

# Long-term down-regulation of BDNF mRNA in rat hippocampal CA1 subregion correlates with PTSD-like behavioural stress response

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## Abstract

Brain-derived neurotrophic factor (BDNF) and its intracellular kinase-activating receptor TrkB, have been implicated in the neurobiological mechanisms underlying the clinical manifestations of PTSD, especially those related to synaptic efficacy and neural plasticity. BDNF interacts with components of the stress response such as corticosterone, and plays an important role in growth, maintenance and functioning of several neuronal systems. This study employed an animal model of PTSD to investigate the relationship between prevalence rates of distinct patterns of behavioural responses to predator stress, circulating levels of corticosterone and local levels of mRNA for BDNF, TrkB and two other neurotrophic factors in selected brain areas. Animals whose behaviour was extremely disrupted by exposure selectively displayed significant down-regulation of mRNA for BDNF and up-regulation of TrkB mRNA in the CA1 subregion of the hippocampus, compared to animals whose behaviour was minimally or partially affected and to unexposed controls. The response was consistent throughout the entire study only in CA1. The consistent long-term the BDNF down-regulation and TrkB up-regulation associated with extreme behavioural compromise may be associated with chronic stress-induced psychopathological processes, especially in the hippocampus. The corresponding changes in neural plasticity and synaptic functioning may mediate clinical manifestations of PTSD.

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## Introduction

The clinical manifestations of post-traumatic stress disorder (PTSD), the prototypic psychopathological outcome of exposure to extreme stress, pivot on an exaggerated and chronic form of the adaptive response to acute stress, and apparently stem from faulty processing of and/or recovery from the initial response. Unlike processes in which exposure to

repeated stimuli induces a process of learning or conditioning, implying an increased efficiency in processing of data to produce the required response, the psychopathology underlying PTSD produces a paradoxical vulnerability to negative sequelae upon subsequent stress exposure.

The neurobiological mechanisms involved in PTSD thus presumably include changes in synaptic efficacy and plasticity in various brain areas which, rather than improving, in fact impair the ability of the brain to respond adequately to subsequent stress response (Abraham and Tate, 1997). Were it possible to correct or to induce mechanisms which would reinstate adaptive neural plasticity and remodelling, this dysfunction might be overcome.

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Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin (NT) family of survival-promoting molecules, plays an important role in the growth, development, maintenance, and function of several neuronal systems (Hyman et al., 1991). It is known to modulate synaptic plasticity and neurotransmitter release in a variety of neurotransmitter systems, as well as intracellular signal-transduction pathways (Hyman et al., 1991). It regulates axonal and dendritic branching and remodelling (Lom and Cohen-Cory, 1999; McAllister, 1999; Shimada et al., 1998; Yacoubian and Lo, 2000), synaptogenesis in arborizing axon terminals, efficacy of synaptic transmission, and the functional maturation of excitatory and inhibitory synapses (Rutherford et al., 1998; Seil and Drake-Baumann, 2000; Vicario-Abejon et al., 1998).

The role of BDNF and TrkB, a protein-tyrosine kinase receptor for BDNF, in learning and memory processes is suggested by their function in activity-dependent synaptic long-term-potential (LTP), the transcription-dependent electrophysiological correlate of long-term memory (Nguyen and Kandel, 1996; Pang and Lu, 2004). BDNF gene deletion or inhibition produces a deficit in learning and memory (Nguyen and Kandel, 1996), whereas learning and memory significantly increase circulating and brain levels of nerve growth factor (NGF) and BDNF (Rosenblum et al., 2002; Ying et al., 2002).

Growing evidence suggests important roles for BDNF in the pathogenesis of mood disorders and in the mechanism of action of therapeutic agents, such as mood stabilizers and antidepressants (Duman, 2002, 2004; Manji and Duman, 2001). BDNF may also be involved in the pathophysiology of anxiety disorders. Data derived from animal studies have demonstrated that, compared with wild-type mice, BDNF conditional mutants (in which BDNF has been eliminated from the brain after birth through the use of the Cre-loxP recombination system) were hyperactive after exposure to stressors and had higher anxiety levels when evaluated using the light-dark exploration paradigm (Rios et al., 2001). On the other hand, single intra-hippocampal administration of BDNF improves performance in a spatial memory task and has enduring anxiolytic effects (Cirulli et al., 2004).

A potential relationship between behaviourally induced stress (associated with PTSD) and hippocampal plasticity has been reported in several experiments in which hippocampal BDNF expression is reduced after the application of single or repeated immobilization stress (Givalois et al., 2001; Hyman et al., 1991).

Rasmusson et al. (2002) have reported that psychological and unconditioned physical stress decreased hippocampal BDNF mRNA. Although the exact mechanism for stress-associated down-regulation of BDNF is unknown, the adrenal hormone corticosterone might be involved in this response, since exogenous administration of the hormone is known to reduce BDNF mRNA (Nibuya et al., 1999). Based on these findings, BDNF has been suggested to be involved in both adaptive processes and in the pathophysiology of memory impairment, and in anxiety and mood disorders, and hence also in the pathogenesis and pathophysiology of PTSD.

The present study sought to assess the relationship between local neurotrophic factor levels in selected brain areas and magnitude of behavioural change, using an approach to analysing the behavioural response to predator-scent stress (PSS) in an animal model which distinguishes between individuals according to the degree to which their behaviour is affected by the stressor.

Populations of stress-exposed rodents are classified according to the degree of their individual behavioural response, creating two distinct groups entitled 'extreme behavioural responses' (EBR) and 'minimal behavioural response' (MBR), and a middle group of 'partial behavioural response' (PBR) (Cohen et al., 2003, 2004, 2005, 2006a–d; Cohen and Zohar, 2004). The animals are segregated using standardized 'cut-off behavioural criteria' (CBC), allowing focused study and comparison of the distinct groups, in imitation of the principle underlying the criteria applied to clinical studies. Prevalence rates of each group are assessed and correlated to bio-physiological data.

The relationship between behavioural response pattern, plasma corticosterone levels and local levels of mRNA for BDNF, TrkB, nerve growth factor (NGF), and neurotrophin-3 (NT-3) in the frontal cortex (FC) and hippocampal subregions were assessed 7 d post-PSS exposure. BDNF mRNA and behaviours were also assessed at 24 h, 48 h, and 30 d after PSS exposure. The working hypothesis was that single exposure to PSS would bring about long-term changes in the expression of mRNA in these brain areas which would be associated with behavioural disruption and circulating corticosterone levels.

## Materials and methods

All procedures were carried out under strict compliance with the ethical principles and guidelines of the NIH Guide for the Care and Use of Laboratory

**Table 1.** Sample sizes

	Naive	PSS exposure		
		EBR	MBR	PBR
Experiment 1 ( <i>n</i> = 39 animals)				
7 d post-exposure	<i>n</i> = 8	<i>n</i> = 12	<i>n</i> = 3	<i>n</i> = 15
Experiment 2 ( <i>n</i> = 24 animals)				
24 h post-exposure		<i>n</i> = 4	Missing data	
48 h post-exposure		<i>n</i> = 3	Missing data	<i>n</i> = 2
30 d post-exposure		<i>n</i> = 6	<i>n</i> = 3	<i>n</i> = 6
Experiment 3 ( <i>n</i> = 50 animals)				
7 d + protein levels	<i>n</i> = 5	<i>n</i> = 9		

Animals. All treatment and testing procedures were approved by the Animal Care Committee of the Ben-Gurion University of the Negev, Israel.

### Animals

Three-month-old male Sprague–Dawley rats weighing 200–250 g were habituated to housing conditions for at least 10 d, housed four per cage in a vivarium with stable temperature and a reversed 12-h light/dark cycle, with unlimited access to food and water. Animals were handled once daily, i.e. picked up with a gloved hand. All testing was performed during the dark phase in dim red-light conditions.

### Predator-scent stress (PSS)

The stress paradigm consisted of placing the test animals on well-soiled cat litter for 10 min (in use by the male cat for 2 d, sifted for stools). The control animals were exposed to fresh, unused litter for the same amount of time.

### Assessment schedule

Behaviours and biological parameters were assessed 7 d post-exposure, based on the recurrent finding that extreme changes which remain constant at 7 d after exposure represent ‘chronic symptoms’ (Cohen et al., 2004) and persist over the long run (Adamec, 1997; Adamec et al., 1997, 2003; Blanchard et al., 1990a,b, 1993, 1998; Cohen et al., 1996, 1999, 2000, 2003, 2004, 2005, 2006a–d; Cohen and Zohar, 2004). Moreover, BDNF mRNA and behaviours were also assessed at 24 h, 48 h and 30 d after PSS exposure. The number of animals at each sampling time is presented in Table 1.

### Behavioural measurements

#### The elevated plus maze (EPM)

The maze is a plus-shaped platform with two opposing open arms and two opposing closed arms (surrounded by 14-cm high opaque walls on three sides) (File, 1993). Rats are placed on the central platform facing an open arm and allowed to explore the maze for 5 min. Each test is videotaped and scored by an independent observer. Arm entry is defined as entering an arm with all four paws.

#### Behaviours assessed

Behaviours assessed were time spent (duration) in open and closed arms and on the central platform; number of open and closed arm entries; and total exploration (entries into all arms). Total exploration was calculated as the number of entries into any arm of the maze (total arm entries), in order to distinguish between impaired exploratory behaviour, exploration limited to closed arms (avoidance) and free exploration.

#### Acoustic startle response (ASR)

Startle response was measured using two ventilated startle chambers (SR-Lab system, San Diego Instruments, San Diego, CA, USA) calibrated daily. The SR-Lab calibration unit was used routinely to ensure consistent stabilimeter sensitivity between test chambers and over time. Each Plexiglas cylinder rests on a platform inside a soundproofed, ventilated chamber. Movement inside the tube is detected by a piezoelectric accelerometer below the frame. Sound levels within each test chamber are measured

routinely using a sound-level meter (Radio Shack; SR-Lab System, San Diego Instruments, San Diego, CA, USA) to ensure consistent presentation. Each test session started with a 5-min acclimatization period to a background of 68 dB white noise, following by 30 acoustic startle trial stimuli in six blocks (110 dB white noise of 40 ms duration with 30 or 45 s inter-trial interval).

#### *Behavioural assessment*

Assessments were: (1) mean startle amplitude; (2) percent of startle response habituation to repeated presentation of the acoustic pulse. Startle amplitude was averaged over all 30 trials. Percent habituation: the change between the response to the first block of sound stimuli and the last.

Percent habituation = 100

$$\times \frac{\left( \text{average startle amplitude in block 1} \right) - \left( \text{average startle amplitude in block 6} \right)}{\text{average startle amplitude in block 1}},$$

#### *Classification according to CBC*

The classification of individuals according to the degree to which their individual behaviour is affected by a stressor is based on the premise that extremely compromised behaviour in response to the priming trigger is not conducive to survival and is thus inadequate and maladaptive, representing a pathological degree of response. In practice, the procedure requires the following steps.

#### *Verification of overall effect of PSS exposure*

The data must demonstrate that the stressor had a significant effect on overall behaviour of exposed vs. unexposed populations at the time of assessment.

#### *Application of the CBC to the data*

The CBC used herein have been repeatedly validated in a large series of studies in rodents (Cohen et al., 2003, 2004; 2006a–d; Cohen and Zohar, 2004) and in a mathematical model (Cohen et al., 2005).

Two behavioural measures were used to define the CBC: fearful behaviour on the EPM and non-habituated exaggerated startle reaction. These were selected to constitute the basis for the behavioural criteria for a number of reasons: first, each has been shown to be a valid measure of stress responses in numerous studies, and second, each is well-defined and straightforward to score.

In order to maximize the resolution and minimize false positives, extreme responses to both of these paradigms performed in sequence were required for 'inclusion' into the EBR group, whereas a negligible degree of response to both was required for inclusion in the MBR group.

#### *Definition of CBC on each paradigm*

##### *Extreme behavioural response*

- (1) Five minutes (entire session) spent in closed arms and no entries into the open arms on the EPM.
- (2) Mean amplitude of the startle response (at 110 dB) exceeds 800 units and the startle response shows no habituation over time.

##### *Minimal behavioural response*

- (1) 0–1 min spent in closed arms and  $\geq 8$  open-arm entries on the EPM.
- (2) Mean amplitude of the startle response (at 110 dB) does not exceed 700 units and habituation is demonstrated.

##### *Partial behavioural response*

Animals neither EBR nor MBR are termed 'partial behavioural responders' (PBR).

#### *Blood sampling*

Animals were rapidly decapitated and blood from the trunk was collected into plastic tubes. Serum was separated, liquated and frozen at  $-70^{\circ}\text{C}$  until further analysis.

#### *Measurement of plasma corticosterone*

Serum corticosterone was assayed by ELISA (R&D Systems, Diagnostic System Laboratories Inc., Webster, TX, USA) according to the manufacturer's recommendations. The sensitivity of the corticosterone assay was  $12.5 \mu\text{g/l}$ . Within-assay variation was  $<10\%$  at  $100 \mu\text{g/l}$ , and between-assay variation  $<15\%$  at  $100 \mu\text{g/l}$ . The kits have a detection limit of  $<60 \text{ pg/ml}$ . The intra- and inter-assay variability was  $<10\%$ .

#### *Brains*

Brains were immediately removed from the decapitated rats. FC and dentate gyrus (DG), cornu ammonis 1 (CA1) and CA3 subregions of the hippocampus were dissected and frozen at  $-70^{\circ}\text{C}$  for biochemical and molecular biological analysis.

**Table 2.** Details of primers used in the study

Gene	Forward primer	Reverse primer	Sequence location
<i>BDNF</i>	5'-TGGCTGACACTTTTGAGCAC-3'	5'-GCAGTCTTTTTATCTGCCGC-3'	254–545
<i>TrkB</i>	5'-ACTACACCTGATGGCCAAG-3'	5'-TTGAGCAGGAGCAACATCAC-3'	1073–1261
<i>NGF</i>	5'-CTGTGGACCCCAGACTGTTT-3'	5'-ATCTCCAACCCACACACTGAC-3'	510–733
<i>NT-3</i>	5'-TGCAACGGACACAGAGCTAC-3'	5'-GTGTTTGTTCATCAATCCCCC-3'	258–639
<i>β-actin</i>	5'-CTCTTCCAGCCTTCCTTCT-3'	5'-TAGAGCCACCAATCCACACA-3'	68–320

The PCR procedure included initial denaturation at 94 °C for 5 min, followed by 30 amplification cycles, each consisting of denaturation at 94 °C for 30 s annealing at 56 °C for 45 s and extension at 72 °C for 45 s, with an additional extension step at the end of the procedure at 72 °C for 7 min. To obtain PCR results within the linear range of detection, the cDNA products were diluted 1:40 for BDNF and TrkB, 1:20 for NGF and 1:1000 for *β-actin* (an internal standard for the house-keeping gene) NT-3 was used undiluted.

### Neurotrophic factor mRNA analysis

Brain tissues were brought to room temperature, sonicated for 15 s at 50% capacity (Ultrasonic processor, Sonic Vibracell™; Sonics and Materials Inc., Newtown, CT, USA) and total RNA was isolated using Trizol Reagent (Molecular Research Center, Cincinnati, OH, USA), according to the manufacturer's instructions. RNA concentration and purity was quantified according to absorbance at 260 nm and 280 nm (GeneQuant II; Pharmacia Biotech, Piscataway, NJ, USA). RNA was reverse-transcribed into cDNA with Reverse-iT First-strand synthesis kit (ABgene, Surrey, UK) for 45 min at 42 °C in a final 20 μl reaction volume containing 1 μg total cellular RNA. In order to amplify gene-specific sequences, PCR techniques were applied using ReadyMix PCR Master Mix (ABgene) with specific primer sequences (details of primers used in the study are shown in Table 2).

Detection of RT-PCR was performed using the T-gradient thermal cycler system (Biometra Goettingen, Germany). Samples of the PCR products were run on 1% agarose gel with ethidium bromide. The final amount of RT-PCR product for each of the mRNAs species was calculated densitometrically using AIDA 2 (Dinco Co., DNR Imaging Systems Ltd, Kiryat Anavim, Israel) software. Each sample was run in duplicate and balanced between groups. The results were calculated as the intensity of the lane of each transcript over the intensity of the corresponding *β-actin* band and expressed as a mean in arbitrary units (AU).

### Western-blot analysis

Brain samples extracted from rats' CA1 and DG were sonicated for 15 s, 4 °C at 50% power capacity

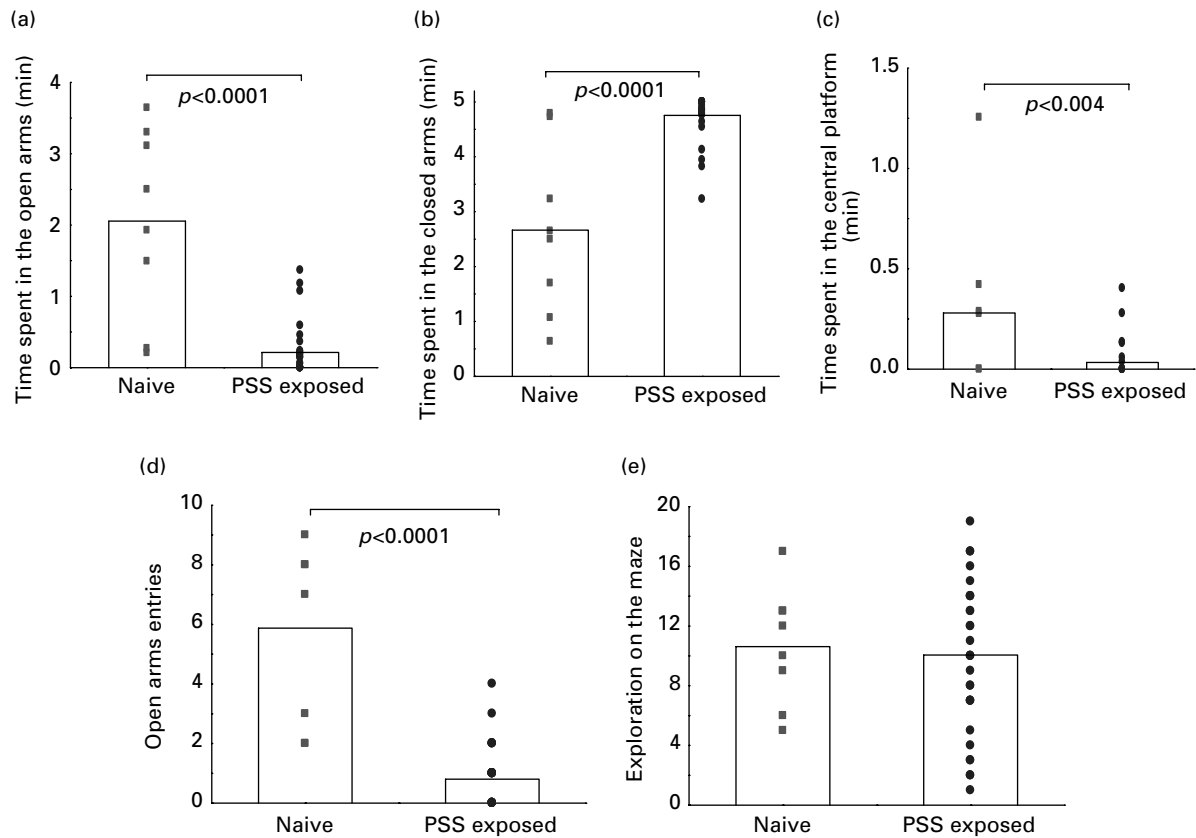
(Heat System Ultrasonic Inc.; Sonics and Materials Inc.) in 300 μl lysis buffer. After centrifugation at 10 000 g for 15 min at 4 °C the supernatant was transferred into new tubes and protein concentration determined (Bradford; Bio-Rad Protein Assay, Munich, Germany). Samples were stored at –20 °C until assayed.

Protein samples (2 μg) were separated on 10% SDS-PAGE, blotted for 2 h on PVDF membrane and blocked with TBS/T (0.61% Tris-HCl, 0.59% NaCl, 1% Tween-20; pH 7.7) containing commercial 5% non-fat dry milk and incubated overnight at 4 °C with BDNF specific antibody (1:1000, Santa Cruz Biotechnology, CA, USA). The membranes were washed with TBS/T and incubated for 1 h with HRP secondary antibody. The specific bands were detected with ECL plus kit (Amersham Bioscience, Piscataway, NJ, USA) and a sensitive film (Kodak Industries, France). Quantification was performed by El Logic 100 imaging system (Kodak) and molecular-weight analysis software (Kodak). To minimize the effect of inter-blot variability, all samples were run in duplicate on the same gel and densitometric comparison were done between control and treated samples from the same gel.

### Statistical analysis

All data were expressed as the mean ± S.E.M. and statistical analyses were performed using one-way analysis of variance (ANOVA). Where significant group effects were detected, Bonferroni test indicated significant post-hoc differences between individual groups.

To gain additional understanding about the relationship between plasma corticosterone and BDNF



**Figure 1.** Anxiety-like response (EPM) at day 7 post-PSS exposure. (a) Time spent in the open arms. (b) Time spent in the closed arms. (c) Time spent in the central platform. (d) Numbers of entries to the open arms. (e) Total exploration in the maze. A single 10-min exposure to PSS significantly enhanced anxiety-like behavioural response and avoidance of open spaces compared to control conditions (unexposed). All data represent group mean  $\pm$  the scatter plot of the data.

mRNA levels, the correlations were computed. Linear regression between BDNF levels in the CA1 and DG hippocampal subregions, and corticosterone levels were assessed for individuals, within and between all groups.

Throughout the study data were analysed for exposed vs. unexposed populations as a whole, and then re-analysed according to the CBC classification separately for EBR, MBR and PBR vs. unexposed controls, and compared to each other.

## Results

### Anxiety-like response (EPM) at day 7 post-PSS exposure

A single 10-min exposure to PSS significantly increased anxiety-like behaviour/avoidance of open spaces compared to unexposed controls. Values for time spent in the open arms (Figure 1a) [ $F(1, 37)=49.0$ ,  $p<0.0001$ ], time spent in the central platform (Figure 1c) [ $F(1, 37)=9.3$ ,  $p<0.004$ ], and entries into open arms

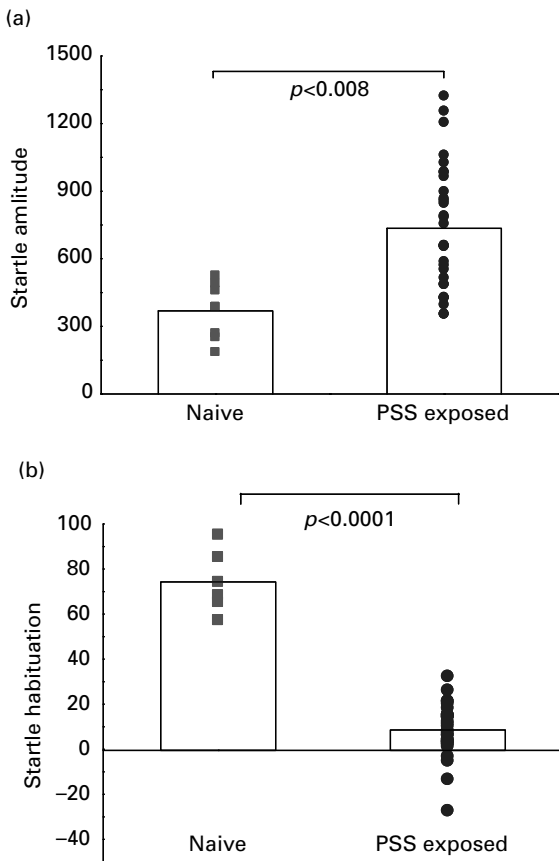
(Figure 1d) [ $F(1, 37)=64.5$ ,  $p<0.0001$ ] were significantly decreased after stress exposure, compared to control conditions. Complementing this, time spent in the closed arms was significantly increased in the exposed group compared to unexposed controls [ $F(1, 37)=49.06$ ,  $p<0.0001$ ].

There were no differences in total exploration of the maze between groups (Figure 1e). This result suggests overall anxiety and avoidance of exploration in the open arms, as opposed to an impairment of locomotion/exploration.

### ASR at day 7 post-PSS exposure

Figure 2a shows the mean startle amplitude of rats in response to 30 startle pulses. Stress exposure significantly increased the mean startle amplitude in exposed rats compared to controls [ $F(1, 37)=13.4$ ,  $p<0.008$ ].

Figure 2b shows that percent habituation of the startle response differed significantly between the groups [ $F(1, 37)=209.2$ ,  $p<0.0001$ ]. Stress exposure



**Figure 2.** Acoustic startle response (ASR) at day 7 post-PSS exposure. (a) Startle response amplitude. (b) Percent habituation of startle response. PSS exposure significantly elevated mean startle amplitude and caused a significant deficit in the habituation of ASR in exposed rats compared to controls. All data represent group mean  $\pm$  the scatter plot of the data.

caused a significant deficit in the habituation of ASR in exposed animals compared to controls.

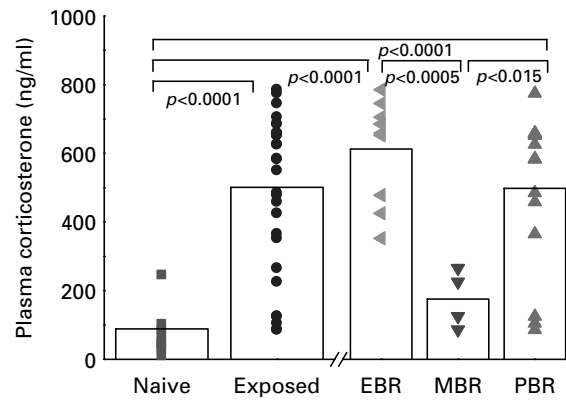
#### **Prevalence of rates of extreme responders at day 7 post-PSS exposure**

In the exposed group 38.7% ( $n=12$ ) fulfilled criteria for EBR, 12.9% ( $n=4$ ) for MBR and 48.4% for PBR ( $n=15$ ).

#### **Circulating levels of corticosterone at day 7 post-PSS exposure**

##### **Exposed vs. unexposed populations**

The exposed population demonstrated significantly increased plasma corticosterone levels compared to controls [ $F(1, 37) = 26.7, p < 0.0001$ ] (Figure 3).



**Figure 3.** Circulating levels of corticosterone at day 7 post-PSS exposure. PSS exposure significantly increased plasma corticosterone levels in exposed animals compared to controls. Stress exposure significantly increased plasma corticosterone levels in EBR individuals compared to the naive (control) group and compared to the MBR group. There were significant differences between the MBR and PBR groups. All data represent group mean  $\pm$  the scatter plot of the data.

##### **Re-analysis according to CBC**

Significant differences in plasma corticosterone levels were demonstrated between the subgroups of exposed animals [ $F(3, 35) = 27.4, p < 0.0001$ ]. Post-hoc Bonferroni test revealed that plasma corticosterone levels of EBR individuals were significantly higher than in MBR individuals ( $p < 0.0005$ ), or controls ( $p < 0.0001$ ). The levels of PBR animals also varied significantly from those of MBR ( $p < 0.015$ ) (and controls). There were no statistically significant differences between naive rats and MBR individuals.

#### **BDNF mRNA levels at day 7 post-PSS exposure**

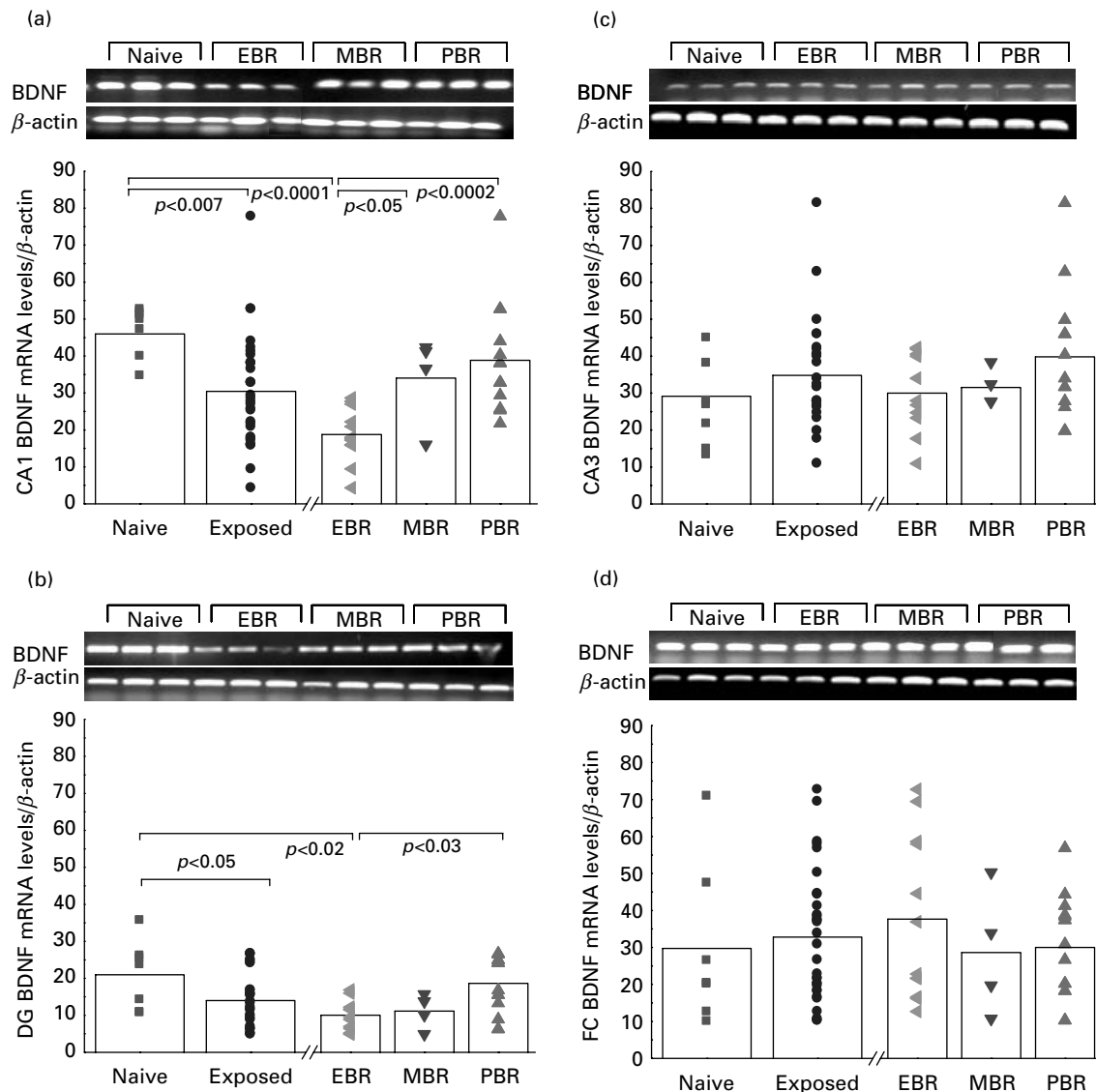
Figure 4 shows the mRNA BDNF levels in the subregions of the hippocampus and FC.

##### **Exposed vs. unexposed populations**

The exposed population demonstrated significantly decreased CA1 (Figure 4a) and DG (Figure 4c) mRNA BDNF levels compared to controls [ $F(1, 37) = 8.2, p < 0.007$ , and  $F(1, 37) = 4.4, p < 0.045$  respectively].

##### **Re-analysis according to CBC**

In the hippocampal subregions CA1 (Figure 4a) and DG (Figure 4c), there were significant



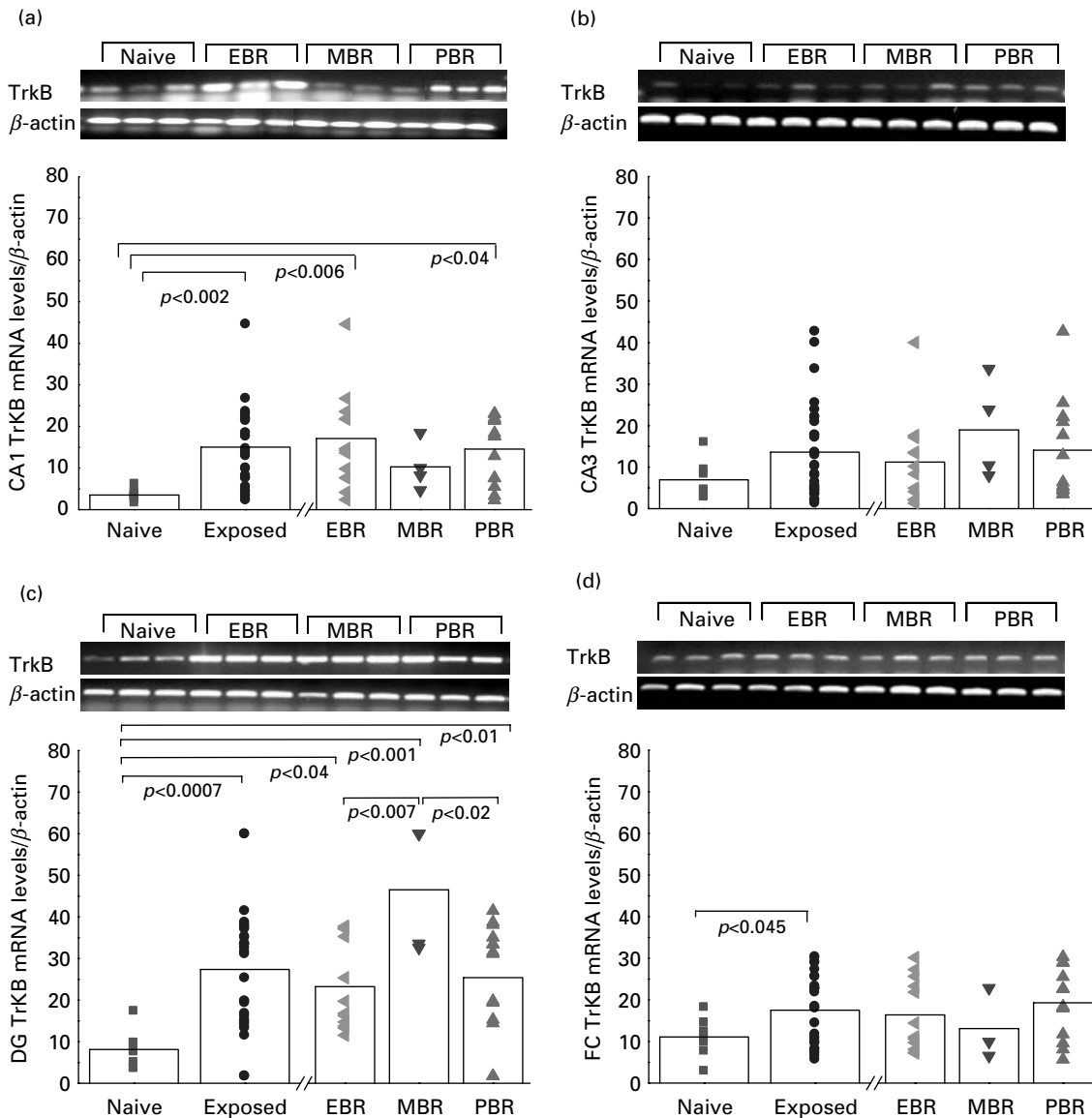
**Figure 4.** BDNF mRNA levels at day 7 post-PSS exposure. RT-PCR analysis of BDNF mRNA expression in representative gels (the upper panel) and densitometry values in the hippocampal CA1 subregion (a), CA3 (b), and DG subregions (c) and frontal cortex (d).  $\beta$ -actin was used as the internal control. Densitometry values are means of 3–5 gels. PSS exposure significantly decreased CA1 and DG mRNA BDNF levels in exposed individuals compared to unexposed controls. CA1 and DG BDNF mRNA levels in the EBR group were significantly decreased compared to controls and to MBR groups. All data represent group mean  $\pm$  the scatter plot of the data.

differences between groups [ $F(3, 35) = 12.1$ ,  $p < 0.0001$  and  $F(3, 35) = 5.3$ ,  $p < 0.005$  respectively]. Post-hoc Bonferroni test revealed that stress exposure significantly decreased CA1 BDNF mRNA levels in EBR individuals compared to controls ( $p < 0.0001$ ) and compared to MBR ( $p < 0.05$ ) and to PBR animals ( $p < 0.0002$ ). In the DG area Bonferroni test revealed that PSS exposure significantly decreased BDNF

mRNA levels in EBR individuals compared to controls ( $p < 0.02$ ) and to PBR animals ( $p < 0.03$ ). There were no significant differences between EBR and MBR individuals in the DG subregion.

In the CA3 subregions (Figure 4b) and the FC (Figure 4d) no significant differences were found between exposed animals and controls. Applying CBC revealed no additional information.





**Figure 5.** TrkB mRNA levels at day 7 post-PSS exposure. RT-PCR analysis of TrkB mRNA expression in representative gels (the upper panel) and densitometry values in the hippocampal CA1 subregion (a), CA3 (b), and DG subregions (c) and frontal cortex (d).  $\beta$ -actin was used as the internal control. Densitometry values are means of 3–5 gels. Stress exposure significantly increased TrkB mRNA levels in CA1, DG and FC compared to unexposed controls. In CA1, stress exposure significantly increased TrkB mRNA levels in EBR animals compared to controls. TrkB mRNA levels were also significantly higher in PBR individuals compared to controls. In the DG, PSS significantly increased TrkB mRNA levels in all the exposed groups compared to the unexposed group. TrkB mRNA levels were significantly higher in the MBR group compared to EBR and PBR animals. All data represent group mean  $\pm$  the scatter plot of the data.

#### TrkB mRNA levels at day 7 post-PSS exposure

Figure 5 shows the TrkB mRNA levels in the subregions of the hippocampus and FC.

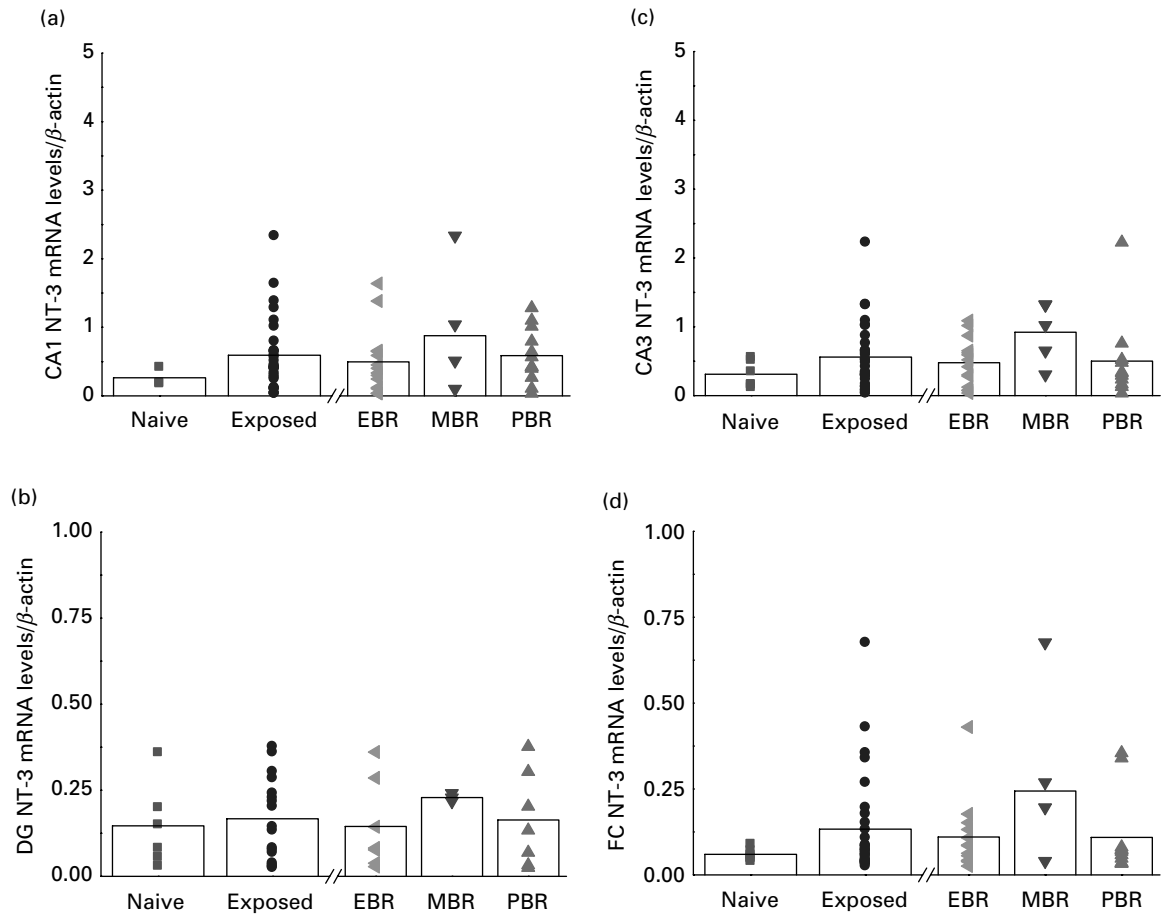
#### Exposed vs. unexposed populations

The exposed population demonstrated significantly increased TrkB mRNA levels in CA1 (Figure 5a), DG

(Figure 5c), and FC (Figure 5d) compared to controls [ $F(1, 37) = 11.8$ ,  $p < 0.002$ ;  $F(1, 37) = 13.7$ ,  $p < 0.00073$ ;  $F(1, 37) = 4.5$ ,  $p < 0.045$  respectively].

#### Re-analysis according to CBC

In the hippocampal CA1 (Figure 5a) and DG (Figure 5c) subregions, there were significant differences in



**Figure 6.** NT-3 mRNA levels at day 7 post-PSS exposure. (a) NT-3 mRNA levels in the CA1 subregion. (b) NT-3 mRNA levels in the CA3 subregion. (c) NT-3 mRNA levels in the DG subregion. (d) NT-3 mRNA levels in the frontal cortex. There were no statistically significant differences between groups in NT-3 mRNA levels in all brain areas, between the unexposed and the exposed groups or amongst the exposed subgroups. All data represent group mean  $\pm$  the scatter plot of the data.

TrkB mRNA levels between groups [ $F(3, 35)=4.69$ ,  $p<0.008$ ;  $F(3, 353)=10.4$ ,  $p<0.00005$  respectively). Post-hoc Bonferroni test revealed that stress exposure significantly increased TrkB mRNA levels in the EBR groups compared to controls ( $p<0.006$ ) in CA1. Moreover, TrkB mRNA levels were significantly higher in the PBR group compared to controls ( $p<0.04$ ). The CA1 TrkB mRNA levels were not significantly different in the MBR group compared to controls.

In the DG, post-hoc Bonferroni test revealed that stress exposure significantly increased TrkB mRNA levels in all the exposed groups compared to controls (EBR,  $p<0.04$ ; MBR,  $p<0.001$ ; PBR,  $p<0.01$ ). The TrkB mRNA levels were significantly higher in the MBR group compared to the EBR ( $p<0.007$ ) and PBR ( $p<0.02$ ) groups.

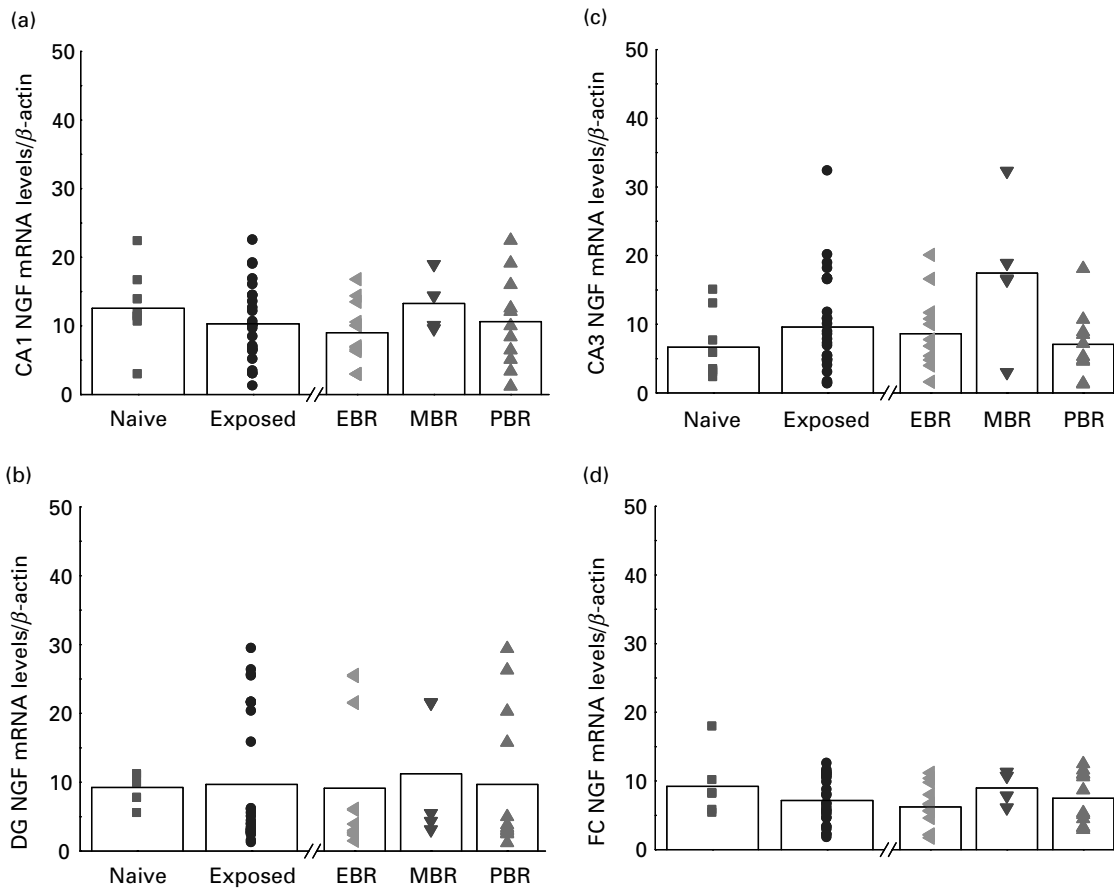
In the CA3 subregion and in the FC, TrkB mRNA levels were not significantly different between the groups.

#### NT-3 mRNA levels at day 7 post-PSS exposure

Figure 6 shows the NT-3 mRNA levels in the subregions of the hippocampus and FC. There were no statistically significant differences between groups in NT-3 mRNA levels in all brain areas, between the naive unexposed and the exposed groups, or amongst the exposed behavioural subgroups.

#### NGF mRNA levels at day 7 post-PSS exposure

Figure 7 shows the NGF mRNA levels in the subregions of the hippocampus and FC. Here too, there



**Figure 7.** NGF mRNA levels at day 7 post-PSS exposure. (a) NGF mRNA levels in the CA1 subregion. (b) NGF mRNA levels in the CA3 subregion. (c) NGF mRNA levels in the DG subregion. (d) NGF mRNA levels in the frontal cortex. There were no statistically significant differences between groups in NGF mRNA levels in all brain areas, between the unexposed and the exposed groups or amongst the exposed subgroups. All data represent group mean  $\pm$  the scatter plot of the data.

were no statistically significant differences between any groups for any of the brain areas.

### Correlations

Figure 8(a, b) shows the linear correlations between plasma corticosterone vs. BDNF mRNA levels in the CA1 and DG respectively. This study revealed no evidence of a significant correlation between BDNF levels in any of the brain areas assessed and circulatory corticosterone levels, within groups or between them.

### BDNF mRNA levels at 24 h, 48 h, 7 d and 30 d post-PSS exposure

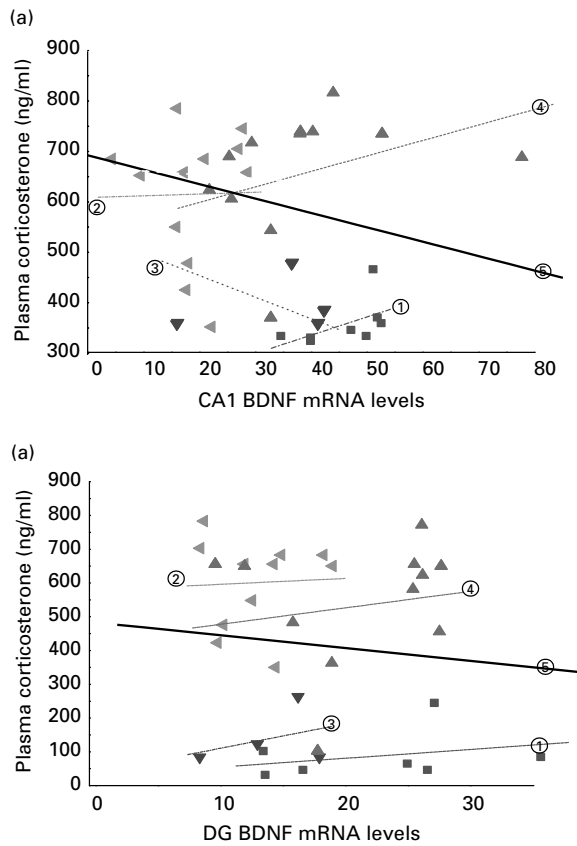
Figure 9(a, b) shows the mRNA BDNF levels in the hippocampal CA1 area in the EBR group. EBR individuals displayed significantly lower BDNF

mRNA throughout the 30 d compared to controls [ $F(4, 26) = 11.36, p < 0.00002$ ]. In contrast, the PBR and the MBR groups displayed no significant changes in BDNF mRNA levels across time (Figure 9b).

Figure 9(c, d) shows the mRNA BDNF levels in the hippocampal DG area in the EBR group. The EBR animals displayed significantly lower BDNF mRNA in the DG 7 d post-PSS exposure compared to controls, but at 30 d post-exposure the expression of BDNF mRNA was increased significantly compared to the other groups ( $p < 0.05$ ). In contrast, the PBR and the MBR groups displayed no significant changes in BDNF mRNA levels across time (Figure 9b).

### BDNF protein levels at day 7 post-PSS exposure

Figure 10 shows the BDNF protein levels in the hippocampal CA1 and DG subregions. EBR



**Figure 8.** Correlations. (a) Linear correlations between plasma corticosterone and hippocampal CA1 BDNF mRNA levels. (b) Linear correlations between plasma corticosterone and hippocampal DG BDNF mRNA levels. There were no significant correlations between BDNF levels in any of the brain areas assessed and circulatory corticosterone levels, within groups or between them. CA1 hippocampal subregion: Panel (a), Unexposed group (■): ① [ $r=0.53$ ,  $F(1,6)=2.3$ ,  $p=0.2$ ]; EBR individuals (◄): ② [ $r=0.02$ ,  $F(1,10)=0.003$ ,  $p=0.95$ ]; MBR rats (▼): ③ [ $r=0.35$ ,  $F(1,2)=0.13$ ,  $p=0.75$ ]; PBR rats (▲): ④ [ $r=0.32$ ,  $F(1,13)=1.5$ ,  $p=0.24$ ]; All rats: ⑤ [ $r=0.3$ ,  $F(1,37)=3.5$ ,  $p=0.09$ ]. DG hippocampal subregion: Panel (b), Unexposed group (■): ① [ $r=0.3$ ,  $F(1,6)=0.5$ ,  $p=0.52$ ]; EBR individuals (◄): ② [ $r=0.05$ ,  $F(1,10)=0.2$ ,  $p=0.9$ ]; MBR rats (▼): ③ [ $r=0.36$ ,  $F(1,2)=0.3$ ,  $p=0.64$ ]; PBR rats (▲): ④ [ $r=0.16$ ,  $F(1,13)=0.32$ ,  $p=0.58$ ]; All rats: ⑤ [ $r=0.1$ ,  $F(1,37)=0.5$ ,  $p=0.49$ ].

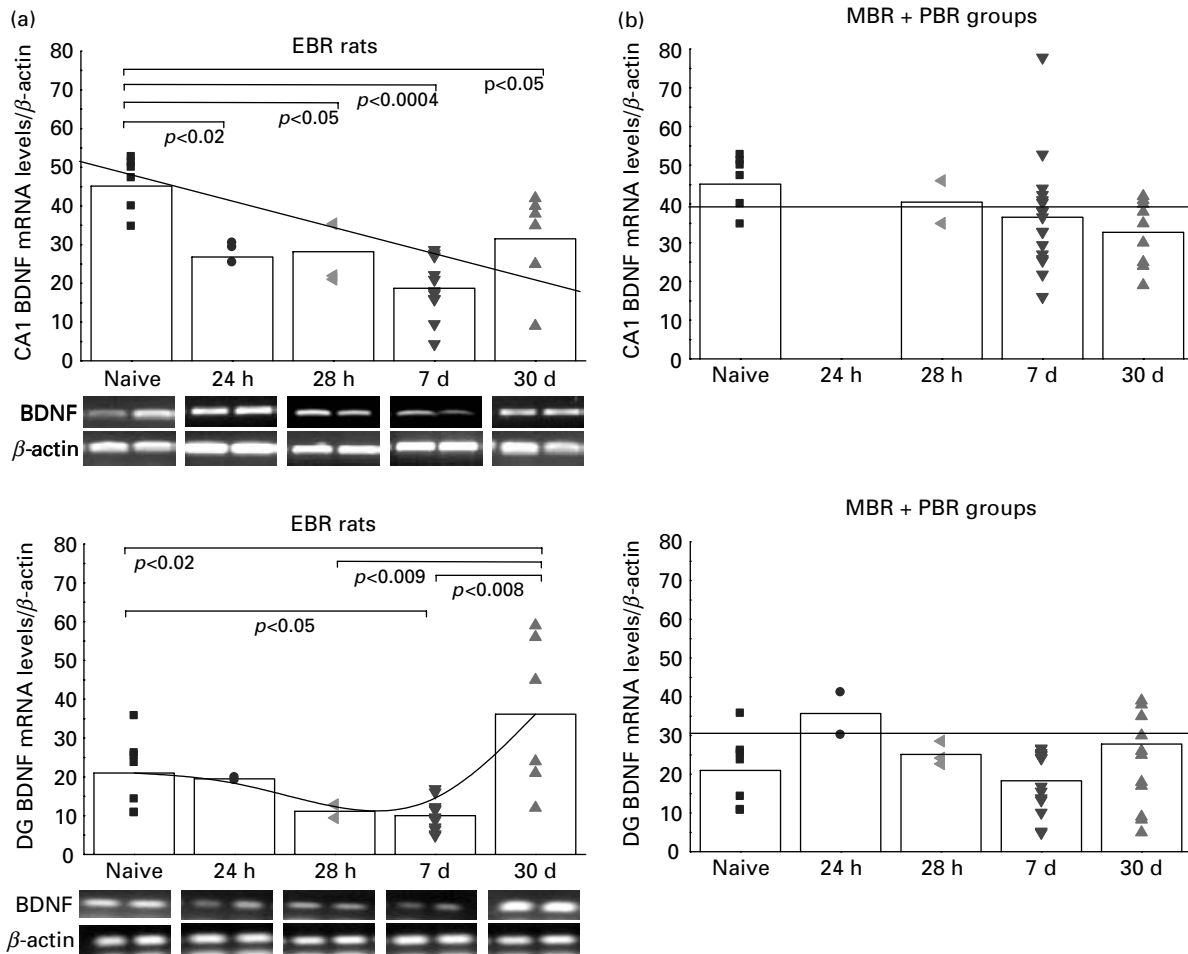
individuals displayed significantly lower BDNF protein levels in the CA1 (Figure 10a, b) and DG (Figure 10c, d) subregions compared to unexposed controls [ $F(1,12)=4.5$ ,  $p<0.05$ ;  $F(1,12)=6.3$ ,  $p<0.03$  respectively].

The summary of overall findings are presented in Table 3.

## Discussion

Overall, compared to unexposed controls, animals exposed to PSS demonstrated a significant degree of anxiety-like and avoidant behaviours on the EPM and exaggerated startle responses with significantly reduced habituation. The increase in anxiety-related behaviour 7 d after exposure was accompanied by significantly elevated plasma corticosterone levels, decreased BDNF mRNA levels in the CA1 and DG hippocampal subregions, and increased TrkB mRNA levels in the CA1 and DG areas of hippocampus and in the FC. These effects were specific for BDNF and TrkB in all groups across all brain areas assessed. Only individuals whose behaviour was extremely disrupted (EBR) in response to PSS continued to demonstrate a persistent significant decrease in BDNF mRNA expression in the CA1 region at day 30, whereas changes in other brain areas were transient. Animals whose behaviour was less severely affected displayed transient changes in BDNF levels.

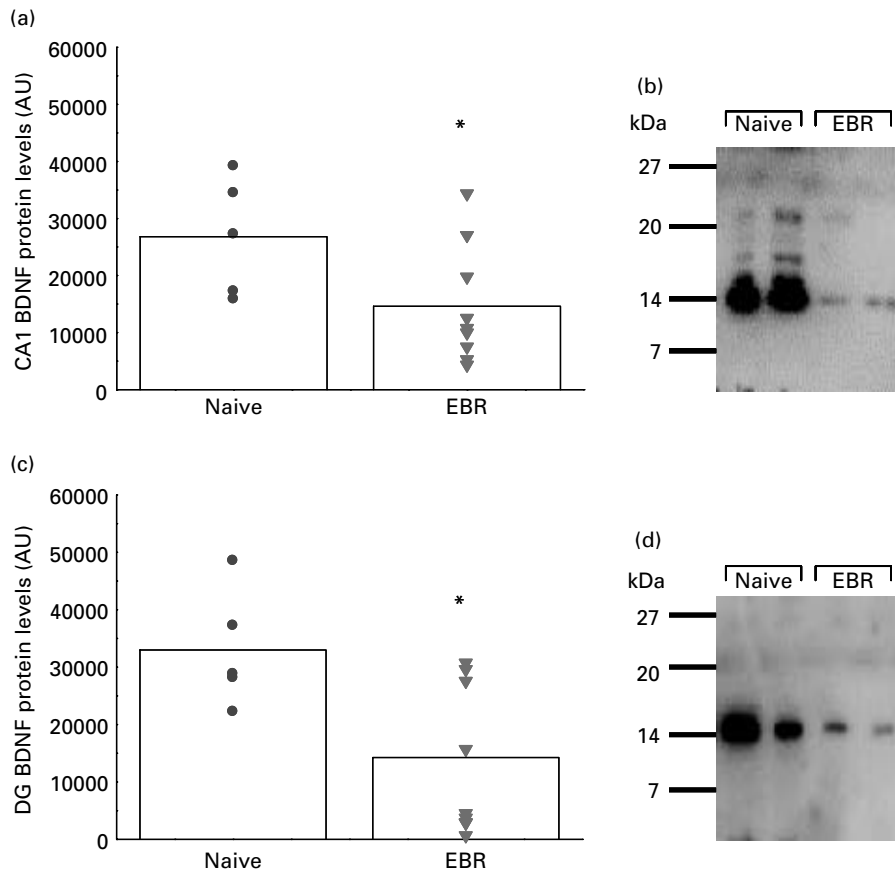
Stress-related changes in levels of BDNF mRNA have been reported quite extensively, for both acute and chronic stress paradigms. The results are not uniform and are difficult to compare accurately due to methodological differences between studies, including degree of resolution of brain areas under study; different stress paradigms, duration of exposure and number of exposures to the stressor and time of assessment; assessment of additional parameters of the stress response (physiological, behavioural, etc). Short periods (15 min) of immobilization stress increase BDNF mRNA expression in whole rat hypothalamus immediately after exposure (Rage et al., 2002); 1 h of immobilization stress increased BDNF mRNA in CA1, CA3, CA4 and DG (Marmigere et al., 2003); the same manipulation, but for 8 h, was reported to decrease BDNF mRNA levels in the hippocampus (Ueyama et al., 1997); single or repeated immobilization markedly reduced BDNF mRNA levels in the DG and hippocampus (Smith et al., 1995); repeated-restraint stress over 1–3 wk caused significant reduction in BDNF mRNA in the hippocampus overall (Murakami et al., 2005); inescapable tail-shock stress significantly increased BDNF mRNA levels in the FC immediately after the stress session (Bland et al., 2005); the same stressor caused a transient decrease in DG BDNF mRNA levels 60 min after the stress session, and BDNF mRNA levels were restored to normal within 48 h (Rasmusson et al., 2002). Thus, although the results are not uniform, it seems quite clear that BDNF levels in the hippocampus, a structure involved in functions related to memory and apparently a key



**Figure 9.** BDNF mRNA levels at 24 h, 48 h, 7 d and 30 d post-PSS exposure. (a) RT-PCR analysis of BDNF mRNA expression in representative gels (bottom panel) and densitometry values in the hippocampal CA1 subregion in the EBR individuals. (b) The densitometry values of BDNF mRNA expression in the hippocampal CA1 subregion in the MBR + PBR groups. EBR animals displayed significantly lower BDNF mRNA throughout the 30 days compared to unexposed rats. The PBR and MBR groups displayed no significant changes in BDNF mRNA levels across time. (c) RT-PCR analysis of BDNF mRNA expression in representative gels (bottom panel) and densitometry values in the hippocampal DG subregion in the EBR individuals. (d) The densitometry values of BDNF mRNA expression in the hippocampal DG subregion in the MBR + PBR groups. In the DG, the EBR animals displayed significantly lower BDNF mRNA 7 d post-PSS exposure compared to controls, but at 30 d post-exposure the expression of BDNF mRNA demonstrated partial recovery.  $\beta$ -actin was used as the internal control. Densitometry values are means of 3–5 gels. All data represent group mean  $\pm$  the scatter plot of the data.

focus for the psychopathology of PTSD, are significantly affected by stress. There are indications of a close relationship between BDNF expression and neurogenesis at the hippocampal level (Cameron et al., 1998). Moreover, impaired neurogenesis has been observed in the DG of chronically stressed rats (McEwen, 2000). It is possible that stress-induced down-regulation of BDNF synthesis in the hippocampus contributes to the pathogenesis of dendritic retraction.

Alterations in TrkB expression have also been reported to be associated with exposure to stress. Whereas Smith et al. (1995) found that single or repeated immobilization stress had no effect on the expression of TrkB. Givalois et al. (2001) reported that immobilization stress elicited a transient decrease in TrkB mRNA signal in the anterior pituitary. In an extensive study of the effect of two different repeated-stress paradigms (immobilization stress for 10 d and repeated unpredictable stress) compared to



**Figure 10.** BDNF protein levels at day 7 post-PSS exposure. Western blot analysis of BDNF protein levels in representative gels (b), and densitometry values in the hippocampal CA1 subregion (a), and in the DG tissue (c, d). PSS exposure significantly decreased CA1 and DG BDNF protein levels in extreme behavioural response individuals compared to unexposed controls. All data represent group mean  $\pm$  the scatter plot of the data.

single-exposure, Nibuya et al. (1999) reported that both paradigms increased the expression of catalytic TrkB mRNA, but did not influence expression of truncated TrkB transcripts in the hippocampus. In contrast, acute immobilization stress did not influence the expression of either catalytic or truncated TrkB mRNA, while the expression of BDNF mRNA was down-regulated by both acute and repeated immobilization. The authors thus suggested that up-regulation of catalytic TrkB mRNA is dependent on the 'chronicity' of the exposure (repeated stress).

In contrast to Nibuya's findings, in our study a single brief exposure to PSS was associated with an up-regulation of the expression of TrkB mRNA 7 d later. Thus, a different cellular response mechanism may be involved in the expression of BDNF and of TrkB mRNA, depending on the intensity, duration and character of the stressor. Nibuya et al. (1999) suggest that the relative up-regulation of TrkB may reflect

an adaptive compensatory response to stress-induced down-regulation of BDNF, possibly related to the well-established finding that long-term administration of corticosterone down-regulates BDNF expression, but does not affect TrkB (Nibuya et al., 1999).

Application of CBC revealed that the prevalence of severely behaviourally affected (EBR) rats amongst the exposed animals was 38.7%, whereas the prevalence of MBR rats was 12.9%. The behaviours of all other rats (48.4%) fell between the CBC for the extreme groups (PBR). The EBR individuals were characterized by a unique pattern of corticosterone and neurotrophic factor levels, significantly different from those observed in other groups; PSS exposure significantly increased plasma corticosterone concentrations only in EBR (behaviourally 'symptomatic'), but not in MBR rats or in unexposed controls. The EBR animals exhibited significantly lower BDNF mRNA levels and higher TrkB mRNA in the CA1 and DG hippocampal

**Table 3.** Summary of overall findings

Parameters levels compared to				
Naive	EBR	MBR	PBR	
<b>7 d post-exposure</b>				
Plasma corticosterone (ng/ml)	↑	—	↑	
BDNF mRNA/ $\beta$ -actin				
CA1	↓	—	—	
CA3	—	—	—	
DG	↓	—	—	
FC	—	—	—	
TrkB mRNA/ $\beta$ -actin				
CA1	↑	—	—	
CA3	—	—	—	
DG	↑	↑	↑	
FC	—	—	—	
NT-3 mRNA/ $\beta$ -actin				
CA1	—	—	—	
CA3	—	—	—	
DG	—	—	—	
FC	—	—	—	
NGF mRNA/ $\beta$ -actin				
CA1	—	—	—	
CA3	—	—	—	
DG	—	—	—	
FC	—	—	—	
BDNF protein/ $\beta$ -actin				
CA1	↓	—	—	
DG	↓	—	—	
<b>30 d post-exposure</b>				
BDNF mRNA/ $\beta$ -actin				
CA1	↓	—	—	
DG	↑	—	—	

subregions at day 7 compared to naive unexposed rats and to the PBR group. These animals also exhibited significantly lower BDNF protein levels in the CA1 and DG hippocampal subregions at day 7 compared to naive unexposed rats, concomitant with BDNF mRNA levels.

The expression of BDNF mRNA was examined over the course of the trial at 24 h, 48 h, 7 d and 30 d post-exposure and compared between behavioural response groups. The EBR individuals displayed lower BDNF mRNA throughout the 30 d in CA1 and a transient down-regulation at day 7 in DG, which recovered at day 30. In contrast, the PBR and MBR groups displayed no significant changes in BDNF mRNA levels. The EBR rats displayed only minimal BDNF mRNA 'recovery' or none at all, throughout the trial period. The differences in long-term

down-regulation of BDNF mRNA in the CA1 as opposed to the DG areas may stem from the differences in apoptosis and neurogenesis in different sub-areas of the adult rat hippocampus (Kozorovitskiy and Gould, 2003; Lucassen et al., 2001; Mirescu and Gould, 2006). The DG region is particularly sensitive to stress, as evidenced by experience-dependent structural changes and thus is more vulnerable to stress-related adverse adaptive modification (Mirescu and Gould, 2006; Warner-Schmidt and Duman, 2006). Restorative neurogenesis may occur in the DG sooner than in the CA1 region.

Long-term BDNF deficiency in the hippocampus could have physiological consequences, inducing damage to hippocampal neurons. Neurotrophins, and particularly BDNF, are known to modulate many aspects of neuronal plasticity (Shieh and Ghosh, 1999; Thoenen, 2000) and the selection of functional neuronal connections in the CNS (Huang and Reichardt, 2001; Mamounas et al., 2000; Poo, 2001). The decreased expression of BDNF mRNA in EBR individuals may decrease synaptic plasticity and impair the stabilization of synaptic connectivity, leading to vulnerability to psychopathology. Endogenous BDNF has been implicated in the process of memory consolidation, although the precise nature of this involvement is unknown. The persistent lowering of BDNF levels in the clearly more extremely affected individuals in this study might reflect or mediate a disturbance which models the anomalous process of memory consolidation observed in PTSD patients (van Praag, 2004).

The present study examined the association between the molecular responses to stress in hippocampal sub-areas and behavioural parameters with a high degree of resolution. The findings are thus of importance, implying a possible association between the molecular findings and psychopathological processes which result in altered behaviour.

The observed alterations in BDNF expression in different brain areas at different points in time may represent normal stress responses aimed at restoring homeostasis. They may equally play a causal role in the pathogenesis of a disordered stress response or may represent markers thereof. Certain of these changes appear to be transient and others more long term. The patterns of these responses across time (and their significance) require further study.

### Limitations

The overall sample size is sufficient for statistical analysis, but larger samples would be preferable.

Animal models are restricted to the assessment of observable behaviours, and thus are not able to reflect criterion B (intrusive recollection) accurately in a single-exposure design.

### Conclusions

There is a clear-cut association between extreme behavioural response to PSS and long-term down-regulation of mRNA for BDNF in the CA1 subregion of the hippocampus. Animals whose behavioural response is less extreme (or minimal) demonstrate only transient changes.

It is unclear whether this finding represents a marker for extreme response or whether it has pathological significance.

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### Statement of Interest

None.

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