Paper III

Chronic mild stress, an animal model of depression, inhibits BDNF expression and CREB activation in the dentate gyrus

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Abstract

Chronic stress is linked to development of depression and may trigger neurobiological changes underlying the disease. Novel theories suggest a dysregulation of the secretory peptide brain-derived neurotrophic factor (BDNF) and the transcriptional regulator calcium/cyclic-AMP responsive binding protein (CREB) in the pathogenesis of depression. This study investigated the effect of chronic mild stress (CMS), an animal model of depression, on the hippocampal BDNF-CREB system. We evaluated BDNF expression and CREB activation in the dentate gyrus and the hippocampus proper. CMS rats consumed less sucrose compared to Control rats, a crucial indicator of anhedonia in rodents, and induced specific inhibition of BDNF expression and CREB activation in the dentate gyrus (-25% and -30%, respectively). No change in the total CREB and no significant effects in the hippocampus proper were observed. In addition, p-CREB levels were positively correlated with sucrose intake. These results emphasize a dysfunction of CREB-dependent gene expression and BDNF expression in the dentate gyrus following chronic mild stress and suggest that CREB activation may be important for adaptation to stress and development of coping mechanisms.

Introduction

Depression has a complex pathophysiology and the cellular and molecular mechanisms underlying the disease are still unknown. Among the most potent factors known to trigger or induce depressive episodes are stressful life events (Anisman and Zacharko 1982; Kendler *et al.* 1999; Paykel 2001). The animal chronic mild stress (CMS) model is claimed to induce anhedonia (diminished capacity to experience pleasure), one of the core symptoms of depression, as reflected in a lower consumption of sucrose in rats (Willner *et al.* 1987). The rodents are exposed to mild and uncontrollable daily stressors (e.g. soiled or tilted cage, food or water deprivation/restriction, paired caging, continuous light, wet bedding). After 4-6 weeks of CMS the animals show a wide variety of symptoms that parallel some features of human depression, contributing to the face validity of the model (Willner 1997; Willner et al. 1987). We have observed that exposure of naive rats to CMS induces, in addition to a lower sucrose intake (reflecting anhedonia), selective changes in sleep (Grønli *et al.* 2004) consistent with those classically observed in human depression (Benca 1996), reduced sexual activity and increased locomotor behavior (Grønli *et al.* 2005).

Traditionally, the monoamine hypothesis of depression suggests a deficiency of serotonin (5-hydroxytryptophan, 5-HT) or noradrenaline in the brain (Schildkraut 1965; Wong and Licinio 2004). However, an emerging hypothesis proposes that problems in information processing within specific neural networks may be the leading cause. In particular, a dysregulation of the neurotrophin brain-derived neurotrophic factor (BDNF) has been suggested in the pathophysiology of depression (Altar 1999; Duman *et al.* 1997; Hashimoto *et al.* 2004; Vaidya and Duman 2001). Levels of serum BDNF are decreased and negatively correlated with the Montgomery-Asberg-Depression Rating Scale in unmedicated major depressive patients (Karege *et al.* 2002) and are associated with vulnerability to develop mood disorders in healthy subjects (Lang *et al.* 2004). In rats, downregulation of BDNF is found in several brain regions following stress paradigms (Fumagalli *et*

al. 2004; Pizarro *et al.* 2004; Rasmusson *et al.* 2002; Roceri *et al.* 2002; Russo-Neustadt *et al.* 2001; Smith *et al.* 1995). Increases in BDNF synthesis and signaling have been implicated in the effect of chronic antidepressant drug treatment (Saarelainen et al., 2003;Castren, 2005;(Altar 1999; Duman 2002; Nibuya *et al.* 1995; Russo-Neustadt *et al.* 1999). Like BDNF, activation of the transcriptional regulator calcium/cyclic-AMP responsive-element binding protein (CREB) is downregulated following stress and upregulated in response to antidepressant treatment (Kuipers *et al.* 2005). Phosphorylation of CREB at its transcriptional regulatory residue Serine-133 is necessary to activate transcription of genes containing a cAMP response element (Montminy *et al.* 1990) this includes CREB-dependent transcription of BDNF (Conti *et al.* 2002; Tao *et al.* 1998). BDNF signaling through its receptor tyrosine kinase, TrkB, is also capable of inducing CREB phosphorylation (Finkbeiner et al., 1997;Ying et al., 2002). BDNF has diverse functions in the adult brain as a regulator of neuronal survival, fast synaptic transmission, and activity-dependent synaptic plasticity (Berninger and Poo 1999; Bramham and Messaoudi 2005). However, the role of BDNF-CREB in the pathophysiology of depression is unknown.

The hippocampus plays an important role in regulation of stress responses and it expresses high levels of BDNF (Conner *et al.* 1997). Stress in rats is associated with reduction of hippocampal BDNF levels (Russo-Neustadt et al. 2001; Shirayama *et al.* 2002). However, there may be a difference between the dentate gyrus and the cornus ammonis (CA) regions of the hippocampus in the stress-induced effect on the BDNF. Immobilization stress in rats is associated with greater impairments in BDNF expression in the dentate gyrus compared to the CA region (Smith et al. 1995). Furthermore, induced overexpression of CREB in the dentate gyrus, but not in the CA1 or CA3 regions, is associated with antidepressant-like behavioral effect (Chen *et al.* 2001).

The present study was designed to evaluate a) if CMS, a valuable animal model of depression, affects the BDNF-CREB system; b) if a specific difference exists between the dentate gyrus and the CA region; c) if such changes are correlated to the level of anhedonia as measured by sucrose intake (the major validation criterion for the CMS model).

Materials and Methods

Ethical evaluation

The experiment has been approved by the Norwegian Animal Research Authority and registered by the Authority. The experiment has thus been conducted in accordance with the laws and regulations controlling experiments in live animals in Norway, i.e. The Animal Protection Act of December 20th 1974, No 73, Chapter VI sections 20-22 and the Animal Protection Ordinance concerning Biological Experiments in Animals of January 15th 1996. Norway has signed and ratified The European Convention for the protection of Vertebrate Animals used for Experimental and other Scientific purposes of March 18, 1986.

Animal handling

Twenty male Sprague-Dawley (Mol:SPD) rats (Møllegaard, Copenhagen, Denmark) were used in this experiment. To minimize stress, the animals were allowed to remain in the transport cage for five days before they were separated and housed individually in conventional Macrolon type III cages. The home cages were placed in a rack allowing visual, olfactory and auditory contact between animals.

The rats had free access to food (Rodent low protein diet, B&K Universal AS, Norway) and water, except when the CMS procedure required deprivation. The ambient temperature was 22 ± 1°C with 52 ± 2 % humidity. Rats were kept on a reversed 12h light/12h dark schedule with gradually increasing lighting from 1800 h and lights fully on at 1900 h. The rats changed to the reversed L/D schedule 10 days before the start of the experiment. Five to seven days are reported to be a sufficient time for establishing a new circadian rhythm in male SPD rats (Hillegaart and Ahlenius 1994).

Additional procedure

All animals were anesthetised and implanted with electroencephalogram/electromyogram electrodes for sleep recording and with a microdialysis cannula in the hippocampus. At least two weeks were allowed for recovery and adaptation prior to start of the experiment. Sleep was recorded before and after the CMS protocol. Dialysates were aquired after CMS. The part of the data relative to these procedures are considered in a separate paper.

Experimental procedure

The animals were divided into two groups. One group was exposed to chronic mild stress (CMS rats, n = 12) and the other group was given ordinary daily care (Control rats, n = 8). The two groups of rats were housed separately in different rooms during the duration of the stress procedure.

Most of the CMS stressors were adapted from the procedure described by Willner and collaborators (Willner et al. 1987) and some stressors were included from Moreau and collaborators (e.g. empty bottle of water, restricted food) (Moreau *et al.* 1992). Each week included 2 h of paired caging, 3 h of tilted cage (45 degrees), 18 h of food deprivation immediately followed by 1 h of restricted access to food (5 micropellets), 2 x 18 h of water deprivation immediately followed by 1 h exposure to an empty bottle, 21 h with wet cage (200 ml water in 100 g sawdust bedding), and 36 h of continuos light. Stressors were presented both during the rats' active (dark) period and during the inactive (light) period. See (Grønli et al. 2005) for details with clock time and duration of the experimental protocol.

In both CMS and Control animals, the sucrose intake (1% sucrose solution) and bodyweight were measured once a week, during a one-hour window after four hours of food and water deprivation. Consumption was measured by comparing bottle weight before and after the one-hour window and expressed in relation to the animal's body weight (ml/kg). Baseline was measured a few days before the start of CMS. Initially, the two groups had a similar preference for sucrose. The

food and water deprivation period preceding sucrose intake measurement may be considered as a further stress applied on top of the CMS protocol. However, Control rats were also exposed to the same procedure, as a part of the sucrose test.

Tissue microdissection and sample preparation

The animals were left undisturbed (stressor free) for two days before being anaesthetised with Isofluran gas (Isofluran Baxter, Norway) and decapitated. The brain was rapidly separated from the skull and divided into the two hemispheres. One hemisphere was rinsed with oxygenated ice-cold artificial cerebral spinal fluid and the dentate gyrus and CA region were rapidly dissected on an ice-cold glass dish, aliquoted into Eppendorf tubes, and stored at –80^{o}C until analysis. The other hemisphere included the site of the microdialysis probe. The side (left or right) for protein analysis or probe localisation was randomly assigned.

Antibodies

Primary antibodies BDNF (N-20) Sc-546 rabbit polyclonal IgG (1:2000) (Santa Cruz), total CREB rabbit polyclonal (1:2000) (Upstate) and phospho-CREB at Serine-133 rabbit polyclonal (1:2000) (Santa Cruz). Secondary antibody goat anti-rabbit IgG (1:5000) (Calbiochem).

SDS-PAGE and Western Blotting

Tissues were hand-homogenized with 15 strokes in 300 µl of Dynal lysis/binding buffer. Protein levels in homogenate samples were determined using the Lowry method. Equal amounts of protein were loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis. Phosphorylated CREB and total CREB on 10% gels and BDNF on 12% gel and run overnight at constant 10 mA. Separated proteins were transferred onto a nitrocellulose membrane (Hybond-C Amersham) at constant voltage of 30 V overnight or 100 V for 1 h. Membranes were blocked on a

gyro-rocker for 1 h at room temperature. Blocking buffer consisted of TBST (Tris-buffered saline/0.1% Tween-20) and 5% albumin from bovine serum (BSA). The primary antibodies were dissolved in blocking buffer containing 3% BSA and the blots for BDNF and total CREB were incubated for 2 h at room temperature and p-CREB was incubated at 4°C overnight with constant shaking. Following three washes with TBST, blots were incubated for 1 h in horseradish peroxidase-conjugated secondary antibody dissolved in TBST. The blots were washed three times with TBST and proteins were visualized using enhanced chemiluminescence (Western Blotting Analysis System, Amersham pharmacia biotech, Norway). Autoradiographs were scanned on a densitometer and quantitated using Phoretics ID plus software.

Statistics

Three animals died before or during the experiment and three more developed epileptic seizures lasting longer than one minute. Thus, for sucrose analysis 17 animals (CMS, n = 11 and Control, n = 6) were included and 14 for protein analysis (CMS, n = 9 and Control, n = 5).

Sucrose and body weight: Analysis of Variance (ANOVA) for repeated measures was performed on sucrose intake and bodyweight with group as independent factor and time as repeated measure. Subsequently, the effect of CMS or Control treatment on sucrose intake over days was further analysed with one-way repeated measure ANOVA. Difference between groups was assessed by multiple comparisons performed by least significant deviation post hoc test.

Proteins: Comparison between the expressions of BDNF, p-CREB, total CREB and the β-actin in the CMS group and in the Control group were made using two sample Student's *t*-test assuming equal variance.

Correlation analysis between sucrose intake and BDNF, p-CREB or total CREB were performed on pooled data, from both Control and CMS rats (n = 14) using Pearson's correlation analysis.

Statistica 5.0 (StatSoft, Inc.) was used for all statistical analysis. Significance was accepted at $p < 0.05$, two-tailed. All values are presented as mean ± S.E.M.

Results

CMS rats consume less sucrose than Controls

The CMS rats drank less sucrose solution than the Controls during the CMS period (Day -5 to Day 38; $F_{(1,14)} = 8.05$, $p = 0.01$). The Control rats increased their consumption ($F_{(5,25)} = 2.65$, $p = 0.047$), while the CMS rats maintained roughly the same level throughout the study ($F_{(5,45)} = 1.01$, $p = 0.42$).

No change in body weight was observed ($F_{(1,15)} = 1.45$, $p = 0.25$) suggesting that CMS rats gained weight at the same rate as their Controls during the CMS period. Sucrose consumption and body weight in Control rats and CMS rats are shown in Figure 1.

Selective reduction of BDNF and p-CREB in the dentate gyrus after CMS

Rats subjected to CMS exhibited a specific inhibition of BDNF and p-CREB levels in the dentate gyrus (Fig. 2). BDNF levels were down-regulated 25% (75.0% ± 3.5%, $p = 0.014$) and p-CREB was down-regulated 30% (70.3% ± 7.3%, $p = 0.013$) relative to Control rats. No difference was present in the CA region ($p = 0.23$ and $p = 0.11$, respectively). The expression of total CREB did not differ between CMS and Control rats, neither in the dentate gyrus nor in the CA region ($p = 0.88$ and $p = 0.60$, respectively).

The protein β-actin, used as a control protein for this study, showed no difference between the two groups of animals in dentate gyrus or CA regions ($p = 0.98$ and $p = 0.97$, respectively).

Expression of p-CREB in the dentate gyrus correlates with sucrose intake

To further investigate CMS effects, we correlated the expression of BDNF, p-CREB and total CREB in the dentate gyrus and the CA region with the animals' sucrose intake.

There was a positive correlation between expression of p-CREB in dentate gyrus and sucrose intake ($r = 0.64$; $p = 0.013$). No correlation was found for sucrose intake and p-CREB in the hippocampus proper ($r = 0.09$; $p = 0.75$). The expression of BDNF or total CREB did not correlate with sucrose intake in the dentate gyrus (r = 0.54; p = 0.14, r = -0.17; p = 0.67, respectively) or hippocampus proper (r = 0.13; p = 0.76, r = -0.27; p = 0.47, respectively). Thus, the rats that consumed less of a palatable sucrose solution (reflecting anhedonia) were those having lowest expression of p-CREB specifically in the dentate gyrus, see Fig. 3.

Discussion

In the present study we used the CMS animal model of depression to investigate the effects of chronic mild stress exposure on BDNF-CREB system in the hippocampus and its correlation with sucrose intake. Chronic mild stress resulted in a significant decrease in the expression of BDNF and p-CREB in the dentate gyrus but these measures were not significantly changed in the CA region. The expression of total CREB and the control protein β-actin were unaffected by CMS. During the CMS protocol, stressed rats consumed less sucrose solution compared to a Control group. This change correlated with p-CREB down-regulation in dentate gyrus. The present results emphasise a dysfunction of BDNF and CREB-dependent gene expression specifically in the dentate gyrus following chronic mild stress and suggest that a decrease in the activated form of CREB in the dentate gyrus is correlated to the severity of stress-induced depression.

The lack of effect of CMS in the hippocampal CA region indicates that dentate gyrus is an area more vulnerable to a prolonged mild stress exposure. Such sensitivity may be reflected in the involvement of limbic and neocortical forebrain structures in the stress-induced depression. In other words a stress-response may be mediated by different stress-sensitive brain circuitry. This is in line with the concept of differential circuitry being involved in the brain processing of psychological (e.g. chronic stress, predator exposure) versus physical stress (e.g. cold, exposure to ether) (Herman and Cullinan 1997). The changes in the levels of BDNF protein and phosphorylated CREB observed after CMS are more likely to be of psychological nature since they were measured 2 days after the final stress session. This delay avoids the confounding effects related to an acute response to stress. Our results differ from a previous study by Kuroda and McEwen (Kuroda and McEwen 1998) that showed no difference in dentate gyrus BDNF mRNA levels following a chronic immobilisation stress protocol. In their study the mRNA levels were measured 21h following the end of the protocol. The difference between their results and ours could reflect regulation of BDNF expression at the post-transcriptional level, which would not be detected in measurements of BDNF

mRNA. The discrepancy could also be related to the different stress procedures used. Both protocols included repeated exposure to stress. However, Kuroda and McEwen used a single type of stress, whereas our CMS protocol included different stressors administered in an unpredictable manner. The latter procedure may be more effective in inducing a feeling of 'helplessness' critical for the establishment of depression. A recent study reported that the level of BDNF in the hippocampus is dependent on adaptation to stress, a decreased level is found in rats that do not learn to avoid electrical shock (Scaccianoce *et al.* 2003). The findings of a decreased BDNF level after exposure to CMS suggest that the animals did not adapt to the procedure, hence, CMS seems to be a more valuable model to study stress-sensitive brain circuits.

The dentate gyrus has emerged as a critical site in the pathogenesis of depression and the action of antidepressant drugs. Recent work suggests that the dentate gyrus functions in fine spatiotemporal separation of novel and complex cues (Kesner et al., 2004;Lee et al., 2005). In so doing, the dentate gyrus may disambiguate stimuli to allow sparse encoding of information (Gould and Cameron 1996). Failure in this function fits with the emotional withdrawal, social isolation, and impaired memory in depressed patients. New granule cells are produced throughout life from a progenitor population in the subgranular zone of the dentate gyrus (Gould and Cameron 1996). Since BDNF promotes the survival of new granule cells and the suppression of granule cell neurogenesis it is likely that it may be involved in the development of depressive episodes (Duman and Charney 1999). In addition to regulation of survival and neurogenesis, BDNF has emerged as a major regulator of activity-dependent synaptic plasticity represented by long-term potentiation (LTP) in the dentate gyrus (Bramham and Messaoudi, 2005). It is therefore likely that downregulation of BDNF expression during CMS impairs the information processing and storage capacity of the dentate gyrus.

Willner and coworkers claim that a reduction in consumption of sweet solutions reflects the anhedonic changes observed in human depression, and may, therefore, serve as a depression-marker in the CMS model (Willner *et al.* 1987). The present study confirms that in our laboratory CMS rats

consume less sucrose compared to Controls and that such consumption is not correlated to their bodyweight. CMS rats consume less sucrose but gain weight in the same manner as the Control group.

The individual expression of p-CREB in dentate gyrus was positively correlated with the animals' sucrose intake. The rats consuming less sucrose solution showed the lowest expression of p-CREB in the dentate gyrus. Hence, p-CREB appears to be a sensitive measure of the pathophysiological changes associated with depression-like symptoms. In an earlier CMS study, we found a correlation between the sucrose intake and depression-like sleep symptoms (Grønli *et al.* 2004). The present study also confirms an individual variability in the effect of CMS the protocol. In general, animals, humans included, differ in their capacity to cope with environmental changes. Inherited individual vulnerability to depression is a well known phenomenon (Kendler *et al.* 1994). Most people subjected to stressful events do not develop depression, and not all cases of depression are a consequence of stressful experiences (Anisman and Zacharko 1982). The present study supports the hypothesis that the levels of hippocampal BDNF may be decreased in depression. However, we did not find a correlation between sucrose intake and BDNF. This might indicate that changes in BDNF protein levels and CREB activation in the dentate gyrus are not necessarily inter-dependent in relation to depression. In depressed unmedicated patients, levels of serum BDNF are decreased and negatively correlated with the Montgomery-Asberg-Depression Rating Scale score for depression (Karege *et al.* 2002). In healthy unrelated volunteers, a low level of serum BDNF is correlated to vulnerability to develop mood disorders (Lang *et al.* 2004).

In conclusion, this study demonstrates a chronic mild stress - induced downregulation of the BDNF-CREB system in the dentate gyrus. The CMS model may therefore prove useful in further exploration of the BDNF-CREB system in relation to stress adaptation and depression.

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Abbreviations

BDNF	brain-derived neurotrophic factor
AMP	adenylmonophosphate
CREB	calcium/cyclic-AMP responsive binding protein
CMS	chronic mild stress
5-HT	serotonin, 5-hydroxytryptophan
TrkB	tyrosine kinase
CA	cornus ammonis
SPD	Sprague-Dawley
TBST	Tris-buffered saline/0.1% Tween-20
BSA	albumin from bovine serum
ANOVA	Analysis of Variance

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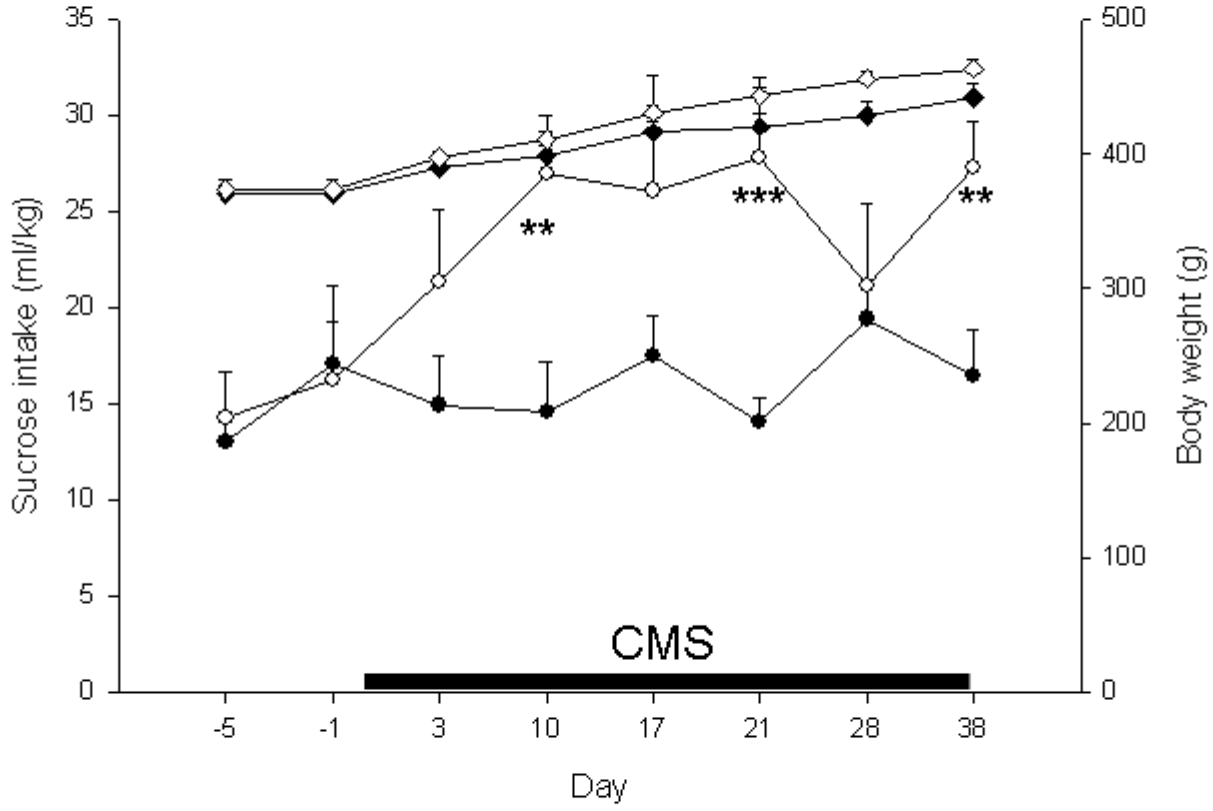
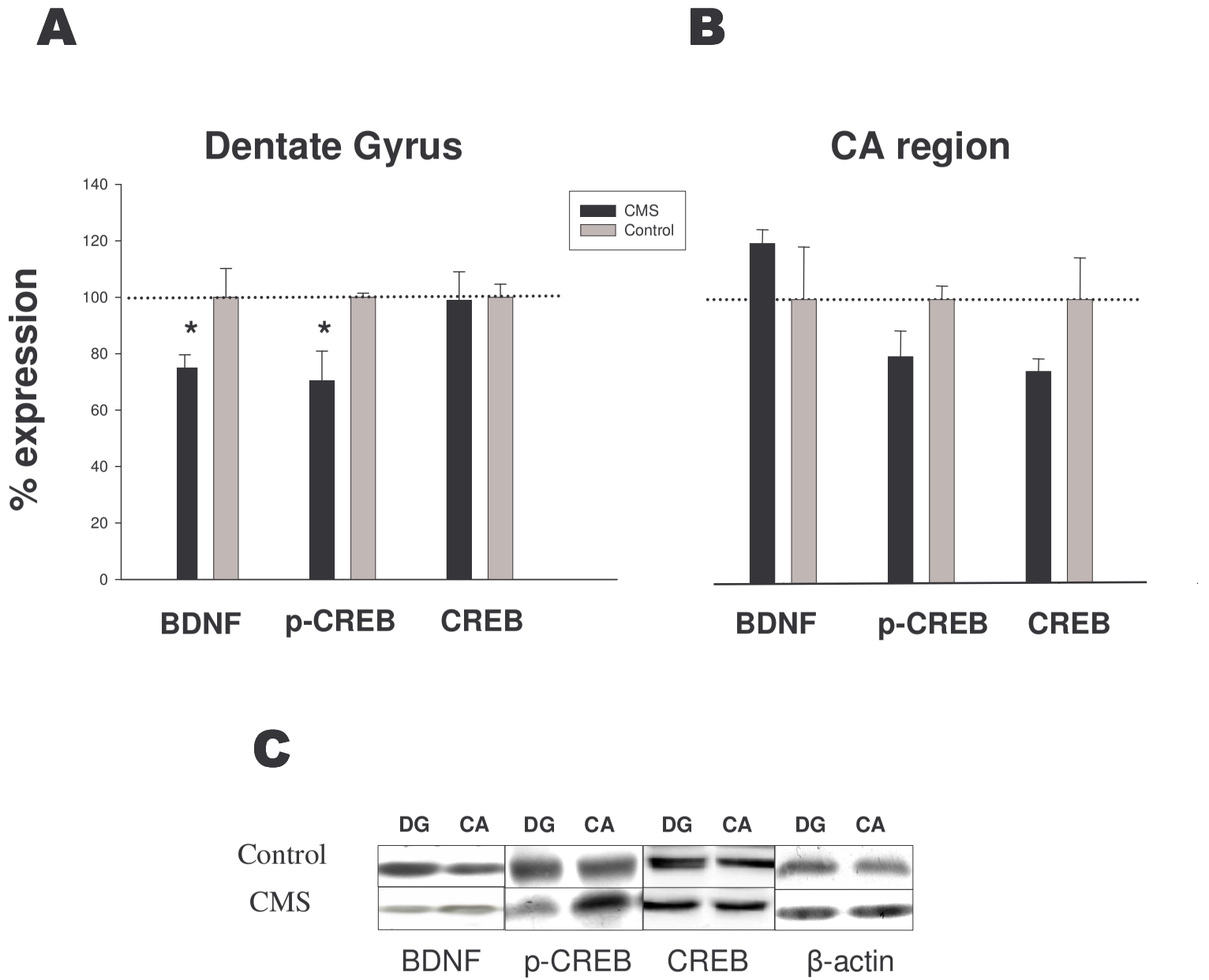
LEGENDS

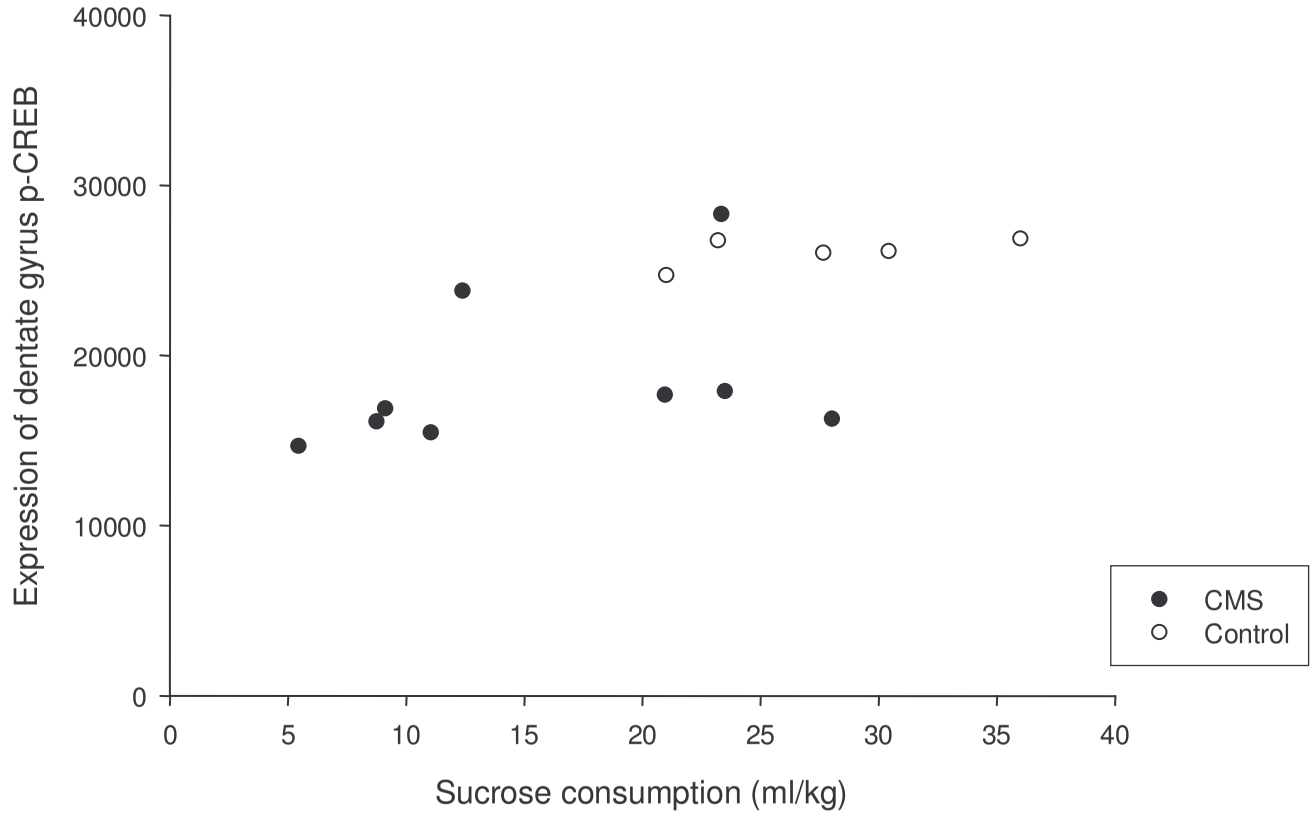
Fig.1. Measure of sucrose intake (ml/kg) and bodyweight (g) before and during the CMS protocol. Circles indicate sucrose intake and diamonds indicate body weight. Open symbols indicate Control rats and filled symbols CMS rats. Results are presented as mean ± S.E.M. ** indicates $p < 0.01$, *** $p < 0.001$ between groups.

Fig. 2. Expression of BDNF, p- CREB and total CREB in the dentate gyrus (Fig. 2A) and the CA region (Fig. 2B) in the CMS and the Control group. Data are shown as per cent of Control group mean ± SEM. * indicates $p < 0.05$ compared to the Control group. Fig. 2C shows the band intensity of Western Blot in one representative animal.

Fig 3. Correlation between sucrose intake (ml/kg) and expression of p-CREB in dentate gyrus ($r = 0.64$; $p = 0.013$; n = 14). Open symbols indicate Control rats and filled symbols CMS rats.


Sucrose intake (ml/kg)
Body weight (g)
Day
CMS
**
***
**
35
30
25
20
15
10
5
0
500
400
300
200
100
0
-5
-1
3
10
17
21
28
38


A
B
Dentate Gyrus
CA region
% expression
140
120
100
80
60
40
20
0
CMS
Control
*
*
BDNF
p-CREB
CREB
BDNF
p-CREB
CREB
C
DG
CA
DG
CA
DG
CA
DG
CA
Control
CMS
BDNF
p-CREB
CREB
β-actin


Expression of dentate gyrus p-CREB
40000
30000
20000
10000
0
0
5
10
15
20
25
30
35
40
Sucrose consumption (ml/kg)
CMS
Control