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FUNCTIONAL ROLE OF CALCIUM-STIMULATED ADENYLYL CYCLASE 8 IN ADAPTATIONS TO PSYCHOLOGICAL STRESSORS IN THE MOUSE: IMPLICATIONS FOR MOOD DISORDERS

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Abstract—The Ca^{2+}/calmodulin stimulated adenylyl cylcase 8 (AC8) is a pure Ca^{2+} sensor, catalyzing the conversion of ATP to cAMP, with a critical role in neuronal plasticity. A role for AC8 in modulating complex behavioral outcomes has been demonstrated in AC8 knock out (KO) mouse models in which anxiety-like responses were differentially modulated following repeated stress experiences, suggesting an involvement of AC8 in stress adaptation and mood disorders. To further investigate the role of this enzyme in phenotypes relevant for psychiatric conditions, AC8 KO mice were assessed for baseline behavioral and hormonal parameters, responses to repeated restraint stress experience, and long-term effects of chronic social defeat stress. The lack of AC8 conferred a hyperactive-phenotype both in home-cage behaviors and the forced swim test response as well as lower leptin plasma levels and adrenal hypertrophy. AC8 KO mice showed baseline "anxiety" levels similar to wild type littermates in a variety of procedures, but displayed decreased anxiety-like responses following repeated restraint stress. This increased stress resilience was not seen during the chronic social defeat procedure. AC8 KO did not differ from wild type mice in response to social stress; similar alterations in body weight, food intake and increased social avoidance were found in all defeated subjects. Altogether these results support a complex role of cAMP signaling pathways confirming the involvement of AC8 in the modulation of stress responses. Furthermore, the hyperactivity and the increased risk taking behavior observed in AC8 KO mice could be related to a manic-like behavioral phenotype that warrants further investigation.

Key words: knock out mice, social stress, hyperactivity, mania, leptin, metabolism.

The ability to cope with environmental changes is fundamental for survival. The functional activation of adaptive responses is aimed at restoring homeostasis, whereas persistent activation of these responses may lead to allostatic overload, predisposing the individual to disease (McEwen, 1998). The nervous system regulates the physiological and behavioral coping responses to daily events and major stressors, and the brain itself is a target of the mediators of these responses through circulating hormones. Experience-driven changes are thought to occur through long-lasting, activity-dependent changes in brain synaptic efficacy; acute and chronic stressor experiences are able to induce long-lasting neuroplastic changes, suggested to be associated with different psychiatric conditions such as major depression, bipolar disorder and drug addiction (reviewed in Calabrese et al., 2009; Kourrich et al., 2007; Manji et al., 2007).

A putative mechanism by which stress could modulate synaptic plasticity involves Ca^{2+}-dependent signal transduction pathways such as Ca^{2+}/calmodulin stimulated adenylyl cyclases, since increased Ca^{2+} influx through voltage-gated channels might influence synaptic modifications such as long-term depression (LTD) (Schaefer et al., 2000). Type 1 and type 8 adenylyl cyclases (AC1 and AC8), the only two Ca^{2+}/calmodulin stimulated adenylyl cyclases expressed in the mammalian brain (Wong et al., 1999), are necessary for different forms of synaptic plasticity (AC1: Wu et al., 1995; Villacres et al., 1998; AC8: Schaefer et al., 2000; Wang et al., 2003). The distinct expression topology of the two enzymes, AC8 having a presynaptic and AC1 a postsynaptic localization (Conti et al., 2007), and the different Ca^{2+} sensitivities (Nielsen et al., 1996) further suggest different roles in the control of synaptic plasticity. Furthermore, AC8 is highly expressed in brain regions (i.e., thalamus, habenula, paraventricular nucleus of the hypothalamus, hippocampal CA1) that are involved in the neuroendocrine and behavioral responses to stress (Xia et al., 1991; Muglia et al., 1999).

AC8 knock-out mice (AC8 KO) have a complete lack of AC8 mRNA and Ca^{2+}-stimulated AC activity in brain regions where AC8 predominates (Schaefer et al., 2000). Interestingly, while no compensation by AC1 seems to occur, AC8 KO mice display significant impairment in hippocampal CA1 LTD, decreased stress-induced phosphorylation of the CREB transcription factor in the hippocampal CA1 area, hippocampal dependent cognitive deficits, decreased anxiety after being challenged with stress and decreased opiate dependence (Schaefer et al., 2000; Zachariou et al., 2008; Zhang et al., 2008). Altogether, these results suggest that AC8 may be involved in the stress-

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Abbreviations: AC, adenylyl cyclase; ACTH, adrenocorticotropic hormone; cAMP, cyclic adenosine monophosphate; DKO, double knock out; FST, forced swim test; GIP, gastric inhibitor polypeptide; HPA, hypothalamo–pituitary–adrenal; KO, knock-out; LTD, long term depression; LTP, long term potentiation; PCR, polymerase chain reaction; PP, pancreatic polypeptide; pYY, polypeptide YY; RIA, radio immuno assay; SEM, standard error of mean; WT, wild type.

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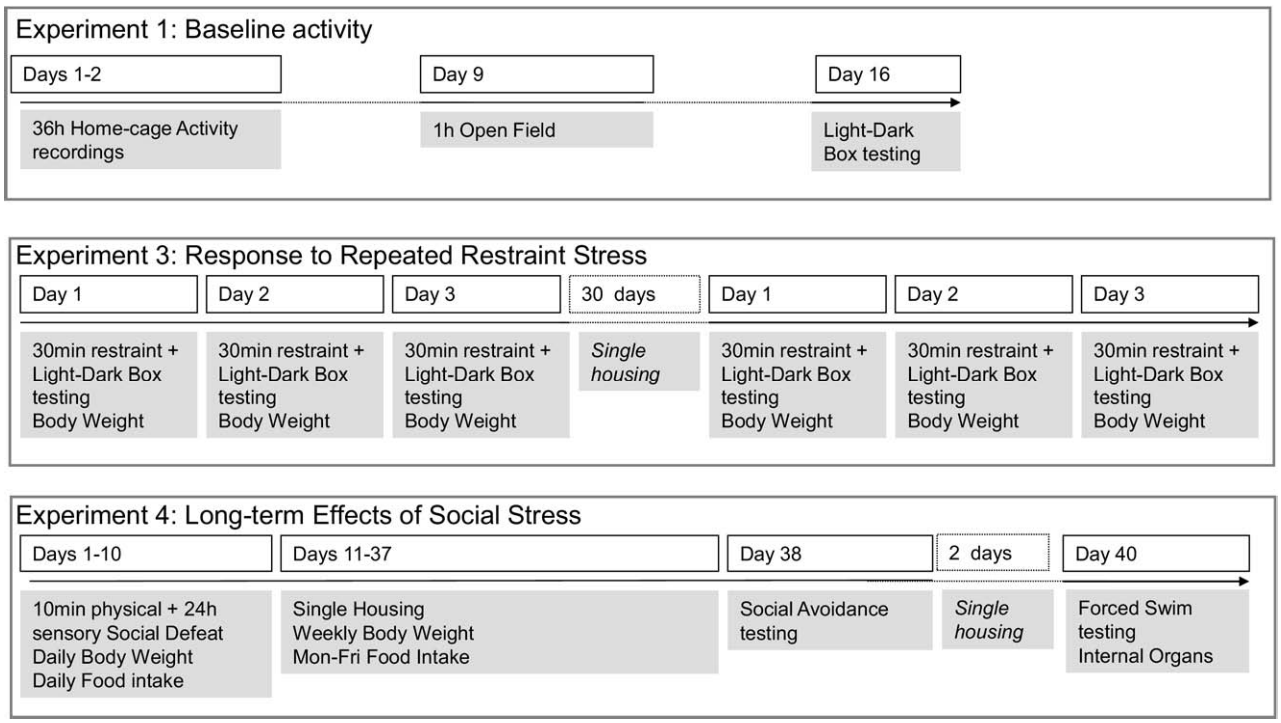

Fig. 1. Experimental procedure (Experiment 2 involved the sampling collection of animals in baseline conditions for peripheral biomarkers assessment and is not represented in this Figure).

coping strategies and, more broadly, in psychiatric conditions associated with impaired synaptic plasticity.

In the present study, the functional consequences of the lack of AC8 were evaluated on a set of behavioral and physiological parameters in baseline conditions as well as following stressful manipulations. For basal characterization, a number of behavioral parameters were evaluated both in novel and familiar environments (i.e. test arenas, home-cage). The consequences of the constitutional lack of AC8 were assessed on endocrine biomarkers reflective of stress as well as metabolic responses. Lastly, stress-related behaviors were tested after repeated restraint and multiple social defeat, the latter considered a chronic psychological stress leading to impaired behavioral and cognitive responses as well as to alterations of hippocampal plasticity (Tornatzky and Miczek, 1993; Von Frijtag et al., 2001; Yap et al., 2006).

EXPERIMENTAL PROCEDURES

Animals

Fully backcrossed homozygous null AC8 mutant male mice, generated and characterized as described by Schaefer et al. (2000), were obtained from Louis J. Muglia, Washington University School of Medicine (St. Louis, MO, USA) and mated with C57BL/6N female mice (Charles River, USA). Heterozygous embryos were extracted and implanted in germ-free CD-1 foster mother mice (Charles River, Italy). Heterozygous null AC8 mutant mice were then mated in order to obtain a colony of homozygous null AC8 mutant mice (AC8 KO) and wild-type littermates (WT). During the breeding, mouse genotype was monitored using a mouse *Adcy8* gene (coding for AC8) specific PCR assay on genomic DNA template isolated from mice tail tips. Mice were housed in individually ventilated cages on a 12 h light/dark cycle (light at 6:00 AM), with free access to food and water.

All experimental procedures were carried out in accordance with Italian law (Legislative Decree no.116, 27 January 1992), which acknowledges the European Directive 86/609/EEC, and were fully compliant with GlaxoSmithKline policy and codes of practice on the care and use of laboratory animals.

General experimental design

Four sets of experiments were conducted in different groups of adult male mice (~4 months of age at the beginning of testing) (see Fig. 1).

In experiment 1, AC8 KO (n=10) and WT (n=9) mice were evaluated for baseline activity in home-cage, open field, and dark–light box test.

In experiment 2, AC8 KO (n=10) and WT (n=9) mice were used for peripheral biomarker evaluation.

In experiment 3, a third cohort of mice (n=12 AC8 KO and n=12 WT) was assessed for stress response and anxiety-related behaviors to repeated testing in the dark–light box test following restraint stress. A further evaluation using the same procedure (restraint stress followed by light–dark box testing) was conducted 1 month later on the same animals.

In experiment 4, additional AC8 KO (n=8) and WT (n=7) mice were subjected to a chronic social defeat procedure, followed by a long-term assessment of behavioral and physiological responses relevant to stress and depressive-/anxiety-like states.

All behavioral procedures, except for the home-cage activity assessment, were conducted between 8.00 AM and 1.00 PM.

Experiment 1—baseline activity

Home-cage activity. Mouse home-cage activity was evaluated by using the LABORAS^{TM} system (Metris b.v., Hoofddorp, The

Netherlands). As previously described (Quinn et al., 2003), the system consists of sensing platforms that convert the animal movements into electric signal, with every movement defined by unique frequency, amplitude and pattern, hence identified into behavioral categories and registered by a computer as the following: locomotion, walking and running (s); climbing, climbing and hanging on the bars of the wire cage lid or food hopper or on the climbing grid in the cage (s); self-grooming, shaking, scratching, wiping or licking of fur, snout, ears, tail or genitals (s); eating (s); drinking (s); and total distance traveled (m).

After about 1 h habituation to the experimental room, mice were placed individually in clean Macrolon® type II cages with wood shavings as bedding and food and water as in their home-cages. Each cage was put on a sensing platform for the automated acquisition of home-cage activity over a period of 36 h, starting at "lights off" (12 h dark phase 1 : 12 h light phase : 12 h dark phase 2).

Open field behavior. Behavioral data were collected using 12 VersaMax Animal Activity Monitors (AccuScan Model RXYZXCM-16, Columbus, OH, USA). Each chamber was 40×40×30.5 cm, made of clear Plexiglas and covered with a Plexiglas lid with air-holes. Infrared monitoring sensors were located every 2.53 cm along the perimeter (16 infrared beams along each side) and 2.5 cm above the floor. Two additional sets of 16 sensors were located 8.0 cm above the floor on opposite sides. Data were collected and analyzed by a VersaMax Analyzer (AccuScan Model CDA-8, Columbus, OH, USA) and were stored on a computer for future analysis.

Animals were transported to the experimental room 1 h before the session-start and then placed individually in the center of the activity chambers for a 60 min recording session. The activity chambers were thoroughly cleansed after each animal. The following variables were quantified: horizontal activity (number of beam breaks in the lower infrared sensors); vertical activity (number of beam breaks in the upper infrared sensors); and total distance traveled (cm).

Light–dark box. The apparatus consisted of an open-topped box 45×27×27 cm, divided into a small (2/5) area and a large (3/5) area by a partition that extended 20 cm above the walls. There was a 7.5×7.5 cm opening in the partition at floor level. The small compartment was painted black and the large compartment white. The white compartment was illuminated by a 75 W bulb and the black compartment by a 60 W red bulb. Nine cm squares were drawn on the floor of the light compartment (nine squares) and on the floor of the black compartment of the apparatus (six squares).

After 1 h adaptation to the dimly illuminated test room, mice were placed individually in the middle of the white area of the apparatus and allowed to explore for 5 min. Mouse behavior was video-recorded for subsequent behavioral analysis by a trained observer, blind to the experimental group. Three parameters were measured: the latency to enter the dark compartment (s), the time spent in the light area (s), and the number of line crossings within the light compartment.

Experiment 2—peripheral biomarkers levels

Sample collection. For this experiment, naive mice of either genetic background were food deprived for 2 h, during which time water was freely available. Samples were collected between 10:00 AM and noon, by sacrificing the experimental subjects by rapid decapitation. Trunk blood (0.5 mL) was collected from each animal in Microtainer K_2-EDTA (Becton Dickinson Italia, Milano, Italy) tubes with addition of 5 μL Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA). The tubes were centrifuged (1900×*g*, 10 min at 4 °C) for plasma separation. Plasma samples were then aliquoted, quickly frozen in dry ice and then stored at −80 °C until use. Glucose levels were measured shortly after sacrifice using a blood glucose meter (EZ Smart, Tyson Bioresearch, ChuNan, Taiwan).

Peripheral biomarkers profiling. All biomarkers were measured using commercially available kits and following the manufacturers' protocols.

Corticosterone concentrations were measured using a radio immuno assay (RIA) kit (RIA Immuchem™ Double antibody 125I RIA kit, MP Biomedical, Billerica, MA, USA).

The plasma levels of insulin, amylin, GIP (gastric inhibitor polypeptide), ghrelin (active, acylated form), leptin, PP (pancreatic polypeptide) and pYY (polypeptide YY) were measured with a Mouse Gut Hormone multiplex panel kit, and adrenocorticotropic hormone (ACTH) by a 3plex Bone Panel, both panels purchased from Millipore (Billerica, MA, USA).

Experiment 3—response to repeated restraint stress

After 1 h acclimation period to the test room, mice were placed individually head-first into a well-ventilated polypropylene tube (2.8 cm diameter×11.5 cm length) for 30 min to deliver restraint. Animals were not physically compressed and did not experience pain. Thirty min following the restraint procedure Light–Dark Box tests were administered as described in Experiment 1. The same experimental procedure was performed for three consecutive days and repeated 1 month later.

Mouse body weight was assessed at the end of each Light–Dark Box test (Fig. 1). Body weight gain data were calculated as differences between the last day of restraint stress and the baseline values (d3–d1).

Behavioral assessments. Mice were tested in the Social Avoidance Test and in the Forced Swim Test to evaluate the long-term behavioral consequences of the social defeat stress that had occurred respectively 28 and 30 days before (Fig. 1).

Social avoidance test. As previously described by Berton et al. (2006), a video-tracking system was used to score approach-avoidance toward an unfamiliar social target. Defeated and Control mice were individually placed in a 45×45 cm arena with an empty wire-mesh cage (10×4.5 cm) located at one end, and their movement was tracked for 2.5 min, followed by 2.5 min in the presence of a confined unfamiliar aggressor; a resident CD-1 male mouse that was introduced into the wire-mesh cage. Between the two sessions, the subject mouse was removed from the arena and placed back into its home-cage for approximately 1 min. The procedure was carried out under red light conditions and video-recordings were performed using a camera equipped with infrared filter. The duration of subject's presence in the "interaction zone" (defined as the 8 cm-wide area surrounding the wire-mesh cage), in the "corners" of the open field opposite to the location of the cage, in the "center" of the open field, and the total distance traveled (cm) was obtained using Ethovision XT software (Noldus Information Technology, Wageningen, The Netherlands).

Forced swim test (FST). Mice were individually placed in an open cylindrical glass container (diameter 10 cm, height 25 cm), containing 10 cm of water at 25±1 °C, for a 6-min duration. The water was changed before the introduction of each animal.

Mouse behavior was video-recorded by a video-camera placed in front of the cylinders. Behavior was scored during the last 4 min of the 6 min test by a trained observer using The Observer XT 7.0 software (Noldus Information Technology, Wageningen, The Netherlands). The following behavioral measures, quantified as duration (s), were included: climbing (upward directed movement of the forepaws along the walls of the cylinder); swimming (horizontal movements throughout all radius of the cylinder); and floating (minimal activity required for the mouse to keep its head above water level).

Internal organ weight. Shortly following the completion of the FST, mice were killed rapidly by decapitation. At autopsy,

internal organs such as testis, seminal vesicles, spleen, adrenals, and thymus, were dissected and weighed (organ weight is presented as relative weight, i.e. absolute weight/body weight).

Experiment 4—long-term effects of social stress

Chronic social defeat stress. Defeat stress was performed using a method similar to that described by Berton et al. (2006). Fifteen CD-1 mice selected on the basis of their attack latency consistency (shorter than 30 s on three consecutive screening tests) were used as aggressive residents. For the social defeat stress, experimental subjects were exposed to an unfamiliar CD-1 resident mouse each day for a 10 min full interaction over 10 days. During this exposure all subject mice showed signs of subordination. After the 10 min unrestricted interaction, subject mice were separated from the aggressive resident by placing a perforated Plexiglas divider with holes into the resident home-cage and were housed in that way for the next 24 h, with food and water provided *ad libitum*. Control mice were housed in pairs, separated by the perforated Plexiglas divider, and handled daily.

Body weight, food intake and feed efficiency. Body weight measures were taken at multiple time points (see Fig. 1).

In all experiments animals were weighed 3 days before the start of the procedure to ensure a balanced distribution between groups.

On experimental days 1 to 10, mice were weighed immediately before being exposed to the social defeat procedure. Additional body weight measures were taken during the weekly change of the home-cage. Finally, animals were weighed 30 days following the last social defeat exposure. Body weight gain data were calculated as differences between the last day of social defeat and the baseline value (d10-d1) and between all body weight measurements during the single housing phase and the value at the end of the social defeat (d19-d10, d26-d10, d33-d10, d40-d10).

Food intake was assessed daily throughout the social defeat procedure (days 1–10) and daily Monday to Friday, from experimental day 11th to 40th, when chow was removed from the food hopper, weighed, and replaced. To minimize food spill, only food pellets weighing more than 5 g were used for replacing the amount of chow available in the food hopper.

Feed efficiency was calculated as body mass gained (g) / cumulative food intake (g) during either the social defeat phase or the single housing phase.

Statistical analysis

Statistical analyses were conducted using Statistica V8 (Statsoft, Inc., Tulsa, OK, USA). When necessary, data were log transformed to satisfy ANOVA's assumptions. Genotype (AC8 KO vs. WT) was the between-subjects factor for all analyses and Social Stress (defeated vs. control) was the additional between-subjects factor for all analyses performed for Experiment 4.

Experiment 1—One-way ANOVA was conducted for data from Open Field and Light–Dark Box tests. Two-way ANOVA containing the within-subjects factor, Light–Dark Phase, was used to analyze data from the Home-cage activity test.

Experiment 2—One-way ANOVA was conducted for peripheral biomarkers data.

Experiment 3—ANOVA for repeated measures, with one within-subject Factor (test day), was used to analyze Light–Dark Box test following restraint data and body weight differences between the first and the final restraint stress days.

Experiment 4—Body weight gain and food intake data were analyzed with two-way ANOVA for repeated measures, with one within-subject Factor (test day). Similarly, social avoidance data were analyzed with two-way ANOVA for repeated measures, with one within-subject Factor (test phase). Feed efficiency, FST and internal organs data were analyzed by means of 2-way ANOVA. When significant main effects or interactions between main factors were highlighted by ANOVA, multiple group comparisons were calculated using Tukey's HSD test.

All results are expressed as mean±standard error (SEM) of raw data.

Differences with P-values<0.05 were considered significant.

RESULTS

Experiment 1—baseline activity

Home-cage activity. Home-cage activity parameters differed significantly between AC8 KO and WT mice (Table 1). The total distance traveled was significantly changed by Genotype ($F_{(1,14)}=10.86$; $P<0.01$), Dark–Light Phase ($F_{(2,28)}=90.01$; $P<0.0001$), as well as their interaction ($F_{(2,28)}=6.98$; $P<0.01$). AC8 KO mice covered a significantly longer distance than WT in the dark phase, (dark phase 1, $P<0.05$; dark phase 2, $P<0.01$), while they did not differ from WT subjects during the light phase. Mouse locomotor activity was significantly altered by Genotype ($F_{(1,14)}=8.72$; $P<0.05$), Dark–Light Phase ($F_{(2,28)}=86.74$; $P<0.0001$), as well by Genotype×Dark–Light Phase ($F_{(2,28)}=5.56$; $P<0.01$), with AC8 KO mice values exceeding those of WT during both dark phases ($P<0.05$ each).

Climbing was significantly influenced by both Dark–Light Phase ($F_{(2,28)}=38.87$; $P<0.001$) and the interaction Genotype×Dark–Light Phase ($F_{(2,28)}=3.94$; $P<0.05$), with AC8 KO showing significantly more climbing than WT mice, mostly during the second dark phase ($P<0.05$).

For the each of the remaining behaviors, AC8 KO did not differ from WT subjects.

Table 1. Home-cage activity characterization over 36 h

	12 h Dark phase 1		12 h Light phase		12 h Dark phase 2	
	WT	AC8 KO	WT	AC8 KO	WT	AC8 KO
Total distance (m)	135.6±8.4	200.7±20.0*	23.3±1.9	23.1±2.7	101.1±13.8	182.0±22.6**
Locomotor activity (s)	2467.0±184.9	3787.8±408.6*	363.3±27.9	438.8±55.2	1756.7±258.5	3065.2±438.1*
Climbing (s)	3722.3±677.1	4479.8±931.6	48.4±15.3	75.9±33.2	2445.8±530.4	5252.0±862.2*
Grooming (s)	4262.0±357.9	3525.2±397.4	2725.3±127.6	2430.4±264.6	4779.9±518.0	3542.8±674.2
Drinking (s)	148.4±72.0	87.1±43.7	18.8±6.2	20.5±5.4	144.3±66.1	92.4±43.1
Eating (s)	1816.6±556.2	1728.5±613.8	432.0±189.1	528.2±258.4	1257.0±456.6	2392.9±516.9

Values represent group mean±SEM.

* $P<0.05$, ** $P<0.01$ Tukey's HSD post hoc tests between AC8 KO ($n=10$) and WT ($n=9$) within each of the three home-cage activity observation phases.

Open field test. No differences were found between AC8 KO and WT mice in any of the parameters (Horizontal Activity counts: WT=14840.3±757.5, AC8 KO=15604.5±1098.6, $F_{(1,17)}$=0.31; n.s.; Vertical Activity counts: WT=1240.3±145.2, AC8 KO=1024.3±94.4, $F_{(1,17)}$=1.37; n.s.; Total Distance: WT=8324.2±452.6 cm, AC8 KO=9749.7±880.3 cm, $F_{(1,17)}$=1.72; n.s.).

Light–dark box test. No differences were found between AC8 KO and WT mice for any of the parameters considered (latency to enter the dark compartment: WT=29.3±6.6 s, AC8 KO=32.1±6.7 s, $F_{(1,17)}$=0.09; n.s.; time in the light area: WT=79.0±6.3 s, AC8 KO=62.4±7.5 s, $F_{(1,17)}$=2.78; n.s.; number of line crossings within the light compartment: WT=47.4±5.3, AC8 KO=39.5±6.4, $F_{(1,17)}$=0.89; n.s.) (Fig. 2—baseline).

Experiment 2—peripheral biomarker levels

Glucose levels were similar in AC8 KO and WT littermate mice (Table 2).

No differences were found in hormonal levels (Table 2) between the two groups, except for leptin levels, that were significantly lower in AC8 KO with respect to WT mice ($F_{(1,17)}$=4.70; P<0.05). PYY and ACTH levels could not be determined since they fell below the detection threshold.

Experiment 3—response to repeated stress

AC8 KO and WT mice were subjected to a 3-day restraint stress and anxiety testing (Light–Dark Box) in two sessions that were carried out 1 month apart from one another, during which no behavioral testing was conducted (see Schaefer et al., 2000).

The latency to enter the dark compartment was significantly longer in AC8 KO than WT mice ($F_{(1,44)}$=4.79; P<0.05) (Fig. 2A). In general mice showed significantly shorter latencies to enter the dark compartment due to the familiarity with the procedure (Test Session: $F_{(1,44)}$=11.59; P<0.01; Day of Testing: $F_{(2,88)}$=11.73; P<0.0001). Overall, AC8 KO mice spent a significantly longer time in the light compartment than WT ($F_{(1,44)}$=6.76; P<0.05) (Fig. 2B), with no influence of either Test Session ($F_{(1,44)}$=1.47; n.s.) or Day of Testing ($F_{(2,88)}$=1.13; n.s.).

AC8 KO mice had significantly higher locomotor activity values (i.e. line crossings count) than WT ($F_{(1,44)}$=4.38; P<0.05) (Fig. 2C), whereas neither Test Session nor Day of Testing had a significant contribution ($F_{(1,44)}$=1.01; n.s.; $F_{(2,88)}$=0.59; n.s.).

Lastly, Genotype significantly influenced the body weight gain measured at the end of the repeated restraint/Light–Dark Box procedure ($F_{(1,22)}$=5.54; P<0.05), with greater body weight loss in WT than in AC8 KO mice (Session 1: WT: −0.51±0.17 g; AC8 KO: −0.18±0.12 g; Session 2: WT: −0.69±0.17 g; AC8 KO: −0.13±0.18 g).

Experiment 4—long-term effects of social stress

Body weight, food intake and feed efficiency. At the beginning of this experimental procedure AC8 KO were found to weigh significantly less than WT mice (24.58±0.43 g and 27.24±0.49 g respectively) ($F_{(1,23)}$=16.67; P<0.001).

Overall, at the end of the social defeat, defeated subject gained significantly more weight than controls ($F_{(1,19)}$=17.95; P<0.001) (Fig. 3A). No significant effects were found due to either Genotype ($F_{(1,19)}$=0.001; n.s.) or its interaction with Social Stress ($F_{(1,19)}$=0.07; n.s.).

During the single housing phase (Fig. 3B), defeated mice gained significantly less body weight than controls ($F_{(1,19)}$=6.12; P<0.05), irrespective of Genotype ($F_{(1,19)}$=0.31; n.s.), but with a close to significant Genotype×Social Stress interaction ($F_{(1,19)}$=4.14; P=0.06). Specifically, while defeated WT mice gained significantly less weight compared to their control (P<0.05), no such difference was observed within AC8 KO mice. Mouse body weight values increased significantly due to Time ($F_{(3,57)}$=5.78; P<0.01) and were also significantly affected by the Time×Genotype interaction ($F_{(3,57)}$=4.27; P<0.01), but not by the Time×Social Stress interaction ($F_{(3,57)}$=0.84; n.s.). While AC8 KO maintained similar body weight gain values throughout the single housing phase, in general WT subjects showed significantly higher final body weight values compared to the start of the procedure (d33-10: P<0.001; d40-10: P<0.05).

At the end of the social defeat phase, food intake was significantly increased in defeated subjects compared to controls ($F_{(1,19)}$=34.02; P<0.0001) (Fig. 4); Genotype ($F_{(1,19)}$=0.38; n.s.) or the Social Stress×Genotype interaction ($F_{(2,19)}$=0.83; n.s.) had no effect. Food intake was also significantly increased by Time ($F_{(9,171)}$=2.07; P<0.05); furthermore a significant Time×Social Stress interaction ($F_{(9,171)}$=3.84; P<0.001) was observed.

During the single housing phase, food intake levels were significantly influenced by Social Stress as well as Genotype ($F_{(1,19)}$=20.48; P<0.001, $F_{(1,19)}$=7.17; P<0.05, respectively); defeated subjects and AC8 KO mice consumed more food than control and WT subjects respectively (Fig. 4). Both Time and the Time×Social Stress interaction had a significant effect on food intake levels (respectively: $F_{(3,57)}$=23.15; P<0.0001; $F_{(3,57)}$=4.89; P<0.01), since defeated mice of either genotype returned to control levels of food intake soon after the end of the social defeats.

Social Stress significantly increased the feed efficiency ($F_{(1,19)}$=18.30; P<0.001) (Fig. 4B) independent of their Genotype ($F_{(1,19)}$=0.015; n.s.) or the interaction Social Stress×Genotype ($F_{(1,19)}$=0.05; n.s.). During the single housing phase, Social Stress tended to influence this index ($F_{(1,19)}$=4.11; P=0.06) (Fig. 4B), with control mice feed efficiency values slightly higher than defeated subjects. No effects of Genotype ($F_{(1,19)}$=0.27; n.s.) or the interaction Social Stress×Genotype ($F_{(1,19)}$=1.83; n.s.) were revealed.

Behavioral assessments

Social avoidance test. The presence in the "interaction zone" was not altered by either Genotype or Social Stress (respectively: $F_{(1,18)}$=0.45; n.s.; $F_{(1,18)}$=3.00; n.s.) (Fig. 5), nevertheless a significant Social Stress×Test Phase interaction was found ($F_{(1,18)}$=5.99; P<0.05); defeated subjects spent a significantly shorter time in the

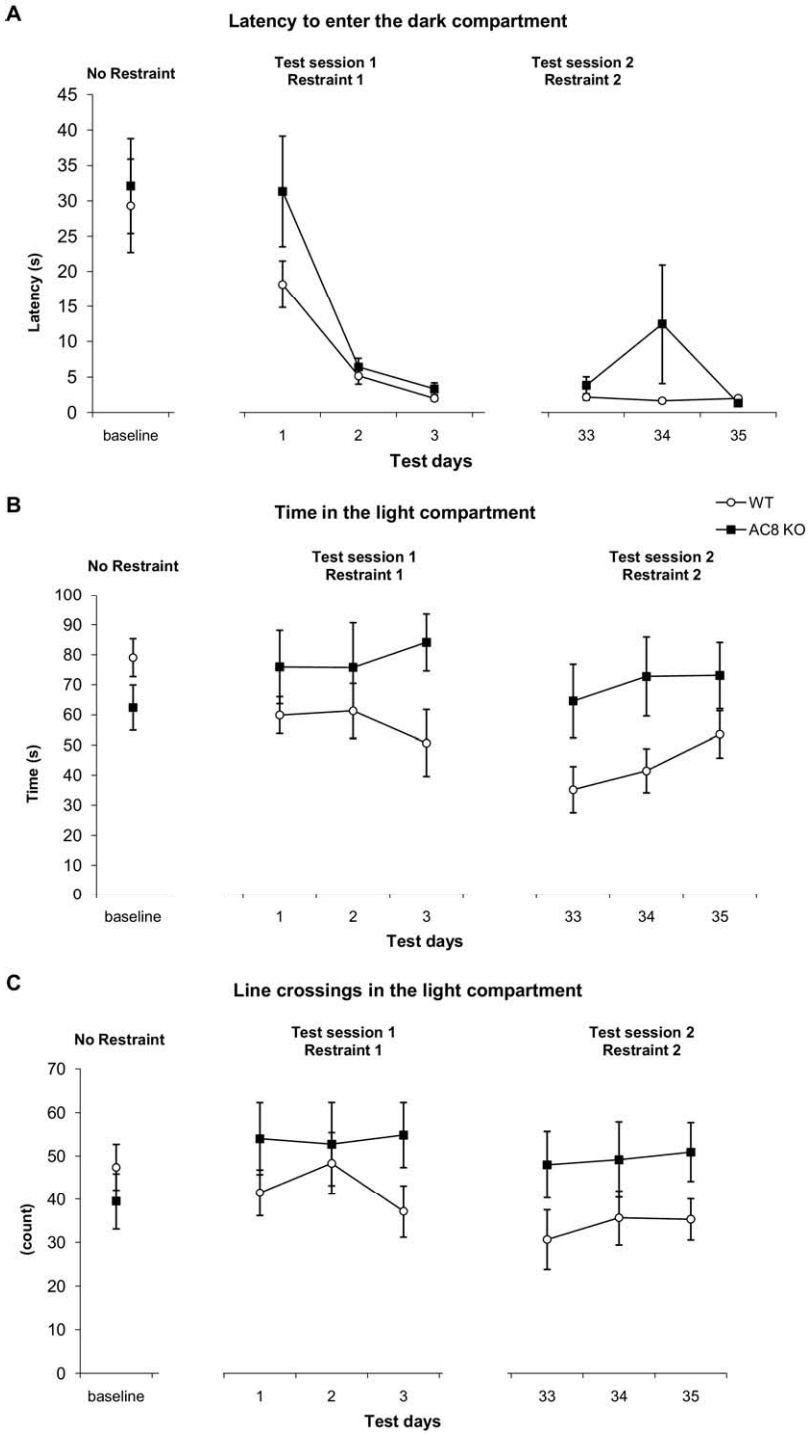

Fig. 2. Light–Dark Box tests following Restraint Stress. Effects on three different variables: (A) latency to enter the dark compartment, (B) time spent in the light compartment, and (C) number of line crossings in the light compartment. For baseline values, $n=9$ WT and $n=10$ AC8 KO were tested (Experiment 1). Mice (AC8 KO: $n=12$; WT: $n=12$) were tested on three consecutive test days after 30 min restraint each day before testing during two sessions, spaced one month apart from each other (Experiment 2).

"interaction zone" in the presence of the aggressor compared to control subjects ($P<0.05$). No statistically significant differences were found in the remaining parameters ("far corners", "center", and "total distance traveled"; not shown).

FST. No effects were found on floating behavior due either to Social Stress ($F_{(1,19)}=0.26$; n.s.) or to its interaction with Genotype ($F_{(1,19)}=0.01$; n.s.), whereas AC8 KO mice exhibited a shorter floating duration than WT subjects ($F_{(1,19)}=4.09$; $P=0.06$) (Fig. 6A). Accordingly, active swim-

Table 2. Plasma glucose and hormone levels under basal conditions

	Glucose mg/dL	Insulin pg/mL	Amylin pg/mL	Leptin pg/mL	Ghrelin (acylated) pg/mL	GIP pg/mL	PP pg/mL	PYY pg/mL	Corticosterone ng/mL	ACTH pg/mL
WT	203.6±11.6	1356±163	141.7±19.2	4955.5±756.5	22.6±7.6	39.6±8	24.2±12.8	n.d.	26.5±13.9	n.d.
AC8 KO	186.5±17.9	1446.5±218.7	115.5±24.2	3065.3±470.9*	15.7±2.6	39±8.3	29.1±17.1	n.d.	15±4.6	n.d.

Data are reported as group mean±SEM and expressed as pg/mL.
* $P<0.05$: ANOVA main factor Genotype: WT ($n=9$) vs AC8 KO ($n=10$).
n.d., not detectable.

ming duration was significantly affected by Genotype ($F_{(1,19)}=5.23$; $P<0.05$); AC8 KO mice swam longer than WT, regardless of Social Stress ($F_{(1,19)}=0.15$; n.s.) or their interaction ($F_{(1,19)}=0.04$; n.s.) (Fig. 6B). No statistically significant differences were found for climbing behavior (not shown).

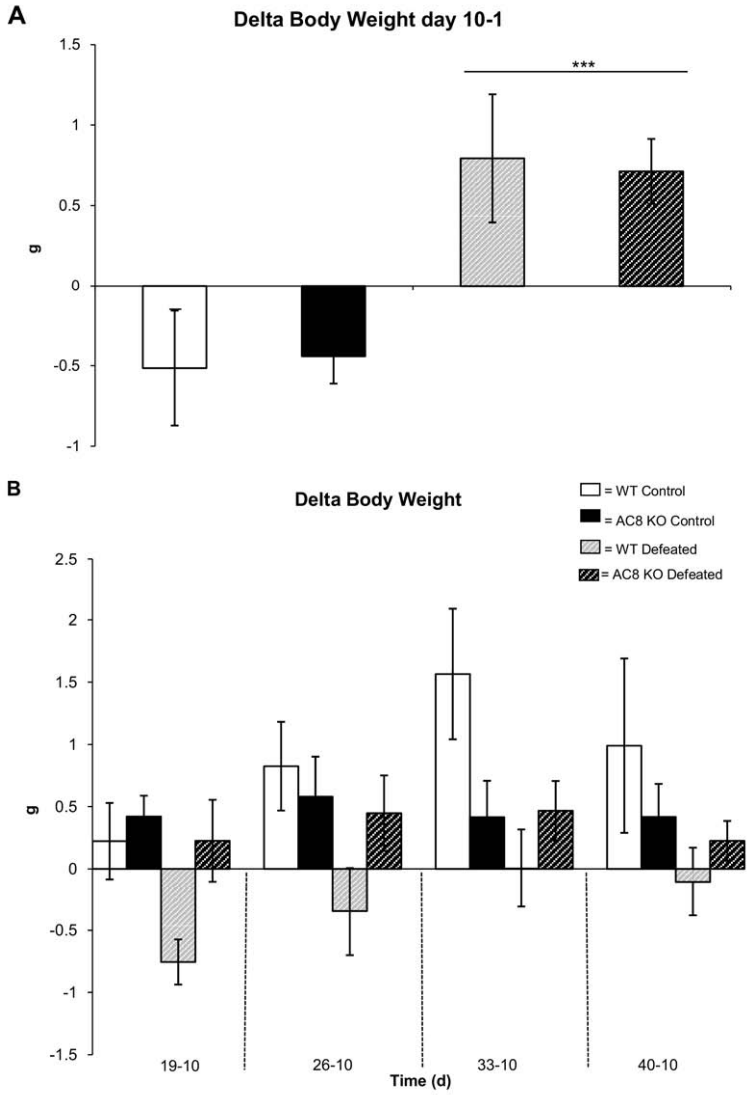

Fig. 3. Body weight changes during 10d Social Defeat (A) and during the following 28d single housing phase (B). Delta body weight were calculated between the first day of the experimental procedure and the last day of the 10 social defeats and between the end of the social defeat stress (d10) and each of the subsequent weekly time-points (d19, d26, d33, and d40). *** $P<0.001$: ANOVA main factor Social Stress. WT: $n=10$; AC8 KO: $n=13$.

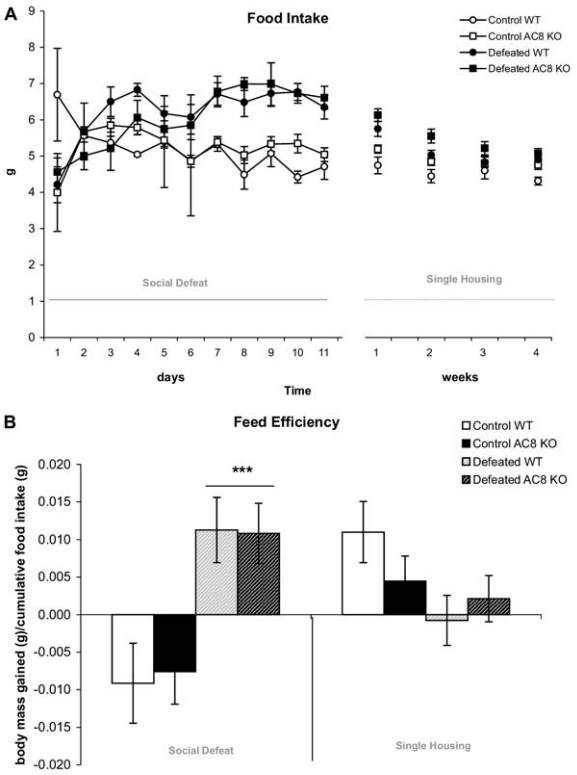

Fig. 4. Food Intake (A) and Feed Efficiency (B) changes during 10d Social Defeat and during the following 28d single housing phase in 10 WT and 13 AC8 KO mice. For Food Intake (A), each point represents the average grams of food consumed within each group of subject±SEM, either daily (social defeat phase) or weekly (single housing phase). For Feed Efficiency (B), data represent group averages±SEM. *** $P<0.001$: ANOVA main factor Social Stress.

Internal organ weight. Several differences were highlighted due to Social Stress, Genotype or their interaction (Table 3). Adrenal weight was significantly increased in AC8 KO mice compared to WT ($F_{(1,19)}=8.87$; $P<0.01$) but it did not vary due to either Social Stress ($F_{(1,19)}=0.01$; n.s.) or Social Stress×Genotype ($F_{(1,19)}=2.22$; n.s.). Spleen weight was not affected by either Social Stress or Genotype ($F_{(1,19)}=0.03$; n.s.; $F_{(1,19)}=1.64$; n.s.), although the Social Stress×Genotype interaction resulted close to significance ($F_{(1,19)}=3.69$, $P=0.07$). Thymus weight tended to be influenced by the Social Stress× Genotype interaction ($F_{(1,19)}=4.07$; $P=0.06$), with increased values in the defeated subjects within the WT background ($P=0.06$) compared to their respective controls. Seminal vesicle weight was significantly decreased in defeated subjects of either genotype (Social Stress: $F_{(1,19)}=17.39$; $P<0.001$; Genotype: $F_{(1,19)}=0.14$; n.s.). No differences were found for either testicle (Social Stress: $F_{(1,19)}=0.001$; n.s.; Genotype: $F_{(1,19)}=0.65$; n.s.) or abdominal fat weight (Social Stress: $F_{(1,19)}=0.44$; n.s.; Genotype: $F_{(1,19)}=0.78$; n.s.).

DISCUSSION

The hypothesis that AC8 might possess a functional role in stress and stress adaptation was assessed in the present series of experiments, subjecting AC8-deficient mice to behavioral procedures relevant to long-term stress-related responses.

Results from the present studies confirm and expand previous data, showing normal basal activity and anxiety in AC8 KO mice, in both open field and light–dark box tests, as well as corticosterone levels similar to WT subjects (Schaefer et al., 2000; Zhang et al., 2008). Consistent with the findings from Schaefer and colleagues (2000), in the present experiments, following repeated restraint stress, AC8 KO displayed an overall lower level of anxiety-like behaviors compared to WT mice. This could be considered suggestive of a greater risk-taking tendency and resilience to the changes induced by the restraint stress in AC8 KO mice, further supported by their minor body weight fluctuations in response to stress compared to WT littermates. When AC8 KO mice were observed in their home-cages, a previously unreported spontaneous hyperactivity was revealed during the dark phase (mouse active phase). Furthermore, while most hormones were unaffected by the lack of AC8, basal plasma levels of leptin were found to be lower in AC8 KO than in WT mice. AC8 KO were undistinguishable from WT littermates for what concerns the outcome of social defeat, a chronic psychological stress that induced similar long-term consequences (i.e. social avoidant behavior, metabolic dysregulation and gonadic impairment) in all defeated subjects. On the other hand, AC8-deficiency determined a variety of alterations, such as a decrease in passive and an increase in active behaviors in the FST, metabolic abnormalities (hyperphagic behavior with minor body weight gain) and adrenal hypertrophy.

AC8, together with AC1, belongs to the Ca^{2+}/calmodulin activated AC subclass, that is thought to play a crucial role in integrating cAMP and Ca^{2+} signaling and that is widely distributed in the brain (Cali et al., 1994; Tang and

Fig. 5. Social Avoidance test 28 days after the end of the social defeat stress; the test comprised two phases, either in the absence (2.5 min) and in the presence (2.5 min) of an aggressor CD1 mouse confined within a small cage, around which the interaction could take place. The time spent in the interaction zone (s) was measured in either phase. Values represent group mean±SEM. Different letters represent significant differences at $P<0.05$ level due to main factor Social Stress. WT Control: $n=4$; WT Defeated: $n=6$; AC8 KO Control: $n=6$; AC8 KO Defeated: $n=7$.

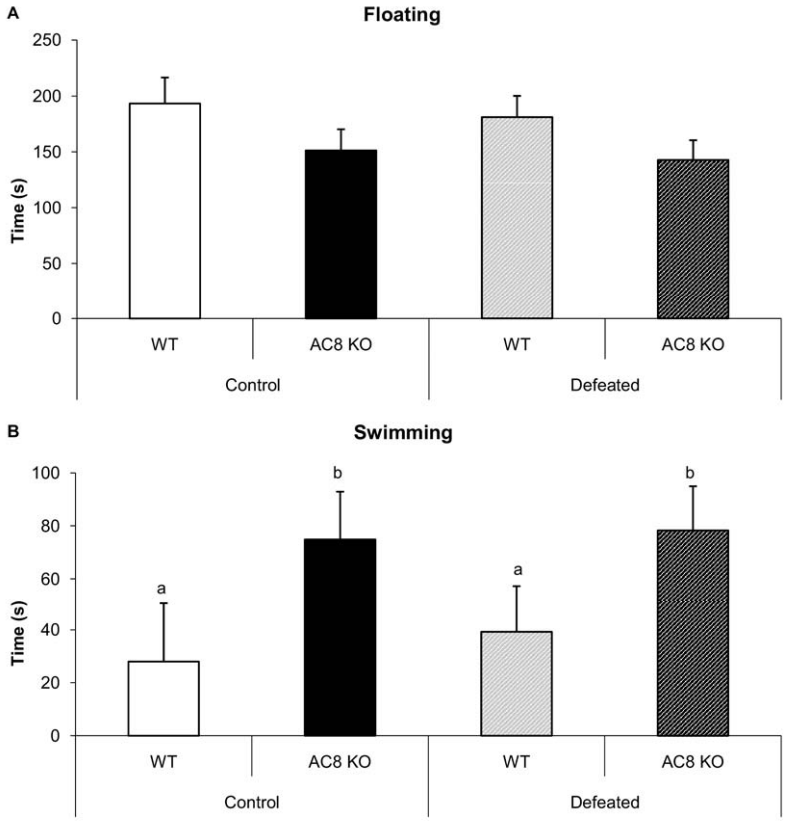

Fig. 6. Forced swimming test behaviors assessed 30d following the last social defeat stress. Data represent the mean±SEM for either floating (A) or swimming behavior (B) during the last 4 of a 6 min test. Different letters represent significant differences at $P<0.05$ level due to main factor Genotype. WT Control: $n=4$; WT Defeated: $n=6$; AC8 KO Control: $n=6$; AC8 KO Defeated: $n=7$.

Gilman, 1991; Wang and Storm, 2003; Xia and Storm, 2005). The functional contribution of AC has been substantially explored utilizing AC1 and AC8 transgenic animals (Villacres et al., 1998; Schaefer et al., 2000; Wang et al., 2003). Most of these studies have been performed on the double AC1/8 KO (DKO), implicating these enzymes in a wide range of responses, such as hippocampal long-term potentiation (LTP), associated learning and memory, olfaction, allodynia and behavioral stress responses, and indicating that AC1 and AC8 are each able to compensate for the loss of other Ca^{2+}-stimulated AC isoform in certain neuronal functions (Wong et al., 1999; Maas et al., 2005; Sindreu et al., 2007; Krishnan et al., 2008; Zachariou et al., 2008; DiRocco et al., 2009). Nevertheless, a partial overlapping of the genes influenced by AC1 and AC8 has been demonstrated (Zachariou et al., 2008), thus further reinforcing the notion that the two isoforms subserve distinct functions. In particular, studies in AC8 KO mice suggest the involvement of this isoform in plastic adaptations underlying such responses as stress responses, hippocampus dependent memory, drug sensitivity, dependence and withdrawal (Wong et al., 1999; Schaefer et al., 2000; Maas et al., 2005; Zhang et al., 2008; Zachariou et al., 2008).

Table 3. Internal organs relative weight

	WT		AC8 KO	
	Control $n=4$	Defeated $n=6$	Control $n=6$	Defeated $n=7$
Adrenals	0.017±0.003	0.020±0.002	0.026±0.004**	0.023±0.001**
Spleen	0.256±0.007	0.294±0.018	0.345±0.049	0.269±0.012
Thymus	0.177±0.013	0.213±0.008	0.181±0.015	0.174±0.007
Seminal vesicles	1.069±0.056	0.866±0.056###	1.050±0.052	0.846±0.026###
Testicles	0.814±0.021	0.813±0.010	0.797±0.023	0.795±0.024
Abdominal fat	1.600±0.296	1.450±0.176	1.389±0.086	1.375±0.087

Relative weights are presented in g/100g BW. Values represent group mean±SEM.
** $P<0.01$: ANOVA main factor Genotype; ### $P<0.001$ ANOVA main factor Social Stress.

Whereas we were able to replicate the anxiolytic profile conferred by AC8 deletion following repeated restraint stress as shown by Schaefer and colleagues (2000), AC8 KO subjects did not differ from WT, at least in the present experimental conditions, in the consequences of social defeat, suggesting that AC8 might not be involved in the modulation of the responses coping with social stress. On the other hand, the present results could be explained by the occurrence of a functional compensation by AC1 overruling the contribution of AC8 on these social behaviors. Social behaviors are in fact complex resulting from the interaction of several components, such as anxiety, social dominance, and motivation (Sousa et al., 2006). Specifically, the simultaneous deficiency of AC1 and AC8 was shown to confer a facilitating effect on social behavior in DKO mice with no previous history of social defeat (Krishnan et al., 2008), while the susceptibility to this type of stress was related to the up-regulation of a different class of AC (Krishnan et al., 2007).

Conversely, a spontaneous hyperactivity was observed in AC8 KO mice only during the mouse activity phase (i.e. dark phase), thus not confounding the results of other assays that were conducted during the light phase of the dark:light cycle, when AC8 displayed normal activity levels. Importantly, a greater reactivity to the FST, one of the most commonly used methods to examine depression-like behavior in laboratory rodents, was also manifested by AC8 KO mice. Whereas we can't exclude the occurrence of such hyperactivity in acute traditional procedures (i.e. open field, light–dark box tests) if they were to be performed during the dark phase, nonetheless the behavioral phenotype highlighted so far for AC8 KO mice resembles more traditional mouse models of "manic-behavior," based upon increased locomotor activity, risk-taking behavior, and "anti-depressant" responses (Malkesman et al., 2009). Recently de Mooij-van Malsen and colleagues (2009) identified quantitative trait loci for mouse home-cage behavioral traits (increased sheltering preference) in association with the AC8 coding gene *Adcy8*. Levels of expression of AC8 mRNA were found to differ in specific brain regions related to the mouse behavioral phenotype such as piriform cortex and hypothalamus, offering further insight into the role played by AC8 in the modulation of behavior. Interestingly, the same authors identified *Adcy8* as a candidate gene connecting human bipolar disorder to specific mouse behaviors utilizing interspecies trait genetics, on the base of the highlighted genetic association between *Adcy8* and bipolar disorder and of the efficacy of the human mood stabilizer carbamazepine (that acts via AC activity) on the *Adcy8*-related mouse avoidant behavior (de Mooij-van Malsen et al., 2009).

In the current studies the lack of AC8 was also accompanied by metabolic disturbances, represented by resiliency to stress-induced body weight fluctuations, hyperphagic behavior and hypoleptinemia. These results complete and help the understanding of earlier reports of increased ingestive behavior observed in AC8 KO mice (Schaefer et al., 2000; Maas et al., 2005). Leptin is a hormone produced in the adipose tissue and communicates to the brain the amount of peripheral fat to contribute to the regulation of several functions connected to energy homeostasis: food intake, glycemic control, thyroid function, reproduction and mesolimbic dopamine system functions related to food reward (Robertson et al., 2008). Although to our knowledge a mechanism directly linking AC8 to leptin is presently not known, a negative correlation exists between cAMP synthesis and food intake (Sheriff et al., 2003). Furthermore mice lacking AC3, an isoform structurally and functionally similar to AC8, are obese, suggesting a mechanism by which hypothalamic cAMP regulates food intake, body weight and leptin sensitivity levels (Wang et al., 2009). Interestingly, serum leptin levels in bipolar disorder patients with manic episodes are decreased (Atmacaa et al., 2002), while lithium treatment may lead to significant increases in serum leptin levels (Atmacaa et al., 2002; Himmerich et al., 2005). Leptin also regulates neuronal excitability in the hippocampus (Harvey, 2007), by inhibiting hippocampal neurons through activation of BK channels (Shanley et al., 2002) and it modulates synaptic plasticity in the CA1 hippocampal region, either by inducing LTP (Shanley et al., 2001) or an NMDA receptor dependent form of LTD (Durakoglugil et al., 2005). Further studies are needed to investigate the possible role of leptin in the hippocampal synaptic plasticity changes and altered stress response observed in the AC8 KO mice.

Finally, the adrenals were significantly enlarged in AC8 KO mice. Adrenal mass is thought to reflect the glucocorticoid- and mineralocorticoid-synthesizing cortex and catecholamine-synthesizing medulla apparently reflecting the increased activity of the hypothalamo–pituitary–adrenal (HPA) axis (Levine, 1967; Brain, 1972; Benton et al., 1978; Aloe et al., 1986; Levine, 2001). Moreover, a link between affective disorders and HPA axis dysfunction, leading to elevated circulating cortisol levels and glucocorticoid resistance, has been proposed (Pariante, 2006). The observed adrenal hypertrophy had no functional consequences on corticosterone basal levels that could be assessed in the present study. Nevertheless we cannot exclude changes in HPA axis reactivity to stressful conditions, as suggested by previous data showing elevated levels of corticosterone immediately prior the final of a series of restraint stress in AC8 KO mice (Schaefer et al., 2000). Further studies in AC8 KO mice are warranted to best elucidate the functional relevance of the adrenal hyperplasia and the possible involvement of brain structures implicated in the HPA axis regulation.

CONCLUSION

In conclusion, data from our study highlight a set of phenotypes dependent on AC8 signaling pathway that are related to hyperactivity, decreased susceptibility to specific stressors, and anti-depressant behavior, together with physiological alterations (metabolic parameters and adrenal size) that could be considered reminiscent of "manic-like" animal models. Recent data identified *Adcy8* as a candidate gene connecting human bipolar disorder to specific mouse behaviors (de Mooij-van Malsen et al., 2009).

Furthermore, while the implication of AC8 in multiple aspects of addictive behaviors has been repeatedly demonstrated (Maas et al., 2005; Zachariou et al., 2008; DiRocco et al., 2009), it is worthwhile to emphasize the strong relationship between bipolar and substance-use disorders (Swann, 2010). Altogether with our findings, these evidences can be considered indicative of a valid case to further investigate the role of AC8 as a potential target for mood disorders.

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