine TNF-α ELISA (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions. The sensitivity of the assay was 5.1 pg/ml and intra- and interassay CV values were 6.5 and 8.6%, respectively.

Statistical analysis

Behavioral, neuroendocrine and immune measures were evaluated by two-way analyses of variance (ANOVA) using the GLIMMIX procedure of SAS System for Windows, release 9.2 (SAS Institute Inc., 2008, Cary, NC, U.S.A.). ANOVA models for these traits comprised the fixed classification variables strain (C57BL/6J and UCP2−/−) and treatment (control, SDR, EPM and SDR + EPM). All pair-wise differences of the least-squares (LS) means were tested using the Tukey–Kramer method. Significance was defined as $p<0.05$. Results are expressed as LS means ± standard errors (SE).

Results

Anxiety-like behavior

To assess anxiety-like behavior, we tested C57BL/6J mice and UCP2−/− mice with or without prior SDR experience on the EPM. We found significant effects of the mouse strain for the time in the open arms ($F(1, 43)=25.82$, $p<0.001$), distances the mice walked in the open arms ($F(1, 43)=24.17$, $p<0.001$), total arm entries ($F(1, 43)=110.89$, $p<0.001$), entries into closed arms ($F(1, 43)=33.00$, $p<0.001$), time spent in the central position ($F(1, 43)=16.49$, $p<0.001$), entries into the central position ($F(1, 43)=99.85$, $p<0.001$) and time in closed arms ($F(1, 43)=31.00$, $p<0.001$). We found significant interactions of strain × treatment on total arm entries ($F(1, 43)=11.77$, $p=0.001$), entries into closed arms ($F(1, 43)=6.4$, $p=0.015$) and entries into the central position ($F(1, 43)=23.73$, $p<0.001$). Pair-wise comparisons revealed that UCP2−/− mice were significantly more anxious than C57BL/6J mice at baseline, i.e., without SDR stress (Fig. 1A). They entered the open arms of the maze less often than C57BL/6J mice ($p<0.001$), spent less time in the open arms ($p=0.010$) and more time in the closed arms ($p=0.003$) and walked shorter distances in the open arms than C57BL/6J mice ($p=0.039$). Total arm entries were reduced in UCP2−/− mice compared to C57BL/6J mice ($p<0.001$), whereas the distances that the mice walked in closed arms did not differ. After SDR stress, UCP2−/− mice spent less time in the open arms ($p=0.002$) and more time in the closed arms ($p<0.001$), walked shorter distances in the open arms ($p<0.001$), and showed less total arm entries ($p<0.001$) than C57BL/6J mice. Entries into closed arms were reduced after SDR stress in UCP2-/− mice compared to controls ($p=0.018$), as well as stressed C57BL/6J mice ($p<0.001$) (Fig. 1B). UCP2−/− mice were also more cautious than C57BL/6J mice; the UCP2−/− mice entered the central position less often (control: $p<0.001$; SDR: $p<0.001$) and spent less time there (control: $p=0.044$; SDR: $p=0.021$) compared to wildtype mice. UCP2−/− mice showed significantly more head dips from the closed arms into the central position than C57BL/6J mice at baseline ($p<0.001$). Head dips were increased by SDR in C57BL/6J mice ($p=0.008$) and reduced by SDR in UCP2−/− mice ($p=0.001$; Fig. 1C).

Corticosterone response

The effects of UCP2 deficiency on the reactivity of the HPA axis were determined on the basis of serum corticosterone concentrations either immediately after SDR or after the EPM test with or without preceding SDR. Stress treatment affected serum corticosterone concentrations independent of the mouse strain ($F(3, 94)=52.53$, $p<0.001$). Also, there was a significant effect of the mouse strain ($F(1, 94)=11.26$, $p=0.001$). In addition, we found a significant effect of the interaction strain × treatment ($F(3, 94)=4.36$, $p=0.006$). Whereas SDR had no significant effect, pair-wise comparisons showed that the EPM significantly increased corticosterone concentrations of control mice of both strains (C57BL/6 J: 46.8 ± 16.5 ng/ml vs. 298.8 ± 16.5 ng/ml; $p<0.001$; UCP2−/−: 46.1 ± 18.5 ng/ml vs. 222.9 ± 22.6 ng/ml; $p<0.001$). SDR + EPM significantly increased corticosterone concentrations in C57BL/6 J ($p<0.001$) and UCP2−/− mice ($p=0.009$) compared to controls. SDR + EPM induced significantly higher corticosterone concentrations than SDR alone in C57BL/6J mice ($p<0.001$) (Fig. 2). Interestingly, the corticosterone response to SDR + EPM of UCP2−/− mice was significantly lower than that of C57BL/6 J mice ($p=0.003$) (Fig. 2).

Influence of corticosterone on UCP2 expression

To search for a direct link between corticosterone and UCP2, we studied UCP2 expression in vitro at the cellular level under the influence of corticosterone. We added physiological concentrations of

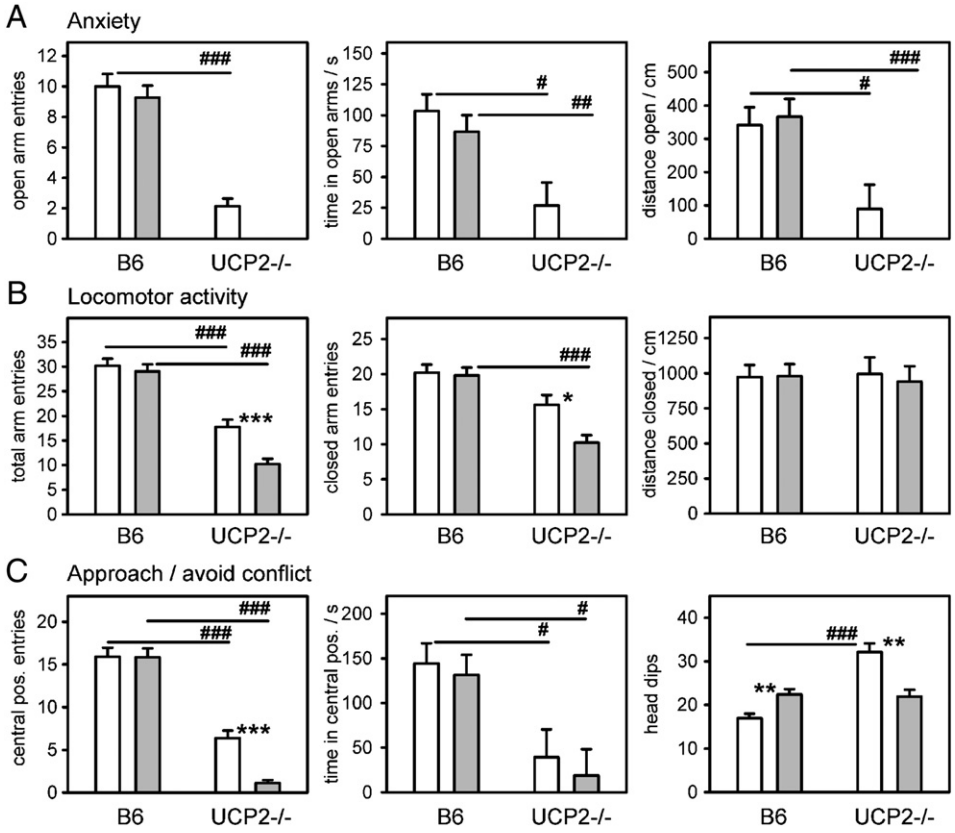

Fig. 1. *UCP2−/− mice display increased anxiety-like behavior.* C57BL/6J (B6) and UCP2−/− mice with (gray bars) and without (white bars) prior exposure to SDR stress were tested on the elevated plus-maze. (A) Anxiety was tested as number of entries into open arms, time spent on open arms and distance walked on open arms. (B) Locomotor activity was recorded as total entries into open and closed arms, the number of times mice entered closed arms and the distance mice walked on closed arms. (C) The conflict between the tendency to explore and avoidance of risk ("approach/avoid conflict") was assessed as the number of entries into the central position ("pos."), the time spent in the central position and head dips from a closed into an open arm. Data represent LS means and SE of 8–15 mice per strain and treatment. Crosses indicate significant differences between mouse strains (#$p<0.05$; ##$p<0.01$; ###$p<0.001$). Asterisks indicate significant differences between treatments within the same mouse strain (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

corticosterone to cultured splenocytes. Western Blot analyses demonstrated a very strong downregulation of UCP2 expression by corticosterone in vitro at 0.1 μM (34.6 ng/ml) (Fig. 3).

Brain neurotransmitter response

To obtain a first indication whether UCP2 may affect brain neurotransmitter systems, we analyzed whole brain homogenates by HPLC. In the brain, we observed significant effects of mouse strain on NA ($F(1, 78) = 7.16$, $p = 0.009$) and 5-HIAA ($F(1, 78) = 4.32$, $p = 0.041$), indicating differences between C57BL/6J and UCP2−/− mice. SDR stress increased HVA ($p = 0.003$) and 5-HIAA ($p<0.001$) levels in both strains compared to untreated controls. Also, the EPM test caused an increase in HVA ($p<0.001$), whereas SDR + EPM increased HIAA ($p = 0.014$) in both strains. We found significant effects of the interaction strain × treatment on HVA ($F(3, 77) = 2.19$, $p<0.001$) and 5-HIAA ($F(3, 78) = 3.40$, $p = 0.022$). Pair-wise comparisons

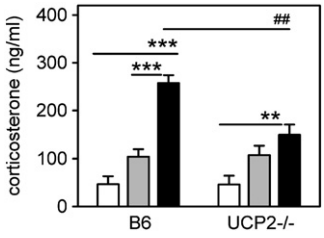

Fig. 2. *Serum corticosterone concentrations of C57BL/6J (B6) and UCP2−/− mice.* Data from mice with and without exposure to SDR stress are indicated by gray and white bars, respectively. Data from mice which underwent behavioral testing following SDR stress are indicated by black bars. Data represent LS means and SE of 9–15 mice per strain and treatment. Crosses indicate significant differences between mouse strains (##$p<0.01$). Asterisks indicate significant differences between treatments within the same mouse strain (**$p<0.01$; ***$p<0.001$).

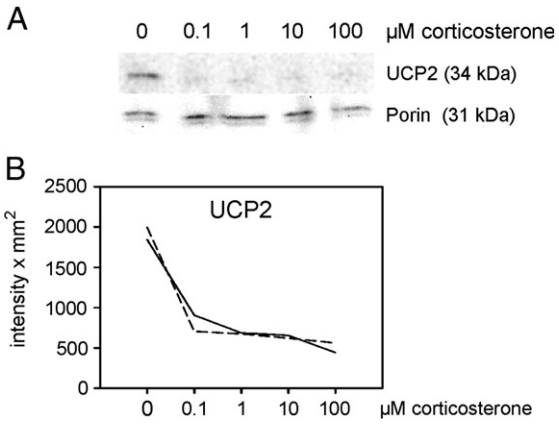

Fig. 3. *Corticosterone downregulates UCP2 expression.* C57BL/6J splenocytes were cultured in the presence of increasing concentrations of corticosterone. A. Data show representative results of one of two independent Western blot analyses. Please note that 1 μM corticosterone corresponds to 346 ng/ml. Porin was immunostained to demonstrate equal mitochondrial protein content of the samples. B. UCP2 protein expression was quantified by densitometry measurement. The graph shows data from two independent experiments after blank subtraction as two lines.

revealed significant increases in HVA ($p<0.001$) and 5-HIAA ($p=0.020$) by SDR + EPM in UCP2−/− mice compared to untreated controls (Fig. 4A and C).

T cell activation

UCP2-dependent effects on the adaptive immune system were studied by measuring T cell activation in splenocyte cultures. There were effects of mouse strain on the expression of CD25 after stimulation with concanavalin A and LPS ($F(1, 46)=24.76$, $p<0.001$; and $F(1, 46)=23.45$, $p<0.001$, respectively). In addition, there were significant effects of mouse strain on the expression of CD69 after stimulation with PMA, concanavalin A and LPS ($F(1, 45)=42.20$, $p<0.001$; $F(1, 45)=17.51$, $p<0.001$, and $F(1, 46)=4.47$, $p=0.040$, respectively) and on expression of CD71 after stimulation with PMA and concanavalin A ($F(1, 46)=8.01$, $p=0.007$ and $F(1, 46)=12.28$, $p=0.001$, respectively). We found significant effects of the interaction strain × treatment on CD25 expression after stimulation with concanavalin A ($F(3, 46)=4.37$, $p=0.009$) and on CD69 expression after stimulation with PMA ($F(3, 46)=3.30$, $p=0.029$). Pair-wise comparisons did not reveal any differences in activation marker expression in T cells of mice without stress. Also, neither SDR stress nor EPM alone affected T cell activation by mitogens. In contrast, SDR + EPM suppressed concanavalin A-induced CD25 expression compared to control ($p=0.012$) and SDR-stressed ($p=0.002$) UCP2−/− mice. Following SDR + EPM, CD25 expression after stimulation with concanavalin A ($p<0.001$) and LPS ($p=0.003$), and CD69 expression after stimulation with PMA ($p<0.001$) were lower in UCP2−/− T cells than in C57BL/6J T cells (Fig. 5).

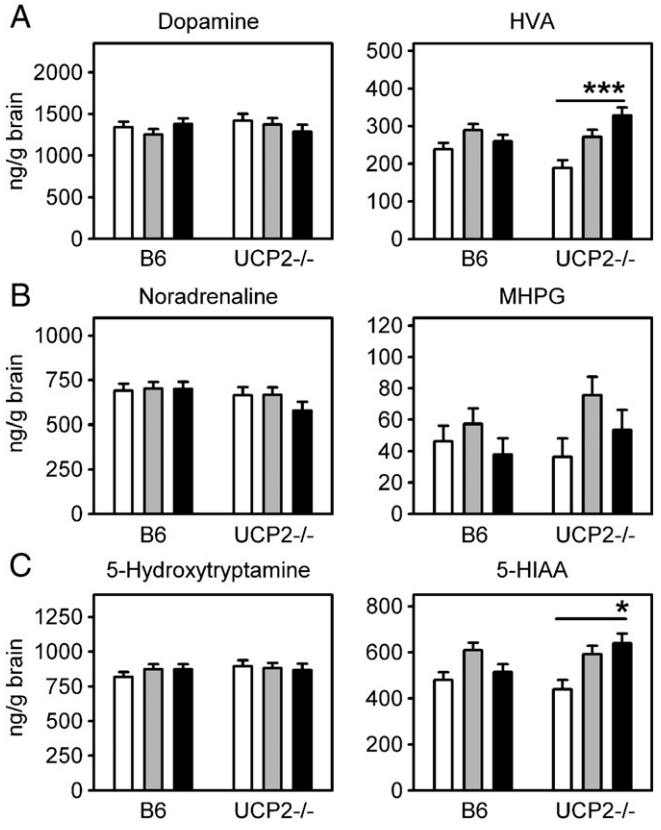

Fig. 4. *Brain neurotransmitters and neurotransmitter turnover.* Neurotransmitters and their metabolites were analyzed in whole brain homogenates from C57BL/6J (B6) and UCP2−/− mice. Data from mice with and without exposure to SDR stress are indicated by gray and white bars, respectively. Data from mice which underwent behavioral testing following SDR stress are indicated by black bars. (A) Effects on the dopaminergic system characterized by dopamine (DA) and homovanillic acid (HVA). (B) Effects on the noradrenergic system characterized by noradrenaline (NA) and 3-methoxy-4-hydroxyphenylglycol (MHPG). (C) Effects on the serotonergic system characterized by 5-hydroxytryptamine (5-HT) and 5-hydroxyindole-3-acetic acid (5-HIAA). Data represent LS means and SE of 8–13 mice per strain and treatment. Asterisks indicate significant differences between treatments within the same mouse strain (*$p<0.05$; ***$p<0.001$).

TNF-α production induced by LPS stimulation

As a test for the function of the innate immune system, TNF-α concentrations were determined in splenocyte culture supernatants after LPS stimulation. There was a significant effect of mouse strain ($F(1, 24)=5.29$, $p=0.030$), indicating altered TNF-α production in UCP2−/− splenocytes compared to C57BL/6J splenocytes. While there was a significant effect of treatment ($F(3, 24)=4.09$, $p=0.018$) independent of the strains, pair-wise comparisons showed that SDR stress did not affect TNF-α production in either of the strains. However, SDR + EPM significantly increased TNF-α production of UCP2−/− splenocytes (compared to controls: $p=0.020$; compared to SDR-stressed mice: $p=0.006$) but not of C57BL/6J splenocytes (Fig. 6).

Discussion

Because of the strong expression of UCP2 in stress- and anxiety-related brain areas and its association with chronic inflammatory diseases that are modulated by stress, we investigated the influence of UCP2 deficiency on anxiety-like behavior, stress reactivity and stress-induced modulation of immune cell activation in C57BL/6J mice and UCP2−/− mice on a C57BL/6J background. We found that UCP2−/− mice were more anxious in the EPM. After SDR + EPM, UCP2−/− mice had a reduced corticosterone response, an increased dopaminergic and serotonergic turnover, a reduced T cell activation and an increased in vitro TNF-α response to LPS compared to C57BL/6J mice.

In general, C57BL/6J mice were not affected by the SDR stress setup in our experiments. This is likely due to only two SDR sessions and the genetic constitution of C57BL/6J mice. Compared to other inbred strains, this strain was less anxious when tested in the elevated plus maze, the open field and the light–dark test (Lad et al., 2010). Therefore, C57BL/6J mice might also be less susceptible to anxiogenic stress imposed by SDR. In a study with six cycles of social defeat, the C57BL/6J mice exhibited an increased anxiety-like behavior and an increased TNF response to in vitro stimulation of splenocytes with LPS (Kinsey et al., 2007).

At baseline, i.e., without SDR stress, UCP2−/− mice are more anxious than wildtype mice. Also, UCP2−/− mice showed a reduced locomotor activity as total arm entries of control and stressed mice and entries of stressed mice into closed arms were lower in UCP2−/− mice compared to wildtype mice. Total and closed arm entries are considered conventional parameters for measuring locomotor activity (Bale et al., 2002; Komada et al., 2008; Rodgers and Dalvi, 1997; Schmitt and Hiemke, 1998; Walf and Frye, 2007). However, we believe that these parameters may be biased by anxiety because in order to enter an open or closed arm, the mice have to leave the "safe" environment of the closed arm they are sitting in. Thus, SDR-induced reductions of total and closed arm entries and central position entries may be interpreted as exacerbation of the anxious phenotype of UCP2−/− mice. By contrast, the distances the mice walk on closed arms are independent of anxiety and did not differ between UCP2−/− and wildtype mice. These distances are also considered a parameter of locomotor activity (Watt et al., 2009). Therefore, we agree with others (Walf and Frye, 2007) that arm entries made in the maze may not be an optimal measure of motor activity. However, a reduced locomotor activity has also been shown for UCP2−/− mice using an open-field test (Andrews et al., 2006). From our data, we cannot judge to which extent the reduced locomotor behavior is due to anxiety or due to motor deficits resulting from neurochemical

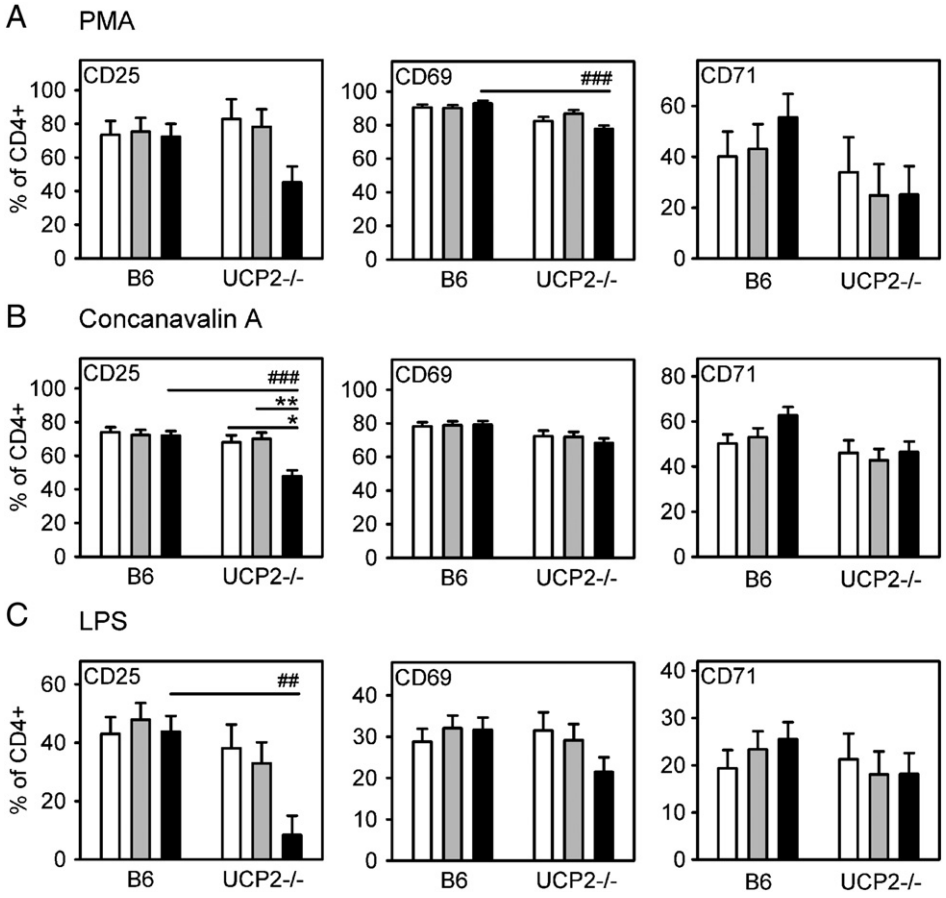

Fig. 5. *Activation marker expression of CD4+ T cells.* Splenocytes of C57BL/6J (B6) and UCP2−/− mice were stimulated with mitogens for 24 h. Data from mice with and without exposure to SDR stress are indicated by gray and white bars, respectively. Data from mice which underwent behavioral testing following SDR stress are indicated by black bars. Mitogens used were (A) phorbol myristate acetate (PMA), (B) concanavalin A and (C) lipopolysaccharide (LPS). Data are shown as mean fluorescence intensities of CD25, CD69 or CD71 staining of CD4+ T cells in culture. Data represent LS means and SE of 4–9 mice per strain and treatment. Crosses indicate significant differences between mouse strains (##$p<0.01$; ###$p<0.001$). Asterisks indicate significant differences between treatments within the same mouse strain (*$p<0.05$; **$p<0.01$).

deficits in the dopaminergic system as shown by Andrews et al. (2006).

Corticosterone analyses showed that the EPM test clearly exposes mice to a challenging situation in a novel environment and must be considered a psychologically stressful event. Thus, mice that had been exposed to SDR stress experienced another challenge on the EPM. Baseline corticosterone concentrations of control UCP2−/− and wildtype mice did not differ, and both UCP2−/− and wildtype mice showed a similar corticosterone response to SDR stress. In contrast, UCP2−/− mice responded to SDR+EPM with significantly lower corticosterone concentrations than wildtype mice. Our results indicate a modulating role of UCP2 for the HPA axis response under stressful conditions. Interestingly, it was shown that only corticotropin-releasing hormone (CRH)-producing neurons of the paraventricular nucleus (PVN) of the hypothalamus (but not other regions) express UCP2. These neurons project from the PVN to the median eminence where they control the activity of the pituitary–adrenal axis (Richard et al., 2001) and to NA-producing neurons in the LC (Reyes et al., 2005). CRH is known to be involved in anxiety and stress sensitivity (Bale et al., 2000; Butler et al., 1990).

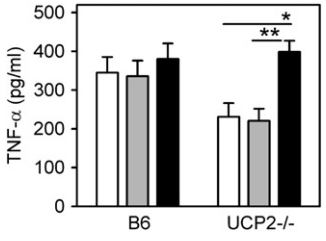

Fig. 6. *TNF-α production of splenocytes.* Splenocytes from C57BL/6J (B6) and UCP2−/− mice were stimulated with LPS for 72 h in vitro. Data from mice with and without exposure to SDR stress are indicated by gray and white bars, respectively. Data from mice which underwent behavioral testing following SDR stress are indicated by black bars. Data represent LS means and SE of 3–6 mice per strain and treatment. Asterisks indicate significant differences between treatments within the same mouse strain (*$p<0.05$; **$p<0.01$).

Neurotransmitter analyses in whole brain homogenates revealed a stronger serotonergic and dopaminergic response to SDR+EPM of UCP2−/− mice compared to wildtype mice as shown by an increased turnover with elevated HVA and HIAA concentrations. Alterations in DA release but not total DA content were observed in the striatum of UCP2−/− mice and were linked to behavioral dysfunctions (Andrews et al., 2006). UCP2 immunoreactivity was found in dopaminergic and noradrenergic terminals within discrete brain regions and it is hypothesized that UCP2 could play an important role in modulating neurotransmission by dynamic regulation of volume transmission processes (Fuxe et al., 2005; Rivera et al., 2006). Serotonergic, dopaminergic and noradrenergic neurotransmission are altered by stress (Charney, 2004) and during depression and anxiety disorders (Morilak and Frazer, 2004; Ruhe et al., 2007). However, whether the increased serotonergic and dopaminergic turnover after SDR+EPM may be interpreted as an increased stress reactivity or as reflecting an anxious phenotype of UCP2−/− mice can only be determined after region-specific neurotransmitter studies.

The reduced T cell activation in UCP2−/− compared to wildtype mice after SDR+EPM suggests an impaired T cell function in UCP2

−/− mice under acute psychological stress. It has been shown that an acute stress suppressed T cell responses while increasing innate immune responses (Fleshner et al., 1998). However, T cell proliferation was unaltered in our mice (data not shown), and SDR stress did not affect T cell activation in either of the mouse strains. Other groups have observed decreased lymphocyte proliferation in response to concanavalin A after social defeat in rats (Stefanski, 1998) and mice (Merlot et al., 2004). This difference may be due to the duration of stress. While those studies applied SDR stress repeatedly, our setup included only two sessions of SDR stress.

SDR + EPM increased LPS-induced TNF-α production of UCP2−/− but not C57BL/6J mouse splenocytes. Recent studies using a stronger SDR model (6 cycles) showed an increased TNF-α production combined with an increased anxiety (Bailey et al., 2007; Bailey et al., 2009). This is in line with our results of UCP2−/− mice which showed a stronger TNF-α response along with higher anxiety. Avitsur et al. (2003) showed that TNF-α secretion of LPS-stimulated C57BL/6 splenocytes was increased by 6 cycles of SDR. Addition of corticosterone was able to suppress this secretion although not to control levels. We cannot exclude that our experimental treatment of two SDR cycles was not enough to induce an increased TNF-α production. While SDR + EPM was definitely a stronger stressor than SDR alone, the significant increase in corticosterone compared to controls or SDR alone was probably due to the EPM. Hence, it occurred in the last 10 min before animals were sacrificed. Unless the produced corticosterone had a sustained effect on the LPS-stimulated splenocyte cultures, one would not expect an influence on TNF-α production. Alternatively, the stress-induced increase in TNF-α production in LPS-stimulated cultures could have been counteracted by the stress-induced increase in corticosterone which was lower in UCP2−/− mice. Thus, TNF-α production of UCP2−/− splenocytes would have been less suppressed than TNF-α production of C57BL/6J splenocytes. While corticosterone is able to suppress TNF-α production, our data show that it also downregulates UCP2. Thus, if we assume that SDR + EPM had a sustained effect on splenocytes via corticosterone, UCP2 would have been downregulated in C57BL/6J splenocytes. Judging from a higher propensity of UCP2−/− mice to produce TNF-α (Vogler et al., 2006), this might result in an increased TNF-α response to LPS. That the TNF-α production in UCP2−/− splenocytes after SDR + EPM is higher than in C57BL/6J splenocytes could then be explained by the short-term influence of corticosterone on C57BL/6J splenocytes compared to the permanent deficiency of UCP2 in UCP2−/− splenocytes. The increased TNF-α production of UCP2−/− splenocytes after stress may also explain the protective role of UCP2 in chronic-inflammatory diseases by controlling inflammatory cytokine production (Yu et al., 2009b).

UCP2 is moderately to strongly expressed in neurons of brain regions that are essentially involved in HPA axis reactivity and anxiety-like behavior, and in neurons that are distinct sources of the neurotransmitters NA, DA and 5-HT (Richard et al., 1998). While the absence of UCP2 in these regions may account for changes in stress reactivity and anxiety, mechanisms explaining the observed effects are still unknown. However, a correlation between intracellular ROS levels and high anxiety has been found in mice (Bouayed et al., 2009; Hovatta et al., 2005; Kromer et al., 2005; Rammal et al., 2008a; Rammal et al., 2008b). It has been hypothesized that oxidative stress might be responsible for an immune perturbation in highly anxious mice (Rammal et al., 2010). Also, psychological stress induces oxidative stress by reducing antioxidant defenses (Zafir and Banu, 2009). We recently demonstrated that mice carrying alterations in mitochondrial proteins that lead to increased ROS production (Yu et al., 2009a), exhibit increased anxiety and stress reactivity (Gimsa et al., 2009). Indeed, UCP2−/− mice have been shown to have elevated ROS levels (Arsenijevic et al., 2000; Vogler et al., 2006). This is especially interesting as UCP2 is highly expressed in substantia nigra and ventral tegmental area, and dopaminergic neurons there show increased ROS levels in UCP2−/− mice (Andrews et al., 2006). Therefore, UCP2 deficiency might exacerbate anxiety and disturb HPA axis regulation, neurotransmitter turnover and immune responses by increased ROS production in the brain.

Conclusions

In summary, our study suggests that the mitochondrial protein UCP2 modulates anxiety and influences HPA axis reactivity, central serotonergic and dopaminergic activity and adaptive as well as innate immune responses under stressful conditions. Whether the observed effects are in a causal relationship to the expression of UCP2 in stress-related brain regions and there may be linked to the regulation of ROS production by UCP2 remains to be investigated. The altered regulation of stress responses in UCP2−/− mice could indicate that the protective function of UCP2 in chronic inflammatory diseases is especially important under stressful conditions.

Conflict of interests statement

The authors declare that there are no conflicts of interest.

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