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CD44 Deficiency Is Associated with Increased Susceptibility to Stress-Induced Anxiety-like Behavior in Mice

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Abstract CD44 is a cell surface adhesion molecule and its principal ligand is hyaluronic acid (HA), a key component of the brain's extracellular matrix. CD44 levels are decreased in the cerebrospinal fluid (CSF) of depressed individuals, and the CD44 gene has been identified in genome wide association study as a possible risk gene in suicidal behavior. In order to define the pathobiological mechanisms by which CD44 may affect behavior, we investigated the role of CD44 using male CD44 knockout (CD44KO) and wild-type mice that underwent chronic mild stress (CMS). Behavior was characterized using the sucrose preference and forced swim tests, open field, novel object recognition, social preference, and the elevated plus maze tests. Gene expression in hippocampus was evaluated using quantitative real-time PCR. Brain monoamines and their metabolites were assessed by high-performance liquid chromatography and serum HA and IL-1β levels were measured using ELISA and electrochemiluminescence assays. CD44KO mice were more susceptible to stress-induced anxiety-like behavior and displayed increased anhedonia and despair than the wild-type controls. The behavioral phenotype of stressed CD44KO mice was associated with reduced cortical serotonergic and striatal dopaminergic turnover. The hippocampal expression of the receptor for HA-mediated motility (RHAMM) was reduced in the non- stressed CD44KO mice compared with WT mice, in a value similar to that observed in WT mice following exposure to stress. Taken together, our experiments suggest that CD44 plays a key role in stress response in mice.

Keywords CD44 · Hyaluronic acid · Extracellular matrix · Stress · Anxiety · Animal model

R. Barzilay and F. Ventorp contributed equally to the study.

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Introduction

CD44 is a cell surface adhesion molecule found on most mammalian cells (Goodison et al. 1999) including neurons (Glezer et al. 2009), astrocytes (Kaaijk et al. 1997) and microglia (Jones et al. 2000). CD44 functions as a ligand-binding receptor by interacting with the extracellular matrix, especially hyaluronic acid (HA) (Jiang et al. 2011). Various immune and neurodevelopmental functions as well as neuronal axon guidance have been reported to be dependent on

CD44 (Ries et al. 2007). In addition, CD44 expression guides the developmental pathway of glial progenitors in vivo (Liu et al. 2004) and plays a crucial role in neural stem cell migration and differentiation (Rampon et al. 2008). Interestingly, CD44–HA interactions have a role in regulating the recruitment of leukocytes and their functioning in response to physical (nerve transection) (Jones et al. 2000) and psychological stress (Inoue et al. 2009).

Accumulating data indicate that the extracellular matrix plays a major role in the molecular underpinning of synaptic plasticity (Frischknecht and Seidenbecher 2008; Frischknecht et al. 2009; Dityatev et al. 2010; Dityatev and Rusakov 2011; Frischknecht and Seidenbecher 2012). Specifically, HA, a key component of the extracellular matrix in the brain, regulates hippocampal plasticity through direct interaction with L-type calcium channel (Kochlamazashvili et al. 2010). Adhesion molecules, which connect cells to the extracellular matrix, are involved in the neural changes that enable structural plasticity (Theodosis et al. 1994). Adhesion molecules were previously suggested to be involved in anxiety and mood disorders (Sandi 2004; Sandi and Bisaz 2007). In addition, accumulating animal studies indicate that adhesion molecules participate in the response to stress (Redwine et al. 2003; Sandi and Bisaz 2007; Chocyk et al. 2010; Gilabert-Juan et al. 2011; Bisaz and Sandi 2012). Furthermore, genetic studies in suicide victims have shown that genes involved in cell adhesion may be risk genes for suicidal behavior (Thalmeier et al. 2008; Galfalvy et al. 2013). Specifically, CD44 (rs1467558) was identified as a possible risk gene for suicide in a suicide post mortem genome wide association study (Galfalvy et al. 2013). This study further reported decreased CD44 gene expression in Brodmann area BA24 (anterior cingulate) and BA9 (dorsolateral and medial prefrontal cortex) brain areas of suicide attempters compared to controls. Two studies examining cerebrospinal fluid recently suggested the relevance of CD44–HA signaling in psychiatric patients, showing reduced CD44 protein levels in depressed patients (Ditzen et al. 2012) and increased HA levels in patients following a suicide attempt (Ventorp et al. 2016) compared to controls.

To date, the role of CD44 in the neurobiological response to stress has not been defined. Based on the above studies, we hypothesized that brain CD44–HA signaling is involved in mediating vulnerability and/or resilience to stress. We therefore investigated the role of CD44 by comparing CD44 knockout (KO) mice with wild-type controls. The mice underwent chronic mild stress (CMS) and behavioral phenotypes were evaluated. Thereafter, alterations in genes related to CD44–HA signaling, HA, cytokines, and brain monoamines levels were examined.

Materials and Methods

Experimental Design

CD44KO mice, previously developed on the background of DBA/1 mice, were used for all experiments (Nedvetzki et al. 2004). Wild-type (WT) mice, descendants of the littermates of the CD44KO mice, were used as controls. Mice were housed in a group of three to five per cage. All mice were kept in a 12-h light/dark cycle and had access to food and water ad libitum except for specific times at which stress protocol was applied. Behavioral training and testing were completed in the light cycle between 8:00 A.M and 5:00 P.M. All animal experiments and protocols were approved by the Committee for Animal Research at Tel Aviv University, Israel.

Two different mice cohorts were used in the study:

In the first cohort, 103 male mice were used. The experimental groups consisted of the following: WT (n = 24, 12 mice for behavior and 12 mice for PCR), CD44KO (n = 31, 19 mice for behavior and 12 mice for PCR), WT + CMS (n = 25, 12 behavior and 13 PCR) and CD44KO + CMS (n = 23, 16 behavior and 7 PCR). After weaning (3–4 weeks old), mice were divided into separate cages in groups of three to five per cage (same experimental group in each cage). At age 5 weeks, mice either underwent CMS for 4 weeks or remained in their standard "home cage" living conditions. The CMS Protocol was based on Schweizer et al. (Schweizer et al. 2009) and is described in details in Supplementary item 1. Thereafter, mice (n = 7–13 from each group) were sacrificed and brains were processed for hippocampal gene expression analysis using real-time PCR. The rest of the cohort (n = 12–19 from each group) were left for a week without any intervention, and then underwent a battery of behavioral tests as depicted in the Supplementary item 2. Mice were sacrificed 3 days after the last behavioral test for further tissue analysis.

In the second cohort, male mice were used to evaluate the interaction effect of multiple tests, employing the same experimental groups as in cohort 1: WT (n = 13), CD44KO (n = 14), WT + CMS (n = 40) and CD44KO + CMS (n = 36). After weaning, mice underwent either CMS or control protocol as described above. A week later, mice underwent the elevated plus maze (EPM) test to evaluate anxiety-like behavior.

Behavioral Tests

The following tests were conducted: sucrose preference test, forced swim test (FST), open field, novel object recognition test, elevated plus maze (EPM), and social preference test. Open field, novel object recognition, EPM, and social preference tests were automatically measured and analyzed by the Noldus EthoVision as previously described (Barzilay et al.

2011). Two independent viewers blinded to the identity of the mice evaluated the FST manually.

Sucrose preference was evaluated through the 4 weeks of CMS after two training sessions in the week prior to CMS. In each session, the regular water bottle was removed and substituted by two tubes, one containing 40 ml of water and another containing 1 % sucrose solution. The animals were allowed overnight access to the tubes. Thereafter, tubes were removed and the amount of liquid in each tube was measured. Measurement in which a tube was found empty and in which the sawdust beneath was wet on the following day, were considered as missing values and excluded from final calculations.

FST was conducted as previously described (Hascoët and Bourin 2009). Briefly, mice were placed in a cylinder containing water for 7 min; the time spent floating was recorded on the last 5 min of the test. For the open field, mice were put in the arena (50 cm^3) and videotaped for 60 min. Novel object recognition test was conducted according to known protocols (Ibi et al. 2009). Social preference test was evaluated as previously reported using the three-chamber paradigm (Nadler et al. 2004). Social preference was calculated as the ratio between the duration of time spent in the unfamiliar mouse chamber compared to the chamber with the inanimate object. EPM was conducted as previously described (Rodgers and Dalvi 1997). Briefly, mice were allowed to explore the arena for 5 min and their behavior was recorded and analyzed for time spent in the open arm.

Tissue Processing

After mice were decapitated, whole blood was collected and brain tissue dissected to specific anatomical regions including the prefrontal cortex, hippocampus, and striatum. Whole blood samples were centrifuged and serum was separated. Tissue and serum were then kept at −80 °C until further processing.

Real-Time PCR

Brain tissue from hippocampus was homogenized with Trizol reagent followed by RNA isolation with RNeasy mini kit and the RNA was transcribed to cDNA using Super-Script III (Invitrogen, Lidingö, Sweden) following manufacturer's instructions. The qPCR reactions were carried out with TaqMan Universal MasterMix (ABI, Carlsbad, CA, USA). We used the geometric mean of three housekeeping genes as endogenous controls: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hypoxanthine–guanine phosphoribosyltransferase (HPRT), and beta-actin. Analysis was first carried out using the ΔΔCT method (Schmittgen and Livak 2008). The ΔΔCT values for the naïve animals were then averaged and used as the control for the percent control conversion. The sequences of primers employed in this study are detailed in supplementary Fig. 3.

Electrochemiluminescence Based Immunoassay

Serum levels of interleukin-1β (IL-1β) were quantified with an electrochemiluminescence-based immunoassay (MSD, Rockville, MD, USA). Serum HA was assessed by ELISA (R&D Systems Europe, Abingdon, England). Both assays were conducted according to the manufacturer's protocol. The levels of the analytes were above the detection limits in all mice, except for IL-1β; 6 % of samples in the WT group were below detection limit compared to 22.5 % in the CD44KO group. All samples below detection limit were assigned the same value, corresponding to the detection limit.

High-Performance Liquid Chromatography (HPLC)

Mouse brain tissue from prefrontal cortex and striatum was processed and extracts were examined using HPLC to measure 5-hydroxytryptamine (5-HT) and dopamine and the respective metabolites 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA). To assess the 5-HT and dopamine turnover, 5-HIAA/5-HT, and DOPAC/dopamine ratios were calculated and compared between the groups.

Statistical Analysis

The statistical analysis was performed using SPSS Statistics 21 (IBM, Armonk, New York, USA), RKWard v0.60, R v 2.15.3 (rkward.sourceforge.net) and the *ggplot2* package (v1.0.0 http://ggplot2.org/). CD44KO effect (knock-out gene effect) and CMS effect (chronic mild stress treatment) were analyzed with two-way independent ANOVA. Significant interaction effects were further resolved with Student's *T* test followed by Bonferroni correction (four comparisons: WT to CD44KO; WT + CMS to CD44KO + CMS; WT to WT + CMS; CD44KO to CD44KO + CMS). To determine the strength of both linear and non-linear relationships between data sets, correlations were assessed using Spearman's rank correlations.

Results

Behavioral Endophenotypes in CD44 KO Male Mice

Gross Motor Function

There were no differences in total distance traveled between the groups of mice, as analyzed in the open-field arena (two-

way ANOVA, NS) as well as in the novel object recognition test, EPM, and social preference tests (data not shown).

Depressive-Like Behaviors

We analyzed sucrose preference as a measure of anhedonic-like behavior. Both CD44KO mice and mice exposed to CMS showed significantly lower preference for sucrose than WT mice (Fig.1a , $p = 0.048$ (two-way ANOVA, CD44KO effect) and $p = 0.038$ (CMS effect). The sucrose preference of WT mice was 66.6 ± 12.4 % (mean ± SD) whereas CD44KO mice and CMS exposed WT mice only showed a minor preference for sucrose, CD44KO: 60.10 ± 16.3 % and CMS WT: 59.6 ± 19.9 %. In the CD44KO mice that were subjected to CMS, the preference for sucrose has been extinguished (47.7 ± 18.3 %).

In the FST, there was a strong trend for genotype × CMS interaction ($p = 0.054$). Post hoc tests indicated that immobility was significantly higher in the CD44KO group (171.74 ± 35.27 s; mean ± SD) compared to the WT group (100.26 ± 37.58 s, Student's *T* test with Bonferroni correction, $p = 0.004$). CMS treatment had no significant effect on immobility compared to WT (WT + CMS, 131.48 ± 59.65 and CD44KO + CMS, 143.79 ± 49.69 s).

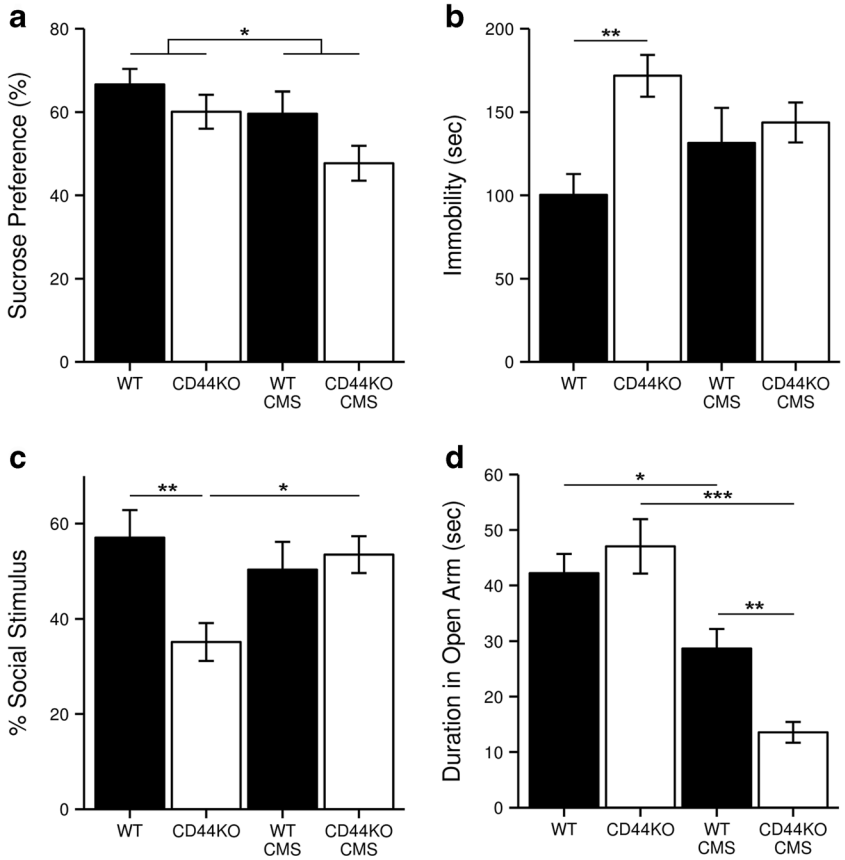

Fig. 1 The effects of CD44KO and chronic mild stress (CMS) on depression-like behavior of male mice. **a** Sucrose preference test. Results represent the intake of sucrose-containing liquid out of the total liquid intake measured over four weekly measurements conducted in each cage. There was a main effect of both genotype ($F_{(1,56)} = 4.09$, $p = 0.048$) and CMS ($F_{(1,56)} = 4.53$, $p = 0.038$) but no significant genotype × CMS interaction. **b** Forced swim test. The time spent immobile represent despair. The tests were discontinued if mice were exceptionally poor swimmers and excluded from the analysis. CD44KO had an effect on the immobility (floating time) ($F_{(1,35)} = 8.21$, $p = 0.008$) but there was a strong trend for genotype × CMS interaction ($F_{(1,35)} = 4.12$, $p = 0.054$). Post hoc tests indicated that immobility was significantly higher in CD44KO compared to WT (Student's *T* test with Bonferroni correction, $p = 0.004$). Treatment groups: WT, $n = 11$; CD44KO, $n = 16$); WT + CMS, $n = 14$; CD44KO + CMS, $n = 19$. **c** Social preference test. There was a strong trend for genotype × CMS interaction effect between CD44KO and CMS ($F_{(1,49)} = 4.00$, $p = 0.051$). CD44KO animals preferred the inanimate stimulus over the social stimulus compared to WT (Student's *T* test with Bonferroni correction, $p = 0.012$) but after CMS treatment, this preference disappears. A few mice were excluded from video analysis due to data-file difficulties. **d** Elevated plus maze test. There was a significant genotype × CMS interaction ($F_{(1,55)} = 6.31$, $p = 0.015$). Specifically, both WT + CMS and CD44KO + CMS mice showed significantly more anxiety-like behavior compared to WT and CD44KO, respectively (Student's T test with Bonferroni correction, $p = 0.048$ and $p < 0.001$). However, CD44KO + CMS and spent significantly less time in the open arm compared to WT + CMS mice ($p = 0.002$). In all figures, *bars* represent the mean ± SEM, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. The mice were exposed to CMS 1 week prior to behavioral testing. All the data were analyzed using two-way ANOVA. Post hoc analyses were carried out when indicated (Student *T* test with Bonferroni correction)

Cognition

In the novel object recognition test, no differences were observed in the preference toward the novel object following either 3- or 24-h retention between all experimental groups. Thus there was no evidence for any effects on recognition memory in any of the experimental groups (NS, supplementary item 4).

Anxiety-Like Behavior

In the three-chamber social preference test, CD44KO animals preferred the inanimate stimulus over the social stimulus compared to WT (0.35 ± 0.17 ratio vs. 0.57 ± 0.20 ratio, mean ± SD ratio represents time spent near social/ time spent near inanimate stimulus), but after CMS treatment, this preference in the CD44KO group disappeared (WT + CMS: 0.50 ± 0.19 and CD44KO + CMS: 0.53 ± 0.15, Fig.1c).

Both WT and CD44KO mice showed more anxiety-like behavior following CMS exposure compared to WT left in home cage conditions, in the EPM test, as observed by decreased duration of time spent in the open arm (Fig.1d). The stressed CD44KO mice spent the shortest time in the open arm (13.5 ± 7.5 s, mean ± SD) compared to all groups. This was significantly less than the WT + CMS mice (28.7 ± 12.2 s), as well as the WT (42.2 ± 12.0 s) and CD44KO mice (47.0 ± 21.4 s). Thus, CD44KO mice display an increased susceptibility to CMS-induced anxiety ($p = 0.002$). Analysis of interaction revealed genotype × stress interaction (Fig.1d).

CD44-Dependent Anxiety-like Behavior Following Chronic Mild Stress Was Not Affected by Multiple Tests Interactions

Several behavioral tests were performed sequentially in the mice as described above, which might introduce a component of stress due to serial testing; we therefore included a naive cohort of mice to confirm the effect of genotype and stress on anxiety-like behavior. Hence, mice were subjected to CMS and underwent EPM testing only. We found that, consistent with the previous cohort, CD44KO mice were susceptible to the CMS protocol. The stressed CD44KO spent the shortest time in the open arm of the EPM (29.4 ± 19.8 s, mean ± SD) compared to CD44KO mice that were not exposed to stress (48.2 ± 26.6 s), and compared to the WT mice exposed to stress (55.8 ± 30.1 s). In WT mice, the CMS protocol did not significantly affect anxiety-like behavior in the EPM. Analysis of interaction revealed genotype × stress interaction consistent with the first cohort (Fig.2).

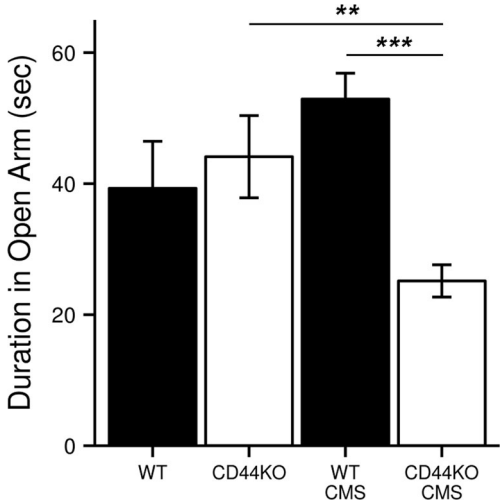

Fig. 2 The effects of CD44KO and chronic mild stress (CMS) on anxiety-like behavior of male mice without the effect of multiple behavioral testing. Mice were exposed to CMS and only subjected to one behavioral test: the elevated plus maze. EPM test was conducted a week following the end of the CMS protocol. There was a significant genotype × CMS interaction ($F_{(1,99)} = 9.214$, $p = 0.003$). Specifically, CD44KO + CMS spent significantly less time in the open arm compared to control CD44 mice without CMS and compared to WT + CMS mice. All the data were analyzed using two-way ANOVA. $^{**}p < 0.01$, $^{***}p < 0.001$. The mice were exposed to CMS one week prior to behavioral testing. All the data were analyzed using two-way ANOVA. Post hoc analyses were carried out when indicated (Student's *T* test with Bonferroni correction)

Tissue Analysis

Analysis Gene Expression in the Hippocampus of Mice immediately Following CMS

We evaluated the expression of CD44–HA signaling genes in the hippocampus to investigate differential effect of stress in the absence of CD44. We found that RHAMM (receptor for hyaluronan-mediated motility, Fig.3a), and L-type calcium channel-1C (Fig.3b) were significantly affected by stress, as was observed by a decrease in RHAMM expression and an increase in calcium channel gene expression. Importantly, there was a genotype × stress interaction in RHAMM expression. There was a main effect of genotype on the HA synthesizing enzyme HAS2 (Fig.3c) and on the pro-inflammatory IL-1β gene (Fig.3d), as expression of both was decreased in the CD44KO mice, with no observed effect of stress. We found no statistically significant effect of either CD44KO or CMS on genes related to hippocampal plasticity (BDNF and MMP-9, Fig.3e–f) and on other genes encoding for adhesion molecules which interact with HA (Tenascin-C, Tenascin-R and Brevican, Supplementary Fig. 5).

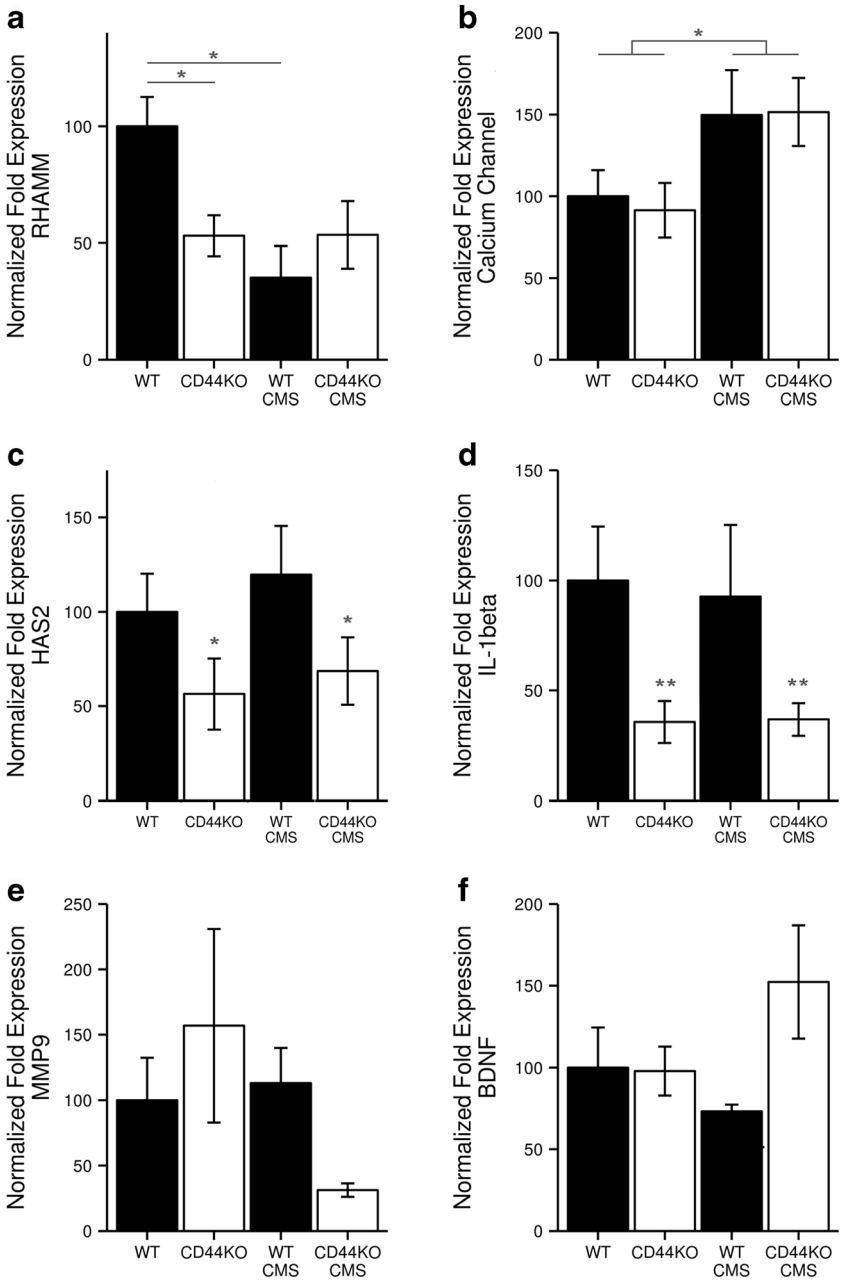

Fig. 3 Analysis of gene expression in hippocampus of mice immediately following CMS using Real-Time quantitative PCR. mRNA expression of each gene is represented in the mean percentage ± SEM as compared to baseline hippocampal expression in the WT mice living in home cage rearing condition without CMS procedure. **a** RHAMM expression, there was a significant stress effect ($F_{(1,23)} = 6.868$, $p = 0.0153$) and a significant genotype × CMS interaction ($F_{(1,23)} = 7.028$, $p = 0.0143$). **b** L-type calcium channel expression, there was a significant stress effect ($F_{(1,39)} = 6.115$, $p = 0.0177$). **c** HAS2 expression, there was a significant genotype effect ($F_{(1,28)} = 4.346$, $p = 0.046$). **d** IL-1β expression, there was a significant genotype effect ($F_{(1,20)} = 8.189$, $p = 0.0087$). **e–f** No significant main effects were found on MMP-9 and BDNF expression. All the data were analyzed using two-way ANOVA. *$p < 0.05$, **$p < 0.01$. Post hoc analyses were carried out when indicated. (Student *T* test with Bonferroni correction)

Analysis of HA and Il-1β 3 Weeks After CMS

The serum levels of the CD44 ligand HA were significantly higher in the CD44KO mice ($p = 0.002$, Fig.4a) and levels of the cytokine IL-1β ($p < 0.001$) was lower in CD44KO mice compared to WT mice (Fig.4b). There was no significant effect of CMS exposure on the serum levels of HA or IL-1β. The IL-1β mRNA expression was decreased in hippocampus of CD44KO animals more than 3 weeks after the CMS in a similar manner as was observed right after the CMS (Fig.4c).

Brain Monoamine Turnover

Cortical and striatal levels of the monoamines 5-HT and dopamine and their respective metabolites 5-HIAA, DOPAC and HVA are shown in Table 1.

Serotonin

CMS-exposed animals had a lower turnover of 5-HT (5-HIAA/5-HT ratio) in the prefrontal cortex compared to WT (CMS effect, $p = 0.003$, Fig.5a). The decrease in 5-HT

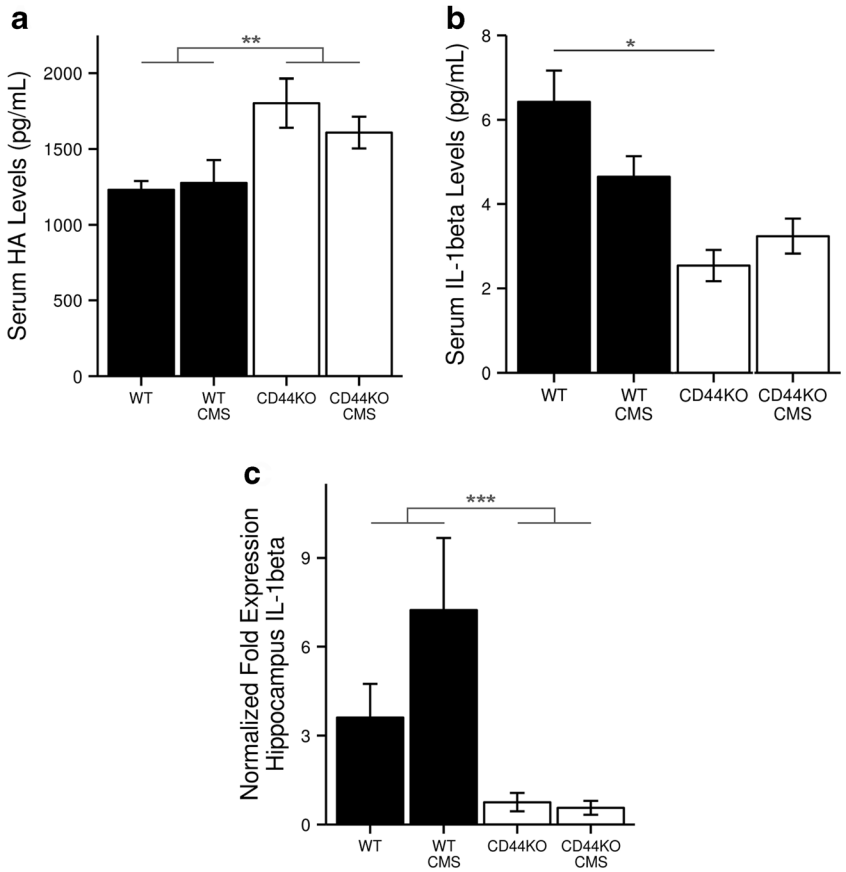

Fig. 4 **a** Hyaluronic acid (HA) levels in serum in ng/mL. CD44KO mice had significant higher levels than WT mice (Two-way ANOVA, genotype effect, $F_{(1,54)} = 10.48$, $p = 0.002$). Treatment groups: WT, $n = 12$; CD44KO, $n = 19$; WT + CMS, $n = 11$; CD44KO + CMS, $n = 16$. **b** Concentrations (pg/ml) of the pro-inflammatory cytokine IL-1β in serum. IL-1β levels were significantly lower in CD44KO mice compared to WT mice. IL-1β also showed a significant genotype × CMS interaction, $F_{(1,54)} = 6.15$, $p = 0.016$, but Bonferroni post hoc test indicated an effect of genotype only ($p < 0.001$). **c** mRNA expression of IL-1β in hippocampus following behavioral tests, more than 3 weeks after the CMS protocol. There was genotype effect (Two-way ANOVA, $F_{(1,55)} = 17.65$, $p < 0.000$). Treatment groups: WT, $n = 12$; CD44KO, $n = 19$; WT + CMS, $n = 11$; CD44KO + CMS, $n = 16$. Bars represent the mean ± SEM. All the data were analyzed using two-way ANOVA. Post hoc analyses were carried out when indicv; 0.05, $^{**}p < 0.01$, $^{***}p < 0.001$

turnover was even more pronounced in CD44KO + CMS mice (0.93 ± 0.51 ratio mean ± SD) than in WT + CMS mice (0.44 ± 0.05, $p = 0.040$).

Furthermore, we found a positive correlation between the 5-HT turnover values and time spent in the open arm of EPM (Spearman's rho = 0.33, $p = 0.025$), indicating a significant association of the decreased 5-HT turnover with anxiety-like behavior. There were no changes of 5-HT or its turnover ratio in the striatum.

Dopamine

Both dopamine and DOPAC levels were decreased in the PFC of CD44KO mice compared to WT mice (DA: $p = 0.037$, DOPAC: $p = 0.014$). There were no group differences in DOPAC/dopamine ratio (dopamine turnover).

There was a negative correlation between dopamine levels in PFC and the time spent immobile in the FST (Spearman's rho = −0.47, $p = 0.009$) and a positive correlation between dopamine levels and the time spent in the open arm (Spearman's rho = 0.31, $p = 0.035$). Thus, the reduced dopamine levels were significantly associated with both despair-like and anxiety-like symptoms in the mice.

In the striatum, dopamine turnover (DOPAC/dopamine ratio) was decreased in mice exposed to CMS (Fig.5b). There was also a positive correlation between dopamine turnover and the time spent in the open arm (Spearman's rho = 0.44, $p = 0.030$).

Discussion

In the current study, we found that CD44 plays a role in mediating the response to stress in male mice, as manifested by increased anxiety- and depressive-like behavior in CD44KO mice compared to wild-type controls. Moreover, we show that

Table 1 Absolute levels of the monoamine neurotransmitters 5-HT and dopamine and their respective metabolites 5-HIAA, DOPAC, and HVA in the prefrontal cortex and striatum (in nanogram per milligram of protein)

Structure	Analyte (ng/mg protein)	Mean ± SD			
		WT	CD44KO	WT + CMS	CD44KO+ CMS
Prefrontal cortex (n = 12, except WT + CMS n = 11)	5-HT$^{\dagger, CMS}$	6.5 ± 1.7	5.7 ± 1.6	6.8 ± 1.8	7.7 ± 1.0
	5-HIAA$^{\dagger, CMS}$	6.2 ± 2.4	6.2 ± 2.4	5.6 ± 2.1	3.4 ± 0.4**
	Dopamine^{CD44KO}	6.9 ± 4.5	5.2 ± 5.1	6.0 ± 4.3	2.3 ± 3.1*
	DOPAC$^{\dagger, CD44KO}$	2.0 ± 1.6	1.7 ± 1.0	2.8 ± 2.7	0.6 ± 0.4
	HVA	1.5 ± 0.8	1.6 ± 0.5	1.3 ± 1.0	1.0 ± 0.7
Striatum (n = 6 in each group)	5-HT	6.4 ± 1.4	5.4 ± 0.6	9.6 ± 7.0	5.9 ± 0.9
	5-HIAA$^{\dagger, CD44KO}$	6.6 ± 0.2	5.8 ± 1.0	7.6 ± 2.8	4.9 ± 1.0
	Dopamine$^{\dagger, CMS*CD44KO}$	131.4 ± 10.4	129.7 ± 12.6	133.8 ± 30.1	177.4 ± 25.9**
	DOPAC$^{\dagger, CMS}$	34.3 ± 11.7	30.5 ± 14.3	26.1 ± 13.8	14.0 ± 2.5*
	HVA	15.4 ± 1.3	13.8 ± 2.7	14.2 ± 1.5	14.4 ± 1.0

$^{\dagger}p < 0.05$ level of significance one-way ANOVA, ^{CMS} and ^{CD44KO} indicate significant CMS effect (two-way ANOVA) and genotype effect, respectively. ^{CMS*CD44KO} indicates significant interaction effect. Dopamine in prefrontal cortex showed a trend toward significance (p = 0.058, one-way ANOVA). Dunnett's post hoc tests, $^{*}p < 0.05$, $^{**}p < 0.01$. Dunnett's t tests treat one group (WT) as a control, and compare all other groups against it

lack of CD44 gene interacts in a statistically significant manner with exposure to stress, manifested by increased sensitivity to develop anxiety-like behavior. Together, the data confirms our hypothesis that CD44 is involved in the neurobiological response to stress. Our study thus supports the notion that CD44–HA signaling is associated with psychiatric phenotypes, as was suggested in three studies in human patients (Ditzen et al. 2012; Galfalvy et al. 2013; Ventorp et al. 2016).

The most robust behavioral indication for CD44KO × stress interaction effect was observed in the EPM test measuring anxiety-like behavior. In the first cohort that underwent multiple testing prior to the EPM, mice that underwent CMS displayed increased anxiety-like behavior, and this was further exaggerated in the CD44KO mice. However, in the second cohort, during which the mice were only subjected to EPM (without prior FST, OF, and NORT testing), neither CD44KO nor CMS were sufficient to induce anxiety effect. Nonetheless, combination of CD44KO with exposure to CMS treatment resulted in an amplified anxiety effect. The EPM results support the notion that CD44KO mice have attenuated resilience to environmental stressful conditions.

We found several biochemical alterations in the KO mice that were associated with the behavioral changes. Both the prefrontal serotonin turnover (5-HIAA/5-HT ratio) and striatal dopamine turnover (DOPAC/DA ratio) were decreased in

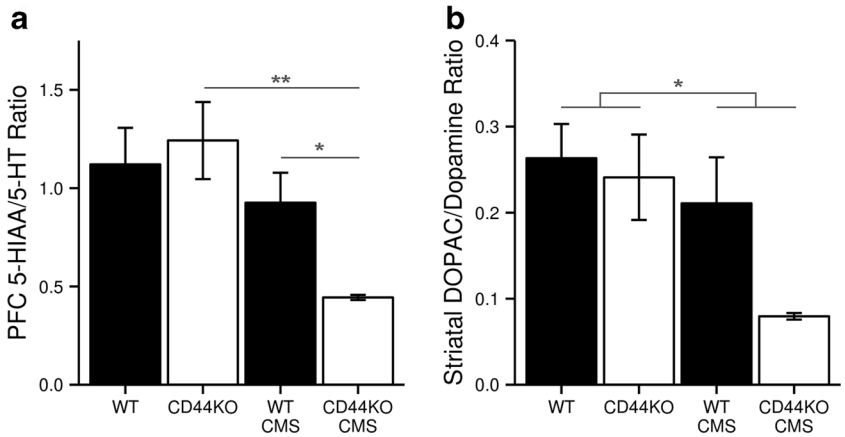

Fig. 5 Monoamine turnover in PFC and striatum. **a** The ratio of 5-HIAA/5-HT in prefrontal cortex (5-HT turnover) in mice. There was an effect of CMS (two-way ANOVA, $F_{(1, 43)} = 10.12$, $p = 0.003$). However there was also a strong trend that failed to reach significance for CD44KO and CMS interaction (two-way ANOVA, $F_{(1, 43)} = 3.76$, $p = 0.059$). There was a significant difference between WT + CMS and CD44KO + CMS (Student's T test with Bonferroni correction, $p = 0.04$), **b** The ratio of DOPAC/dopamine in striatum (dopamine turnover), CMS treatment lowered the dopamine turnover in the striatum (two-way ANOVA, CMS effect, $F_{(1, 20)} = 6.62$, $p = 0.018$, $^{*}p = 0.018$). There was no genotype × CMS interaction effect (two-way ANOVA, NS). $^{*}p < 0.05$, $^{**}p < 0.01$, *bars* represent the mean ± SEM

mice exposed to CMS. The serotonin turnover was lower in the CD44KO + CMS group compared to the WT + CMS group and correlated with the duration of time spent in the open arm of the EPM. This suggests that the increased vulnerability to stress detected in the CD44KO mice is associated with decreased serotonin turnover. Aberrant monoaminergic turnover has previously been described following exposure to various forms of acute and chronic stress in rodents (Konstandi et al. 2000; Rasheed et al. 2010; Ahmad et al. 2010, 2012). Two other studies conducted in rats also reported an association of decreased cortical serotonin turnover with anxiety-like behavior in the EPM (Tokumo et al. 2006) and decreased striatal dopamine turnover with reduced sucrose preference (Bekris et al. 2005). Hence, we can speculate that in the CD44KO + CMS group, the decreased cortical 5-HT turnover accounts for the increased anxiety-like behavior in the EPM, whereas the extinction of sucrose preference in CD44KO mice under stress is related to decreased striatal dopaminergic turnover. This is the first study that associates CD44 with aberrant monoamine turnover. Interestingly, another cell adhesion molecule, the neuronal cell adhesion molecule (NCAM), has been linked with serotonergic and dopaminergic neurotransmission in rodents. NCAM (−/−) mice show increased anxiety-like behavior and functional alterations in 5-HT receptors (Stork et al. 1999) and it was demonstrated that stress-induced changes in dopaminergic circuitry are mediated through NCAM (Chocyk et al. 2010).

HA-dependent signaling has an established role in mediating inflammation, this signaling involves the two main HA receptors, CD44 and RHAMM. Studies have shown that both CD44 and RHAMM play a complex role in regulating inflammation and tumor suppression (Misra et al. 2015). While most studies on the mechanisms of HA-CD44/RHAMM signaling were conducted in cancer and blood cells, CD44 and RHAMM are robustly expressed in the brain and specifically in the hippocampus (Kaaijk et al. 1997; Lynn et al. 2001). HA-CD44 signaling may also affect brain function through influence on blood–brain-barrier permeability, which was previously shown to be directly affected by CD44 (Flynn et al. 2013). In our study, we found that CD44KO mice have lower hippocampal RHAMM expression, higher serum HA, and lower IL-1ß both in serum and hippocampus, as was previously described in CD44KO mice (Wang et al. 2002). HA is known to suppress IL-1ß production (Baeva et al. 2014). In line with this, we found significant higher levels of HA and lower levels of IL-1ß in serum of CD44KO compared to WT mice. Moreover, we found decreased hippocampal IL-1ß expression in the CD44KO mice, as was previously described in CD44KO mice (Wang et al. 2002). This finding may suggest a mechanism through which serotonin turnover is affected, as IL-1ß activates serotonin transporters (Zhu et al. 2006). Taken together, these findings demonstrate the aberrant neuroimmune-response in the CD44KO mice which may underpin their vulnerability to stress.

The diverse functions of CD44 may affect synaptic plasticity and consequently responses to stress (McEwen and Gianaros 2011). This concurs with the notion that neuron-glia-extracellular matrix signaling mediates synapse dynamics and may play a role in the pathophysiology of psychiatric disorders (Frischknecht and Seidenbecher 2008; Dityatev et al. 2010; Dityatev and Rusakov 2011). Specifically, HA has been reported to affect synaptic plasticity through calcium-dependent signaling in the hippocampus (Kochlamazashvili et al. 2010). In our study, we found that stress indeed significantly affected the hippocampal expression of calcium channel gene. However, the CD44KO trait had no effect on either calcium channel nor on other genes we measured as a proxy-measures of plasticity such as BDNF and MMP-9, which was reported to interact with CD44 and affect synaptic plasticity through mechanical modeling of the extracellular matrix around the synapse (Dziembowska and Wlodarczyk 2012). Also, we did not find significant effect of stress on the expression of genes encoding for other adhesion molecules which interact with CD44 (Tenascin-C, Tenascin-R, and Brevican).

There are some limitations to our study. The mice were subjected to several behavioral tests; hence, preceding tests might have affected the outcome of subsequent tests by increasing the stress burden on the animals. Notably, the strong CD44KO × stress interaction in the EPM test was replicated in a second cohort of mice with no preceding behavioral tests. In addition, no single mechanism is suggested to explain the complex role of CD44 in behavioral and psychiatric disorders, rather several possibilities are identified here, i.e., alterations in inflammatory processes, blood–brain-barrier permeability or neuroplasticity. Examination of the role of specific mechanism of CD44–HA signaling in the development of psychopathology merits further investigation.

To conclude, the current study shows that CD44 knockout genotype interacts with stress to induce robust anxiety-like behavior and decreased cortical serotonin turnover, that may underpin the CD44KO mice impaired resiliency to stress. Recently we reported increased HA levels in the CSF of suicide attempters (Ventorp et al. 2016). Together, these studies may suggest that CD44–HA signaling plays an important role in the brain's capacity to regulate the response to stress. Future studies may focus on CD44–HA signaling components as putative biomarkers for mental distress, as previous studies have shown that levels of certain immune mediators may discriminate between suicide attempters and controls (Janelidze et al. 2011; Lindqvist et al. 2011; Ganança et al. 2016). Furthermore, the CD44 signaling pathway could potentially be considered as a novel target for intervention in stress-related mental disorders.

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Compliance with Ethical Standards

Conflict of Interest All authors declare that there are no relevant conflicts of interest.

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