Social deprivation stress is a triggering factor for the emergence of anxiety- and depression-like behaviours and leads to reduced brain BDNF levels in C57BL/6J mice

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Summary Stress is a main risk factor that can trigger psychiatric disorders, including anxiety and major depression. Neurotrophins, such as Brain-Derived Neurotrophic Factor (BDNF), have been identified as neuroendocrine effectors involved in the response to stress and in the neurobehavioural changes associated with depression. Aim of this paper was to study the relationship between neuroendocrine activation (circulating corticosterone and brain BDNF levels) and a wide array of depression- and anxiety-like behaviours (anhedonia, behavioural despair, generalised and social anxiety) resulting from exposure to chronic stress. To this end, 3-month-old C57BL/6J male mice were exposed to either chronic disruption of the social structure (SS), to a stable social structure (SG) or to social deprivation (SD), a condition lacking social stimuli. Results show that, despite not developing anhedonia (decreased preference for a sucrose solution), SD mice were characterised by increased emotionality and hypothalamic—pituitary—adrenal axis reactivity in addition to reduced BDNF levels. By contrast, SG and SS mice showed increased anhedonia accompanied by no alterations in the behavioural and neuroendocrine profile. The results here reported indicate that mice exposed to different social housing conditions use different behavioural strategies to cope with external challenges. In addition they suggest that social deprivation might represent a stressful condition triggering the emergence of both anxiety- and depression-like behaviours and clearly indicate BDNF as a main neurobiological variable mediating these responses.

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1. Introduction

Stress is a main risk factor for psychopathology as chronic exposure to severe physical or emotional stimuli can precipitate major depression in vulnerable individuals (Kendler et al., 1999; Cirulli et al., 2009a). Clinical evidence indicates that in depressed patients, emotional arousal, cognitive abnormalities and vulnerability to psychotic episodes are associated to a hyperactive hypothalamic—pituitary—adrenal (HPA) axis (de Kloet et al., 2007). In addition to glucocorticoids (GCs), neurotrophins, such as Brain-Derived Neurotrophic Factor (BDNF), have been identified as neuroendocrine effectors involved in the response to stress and to neurobiological and behavioural changes associated with depression (Pezawas et al., 2004; Duman and Monteggia, 2006; Castren et al., 2007; Schmidt and Duman, 2007; Brunoni et al., 2008; Cirulli and Alleva, 2009; Cirulli et al., 2009b, 2010b). Indeed, decreased blood BDNF levels characterise subjects diagnosed as major depressives, antidepressants reverting this neurobiological change (Shimizu et al., 2003; Karege et al., 2005; Duman and Monteggia, 2006; Sen et al., 2008). In rodents, chronic stress decreases the expression of this neurotrophin, which can lead to neuronal atrophy in the hippocampus and other brain structures (Schmidt and Duman, 2007), while direct hippocampal infusion of BDNF produces anxiolytic and antidepressant effects (Siuciak et al., 1997; Shimizu et al., 2003; Cirulli et al., 2004). In addition, a very recent study reports that knocking-down BDNF in specific rat's brain sites precipitates behaviours associated with depression such as preference for a sucrose solution (Taliaz et al., 2010).

Progress in understanding the pathophysiology of major depression and treatment development would greatly benefit from appropriate animal models (Nestler and Hyman, 2010). The chronic mild stress (CMS) paradigm has been developed in rodents to simulate unpredictable stress that may occur in everyday life (Willner et al., 1987). This procedure is effective in inducing neurobehavioural disturbances, such as a reduction in responsiveness to a reward, which has been compared to anhedonia, one of the core symptoms of human depression (DSM-IV, American-Psychiatric-Association, 1994). Moreover, all the behavioural alterations induced by CMS can be reversed by chronic antidepressant administration (Willner et al., 1987). Although the CMS paradigm appears to be able to reproduce a fair number of depression-like symptoms, data relating specific physiological changes with the behavioural phenotype resulting from exposure to this procedure are scattered and often conflicting. As an example, Larsen and colleagues, despite a behavioural depressive-like phenotype, found an increase, rather than the expected decrease, in BDNF mRNA expression in the hippocampus of rats exposed to chronic unpredictable stress (Larsen et al., 2010). In addition, results from studies assessing anxiety-related behaviours in CMS animal models of depression are rather ambiguous, since some authors report decreased (Kopp et al., 1999) while others find increased anxiety after this procedure (Kompagne et al., 2008). These discrepancies might depend upon the interplay among different neurobiological variables known to affect anxiety- and depression-like behaviours including HPA axis activation and levels of serotonin (5-HT) and BDNF (Nutt and Stein, 2006) as well as upon the ultimate length of the stress period. Indeed, although there are sufficient and significant differences between anxiety and depression, which support the view that they are independent clinical entities, there is also evidence that these psychopathologies co-exist and that anxiety typically precedes depressive disorders (Ballenger, 1999; Ninan, 1999; Nutt and Stein, 2006). Such co-morbidity is due to the fact that these two pathologies share common neurobiological mechanisms, as increased 5-HT levels, associated with administration of selective 5-HT reuptake inhibitors (SSRIs), are not only able to reduce depressive symptoms but also to reduce anxiety levels (Cryan and Holmes, 2005).

Social stress in humans represents a major etiological factor in the development of emotional disorders, including depression (Leskela et al., 2006; McEwen and Gianaros, 2010). Based on the notion that changing the social structure in a group of mice is a stressful event, especially if this is repeated over time (Avitsur et al., 2002; Schmidt et al., 2010), we chose such paradigm to increase the chance of stimulating behavioural and neuroendocrine responses that have been shaped by evolutionary processes. With this study we aimed at developing a comprehensive and ethologically relevant experimental method to induce chronic stress in mice and to investigate its effects on the interplay among behavioural, neuroendocrine and neurobiological aspects. More in detail, adult male mice (age 3 months), of the C57BL/6J strain, underwent either a Chronic Social Stress (SS), consisting of an experimentally induced disruption of the social structure, or group-housing in a stable social arrangement (SG). It is widely reported that the presence of social odour cues emanating from the mouse body, originating largely from their urine, are involved in the advertisement of dominance over a defend territory (Gosling and McKay, 1990; Hurst, 1990). Therefore by changing the composition of the social group (SS condition) we mimicked the presence of an intruder in the territory, which might lead to a change in the social structure. However, also the maintenance of a stable social structure may represent a source of stress (Avitsur et al., 2003). In fact, it has been shown that group-housed mice show altered behavioural, endocrine and immune indices of stress (Sgoifo et al., 2001). In particular, Bartolomucci et al. (2002) have provided evidence that lack of familiarity/relatedness among cage-mates might be regarded as a main source of stress. Thus, we compared SS and SG to a further condition, i.e. social deprivation (SD). This condition, consisting of prolonged individual housing, is characterised by the lack of social stimuli and, if prolonged, might result in the so-called "isolation syndrome" (Valzelli, 1973).

The effectiveness of such procedures to activate the neuroendocrine system was assessed by measuring the activation of the HPA axis through plasma levels of corticosterone (CORT). We hypothesised that, following stress, a combination of anxiety and depressive-like behaviours might become manifest in the experimental subjects. Thus we selected those tests which might be more appropriate (according to previous data) to discriminate between these two. To this aim we compared the selected stressful procedures in their ability to induce a depressive-like state was assessed by measuring behaviours such as levels of anhedonia (preference for sucrose solution) and behavioural despair (Forced Swim Test). In addition generalised (Elevated Plus Maze),

social anxiety (Social Interaction Test) and the emotional response to a novel environment (Open Field test) were also observed. Levels of BDNF protein were measured in the limbic system as a possible marker of vulnerability to stress-induced depression.

2. Materials and methods

2.1. Animals

Experimental subjects were 44 adult male mice of the C57BL/6J strain that were purchased from a commercial breeder (Charles River, Calco, Italy). Upon arrival all animals were singly housed in the same room provided by air conditioning (temperature $21 \pm 1\,^{\circ}C$, relative humidity $60 \pm 10\%$), in transparent Plexiglas cages (29 cm × 12 cm × 14 cm), under a reversed 12/12 h light/dark cycle with lights off from 0800 to 2000 h. Pellet food (standard diet Altromin-R, purchased from Rieper, Italy) and tap-water were continuously available. Animals were left undisturbed for 1 week before the beginning of the experimental procedure. At the end of this period animals were allowed to habituate to a sucrose solution in order to establish an individual baseline. When the baseline was established mice were assigned to 3 conditions: two group-housing (social stress — SS, $n = 16$; social group — SG, $n = 16$) and one individual-housing condition (social deprivation — SD, $n = 12$); home cages for grouped mice had the following dimensions 37 cm × 21 cm × 19 cm. Sucrose preference was assessed once a week, during 3 weeks (day 0 — baseline, day 7, 14 and 21). At the end of the third week of stress, 8 mice were randomly selected from each social condition to undergo a battery of behavioural tests aimed at assessing the emotional response to a novel environment (Open Field — OF), depressive-like behaviours (Forced Swim Test — FST), generalised (Elevated Plus Maze — EPM) and social anxiety (Social Interaction Test — SIT). During the behavioural assessment the groups/housing conditions (SD, SG and SS) were maintained until the brief (24 h) social isolation preceding the SIT. In particular, on the first day of behavioural testing mice underwent Open Field (0930—1130 h) and four hours later experimental subjects were assessed in the Elevated Plus Maze (1530—1730 h). On the next day a first Forced Swim Test session was performed, followed by a second session 24 h later (0930—1030 h). On the following day mice underwent an acute social challenge (Social Interaction Test) between 1700 and 1800 h. Before the beginning of behavioural tests, all subjects were moved to the testing room (by the experimenter) and left there to habituate for 1 h. Behavioural performances were video recorded and the behavioural analysis was carried out from the videotape by an observer blind to the social condition, using commercial software ("The Observer 3.0"). At the end of each behavioural session apparatuses were cleaned by a cotton pad wetted with a 50% solution of ethanol and water. All behavioural tests were conducted under dim red light (1 lx) between 0930 and 1730 h (i.e. during mice's active-period) except for the Social Interaction Test which was used as a social challenge to assess CORT levels during the circadian trough (i.e. between 1700 and 1800 h). In addition, CORT levels were assessed in response to the

Social Interaction Test. Ten minutes following the end of this test (i.e. 30 min from the beginning) subjects were sacrificed (between 1730 and 1830 h), brains were dissected out and BDNF levels measured in the hypothalamus, hippocampus, frontal cortex, striatum and midbrain. Moreover, adrenal glands were also dissected out, and the ratio of adrenal weight to body weight was calculated in order to provide indirect evidence of hypercorticism-related hypertrophy. All behavioural tests, CORT and BDNF measurements were conducted on 8 subjects per group randomly chosen. The number of subjects was defined on the basis of the distribution of the dependent variables to be assessed and the experimental design, with a level of significance Alpha = 0.05 in a two-tailed test and a power of 1-Beta = 0.80.

Animal handling and experimental procedures were performed in accordance with the EC guidelines (EC Council Directive 86/609 1987) and with the Italian legislation on animal experimentation (Decreto L.vo 116/92).

2.2. Experimental procedures

2.2.1. Sucrose Preference Test — SPT
One week following arrival, experimental subjects underwent 7 days of familiarisation with a novel sucrose solution in order to establish the individual preference. During this phase (familiarisation) mice were housed individually and each cage was provided by two bottles, one containing fresh tap-water and one containing a 4% sucrose solution (Pothion et al., 2004; Lewis et al., 2005). Bottles were daily weighed in order to monitor liquid consumption and switched to balance the effect of side preference in drinking behaviour, which was reported to be of importance for the correct evaluation of sucrose preference (Kant and Bauman, 1993; Strekalova et al., 2004).

The sucrose preference was calculated only on the last day (day 7) of the sucrose exposure period following this procedure: mice were deprived of food and water for 5 h (from 0900 to 0200 h), soon after they were provided again with two bottles either with tap-water or with a fresh sucrose solution for 1 h (in the absence of food). Sucrose preference was then calculated as follows: % sucrose preference = (sucrose solution intake × 100)/(water intake + sucrose solution intake). Sucrose consumption was derived from bottles' weight. Following the establishment of the sucrose consumption baseline (day 0), on the days anhedonia was assessed (day 7, 14 and 21), SG and SS mice were singly housed for 5 h in order to score individual consumption, while SD mice did not change their social condition.

2.2.2. Chronic Social Stress — CSS
Mice were assigned to 3 different conditions: SS, SG or SD. More in detail, SS mice ($n = 16$) were ear-marked and housed into 4 cages (4 mice/cage) and social structure was disrupted twice a week for three weeks by replacing one mouse with a novel unfamiliar one selected randomly from another cage. Sawdust was replaced at the same time in each cage. Mice in the SG condition ($n = 16$) were also housed in groups of 4 mice/cage however members of each group remained always the same, possibly leading to a stable social structure. Cages were cleaned and sawdust replaced twice a week mimicking

the handling procedure which the SS group was subjected to. SD mice (n = 12) were individually housed and cages also cleaned and sawdust replaced once a week.

2.2.3. Open Field — OF

Mice were individually placed in the centre of a cubic arena (open field box 44 cm × 44 cm × 44 cm) made of grey Plexiglas and allowed to freely explore for 20 min; during the last 5 min of the test a novel object (a 50 ml glass beaker) was introduced in the centre of the arena. The open field box was ideally divided into 25 squares and ideally partitioned into a central portion (26.4 cm × 26.4 cm) and a peripheral one, identified as the remaining part of the arena. When data were analysed, each session was subdivided in 4 time blocks (tb) and the time spent in each portion of the arena as well as latency, frequency and duration of locomotion (*crossings* of squares), exploratory activity (*sniffing* and *rearing*), risk assessment (*stretch-attend posture* — *SAP*), self-directed behaviours (*self-grooming*) and object exploration (*sniffing* and *touching*) were scored. Behaviours were defined as follows:

- *crossing*, crossing the square limits with all paws;
- *sniffing*, self explanatory;
- *rearing*, standing on the hind paws;
- *stretch-attend posture*, exploratory posture in which the body is stretched forward and then retracted to the original position without any forward locomotion;
- *self-grooming*, rubbing the body with paws or mouth and rubbing the head with paws.

When the stimulus object was present, latency to the first *contact* and *sniffing* and frequency of *contacts* and *sniffing* of the object were also scored.

2.2.4. Elevated Plus Maze — EPM

The Elevated Plus Maze was made of two open arms (30 cm × 5 cm × 0 cm) and two closed arms (30 cm × 5 cm × 15 cm) that extended from a common central platform (5 cm × 5 cm). Each arm was ideally divided into 4 portions in order to facilitate scoring of locomotor activity (*crossings*, see below). The apparatus, made of Plexiglas (grey floor, clear walls), was elevated to a height of 60 cm above the floor. Mice were individually placed on the central platform facing an open arm and allowed to freely explore the maze for 5 min. Behavioural parameters observed were: frequencies of total, open and closed entries (arm entry = all four paws into an arm), % open entries [(open/total) × 100], and time spent in open and closed parts of the maze (File, 2001). Furthermore, we also scored latency, frequency and duration of *crossing, sniffing, rearing, self-grooming* (for a detailed description see Section 2.2.3), *head dipping* (*HED*, exploratory movement of head and shoulders over the edge of the maze) and *SAP*.

2.2.5. Forced Swim Test — FST

Mice were tested according to the Porsolt's procedure (Porsolt et al., 1977). Each experimental subject was gently placed into a cylindrical glass (20 cm Ø, 40 cm height), filled with 25 cm of water at a temperature of 26 ± 1 °C for 6 min on 2 consecutive days with a dim light illumination (1 lx). When removed from the water, mice were allowed to dry for 5 min under red light. Twenty-four hours later, a second session took place and latency, frequency and duration of the following behavioural responses were scored: *struggling* (vigorous attempts at climbing the walls of the cylinder); *swimming* (active swimming around) and *floating* (total absence of movement).

2.2.6. Social Interaction Test — SIT

A Social Interaction Test was used as a social stressful challenge in order to test the activity of the HPA axis and to assess social anxiety. The night before the test all subjects were placed in a holding cage to stimulate social interactions (Terranova et al., 1993; Cirulli et al., 1996; Panksepp et al., 2007). Experimental subjects were placed in a novel cage, identical to the holding cage, ideally subdivided in three equal parts, with an unfamiliar conspecific of the same strain, weight and sex that had been previously isolated (standard opponent). Standard opponents were marked by a yellow, scentless and nontoxic paint 30 min before testing, in order to discriminate the experimental subject from the unfamiliar conspecific during data collection. The maximum length of social encounters was 20 min; by the time of behavioural observation, each session was subdivided in 4 tb and frequency and duration of *environmental exploration* (sum of *crossing, sniffing* and *wall rearing*), *social investigation* (sum of *body, nose* and *ano-genital sniffing*), *affiliative behaviours* (*allogrooming* and *following*) and *displacement/ de-arousal behaviours* (sum of *digging* and *self-grooming*) were scored. Behaviours were defined as follows:

- *wall rearing*, animal stands on its hind limbs and touches the walls of the cage with the forelimbs;
- *body sniffing*, sniffing any other area of the body of the opponent;
- *nose sniffing*, sniffing the head and the snout region of the opponent;
- *ano-genital sniffing*, sniffing the ano-genital area of the opponent;
- *allogrooming*, grooming the opponent;
- *following*, experimental subject follow the standard opponent;
- *digging*, animal digs the sawdust with the forelimbs, often kicking it away with the hind limbs.

For crossing, sniffing and self-grooming see Section 2.2.3.

Animals were blood sampled by tail nick, for basal CORT levels, the night (1730–1830 h) before the last Sucrose Preference Test. At the end of the social challenge (1730–1830 h), all subjects were sacrificed, trunk blood was collected for CORT assessment and tissues dissected out (brain regions and adrenals) and immediately frozen until quantification of BDNF.

2.2.7. Radioimmunoassay for corticosterone determination — RIA

Blood samples (100 μl, approximate volume) were collected individually in potassium EDTA coated tubes (1.6 mg EDTA/ml blood, Sarstedt, Germany). All samples were kept on ice and later centrifuged at 3000 rpm for 15 min at +4 °C. Blood plasma was transferred to Eppendorf tubes for CORT determination and stored at −20 °C until further analysis. CORT

was measured using a commercially available radioimmu-noassay (RIA) kit containing [125]iodine labelled CORT; 5 μl of plasma were sufficient to carry out CORT measurement.

Sensitivity of the assay was 0.125 μg/dl, inter- and intra-assay variation was less than 10 and 5%, respectively (MP Biomedicals Inc., CA, USA). Vials were counted for 2 min in a gamma-scintillation counter (Packard Minaxi Gamma counter, Series 5000).

2.2.8. BDNF measurement

BDNF evaluation was carried out with an enzyme-linked immunosorbent assay kits (Emaxtm ImmunoAssay System number G6891, by Promega, Madison, Wisconsin, USA) following the instructions provided by the manufacturer. Following sacrifice brains were quickly removed and the frontal cortex, hippocampus, hypothalamus, striatum and midbrain were dissected out and immediately stored at −80 °C until used. Brain tissues were homogenised in a lysis buffer prepared according to the kit instructions and centrifuged at 8500 rpm, and the supernatant was used for BDNF analyses. Briefly, BDNF standard and brain samples were distributed in 96-well immunoplates precoated with monoclonal anti-mouse BDNF antibody (100 μl/well) and incubated for 2 h at room temperature. After washing plates were incubated with an anti-human BDNF antibody for 2 h at room temperature. The plates were washed again and then incubated with an anti-IgY horseradish peroxidase (HRP) for 1 h at room temperature. Tetramethylbenzidine (TMB)/peroxidase substrate solution was added to the wells to produce colorimetric reaction measured at 450 nm with a microplate reader (Dynatech MR 5000, Dynatech Laboratories, Chantilly, Virginia, USA). BDNF concentrations were determined from the regression line for the BDNF standard incubated under similar conditions in each assay. The sensitivity of the assay was about 15 pg/mg of BDNF, and the cross-reactivity with other related neurotrophic factors (NGF, NT-3, and NT-4) is considered nil (Aloe et al., 1999).

2.2.9. Statistical analysis

Data were analysed using parametric analysis of variance (ANOVA) with "social condition" as between-subjects factor and "time blocks" and "zone" as within-subject repeated measures, when appropriate (Sucrose Preference Test, Open Field, Elevated Plus Maze, Forced Swim Test, Social Interaction Test, CORT assessment and BDNF). *Post hoc* comparisons were performed using the Tukey's test. In analysing data on social investigation (Social Interaction Test), this test was used in the absence of significant ANOVA effects according to the indications given by Wilcox (Wilcox, 1987). Statistical analysis was performed using Statview II (Abacus Concepts, CA, USA). Data are expressed as mean + SEM. A significance level of 0.05 was chosen.

3. Results

3.1. Sucrose Preference Test — SPT

Social condition affected the preference for a sucrose solution both in the SG and SS groups but not in SD mice (main effect of the social condition: $F_{(2,41)} = 253.791$; $p < 0.0001$, *post hoc* SD *vs*. SG and SS $p < 0.01$; SG *vs*. SS $p < 0.05$). The
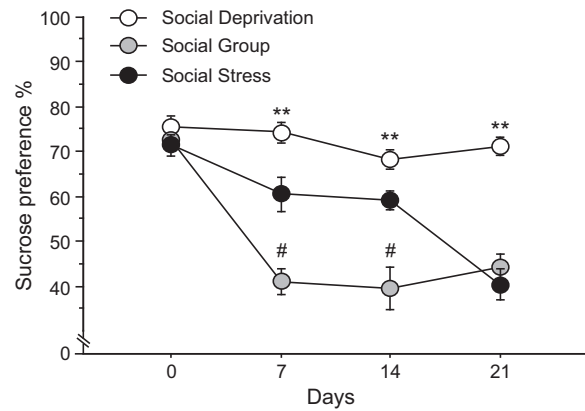

Figure 1　Assessment of anhedonia in Sucrose Preference Test. Anhedonia increased over time in the social group (SG) and social stress (SS) conditions only. Results are presented as mean ± S.E.M. (social deprivation − SD, $n = 12$; SG and SS, $n = 16$), **$p < 0.01$ SD *vs*. SG and SS (days 7, 14 and 21), #$p < 0.01$ SG *vs*. SS (days 7 and 14).

three groups showed the same basal preference for the sucrose solution which did not decrease in the SD group. After the first week the SG group developed a strong anhedonic profile which did not change from day 7 to 21. By contrast, the SS group developed anhedonia only during the last week of the stress procedure (day 21) (interaction between social condition and days: $F_{(6,123)} = 10.561$; $p < 0.0001$, *post hoc* comparisons: SD *vs*. SG and SS $p < 0.01$ on days 7, 14 and 21; SG *vs*. SS $p < 0.01$ on days 7 and 14; see Fig. 1).

3.2. Open Field — OF

All subjects spent significantly more time in the peripheral part of the arena (main effect of the zone: $F_{(1,21)} = 202.673$; $p < 0.0001$), this was particularly evident for the SD group (interaction between zone and social condition: $F_{(2,21)} = 9.093$; $p = 0.0006$, *post hoc* SD *vs*. SG and SS $p < 0.05$ (Fig. 2A). Locomotor activity overall did not differ among groups (main effect of the social condition: $F_{(2,21)} = 0.042$; $p = 0.9585$) and decreased for all subjects from tb 1 to 3 and increased again during tb 4, i.e. when a novel object was introduced in the arena (effect of time: $F_{(3,63)} = 10.416$; $p < 0.0001$, *post hoc* tb 1 *vs*. tb 2 and 3 $p < 0.05$; mean ± standard error: 40.2 ± 2.5; 36.1 ± 2.5; 34.7 ± 2.7; 37.5 ± 2.6, respectively for tb 1, 2, 3 and 4). The SD group performed more *rearing* than the other groups (effect of the social condition: $F_{(2,21)} = 12.112$; 9.204; $p < 0.0001$; $p = 0.0006$, respectively for frequency and duration, *post hoc* SD *vs*. SG and SS $p < 0.01$) and this behaviour persisted over time during the test (interaction between tb and social condition: $F_{(6,63)} = 3.750$; 2.274; $p = 0.0020$; 0.0416, respectively for frequency and duration, *post hoc* SD *vs*. SG and SS tb 2, 3 and 4 $p < 0.05$; mean ± standard error: 51.2 ± 5.2; 65.4 ± 5.3; 60.7 ± 5.2; 36.8 ± 4.2, for the SD group for each tb respectively; 38.5 ± 3.4; 42.3 ± 5.8: 34.5 ± 5.7; 14.1 ± 2.9, for the SG group for each tb respectively; 34.9 ± 4.9; 31.4 ± 5.6; 32.4 ± 7.2; 20.3 ± 4.5, for SS group for each tb respectively). Overall, SD mice also showed a higher *self-grooming* frequency (effect of social condition:
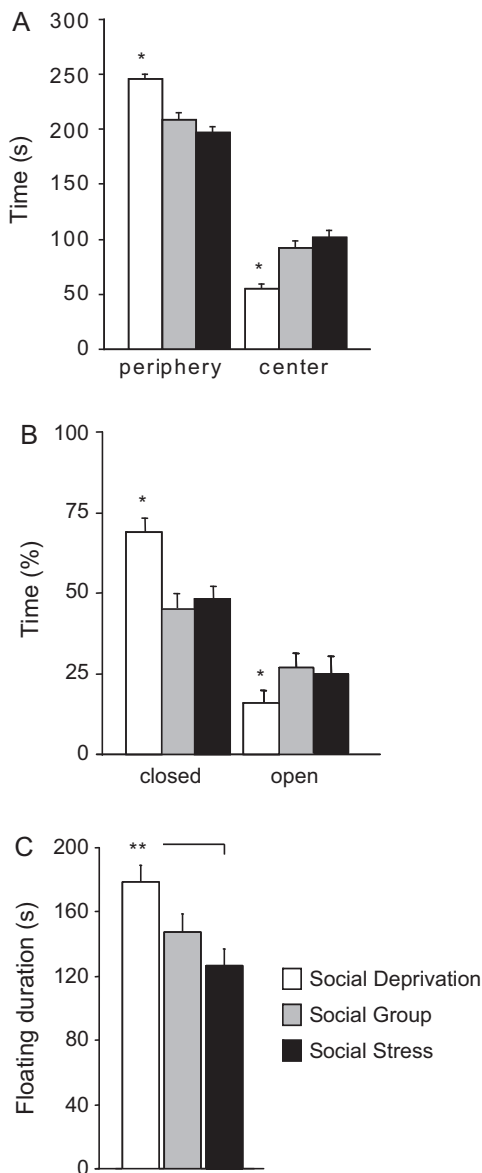
Figure 2 Emotional and depression-like behaviours. Socially deprived (SD) mice were characterised by higher emotional responsiveness towards a novel environment as well as by increased anxiety since they spent more time respectively in the periphery of the Open Field arena (A) and in the closed arms of the Elevated Plus Maze (B). In addition, socially deprived mice showed the longest floating duration in the Forced Swim Test indicating a depression-like behavioural profile (C). Data shown are mean + S.E.M. ($n = 8$ for each experimental group). $*p < 0.05$, $**p < 0.01$.

($F_{(2,21)} = 4.857$; $p = 0.0134$, *post hoc* SD *vs.* SG and SS $p < 0.05$), while the duration of this behaviour was not significant ($F_{(2,21)} = 2.340$; $p = 0.1105$).

3.3. Elevated Plus Maze — EPM

All subjects spent more time in the closed arms of the maze (effect of the zone: $F_{(1,21)} = 36.952$; $p < 0.0001$, *post hoc* closed arms *vs.* open arms $p < 0.01$), however this behaviour was particularly evident for the SD group (interaction

between zone and social condition: $F_{(2,21)} = 4.463$; $p = 0.0185$, *post hoc* SD *vs.* SG and SS $p < 0.05$, see Fig. 2B) that was characterised by a decreased locomotion (effect of social condition: $F_{(2,21)} = 6.125$; $p = 0.0051$, *post hoc* SD *vs.* SG and SS $p < 0.05$), a higher frequency and duration of *rearing* ($F_{(2,21)} = 3.873$; 3.436; $p = 0.0300$; 0.0431, *post hoc* SD *vs.* SS $p < 0.05$, respectively for frequency and duration) and by a shorter latency and higher frequency of *SAP* (effect of social condition: $F_{(2,21)} = 3.355$; 4.337; $p = 0.0461$; 0.0205, respectively for latency and frequency, *post hoc* SD *vs.* SG $p < 0.05$, for latency; SD *vs.* SG and SS $p < 0.05$, for frequency). In addition, SD together with the SS group, also showed a higher frequency and duration of *self-grooming* (effect of social condition: $F_{(2,21)} = 4.694$; 3.134; $p = 0.0154$; 0.0556, *post hoc* SD and SS *vs.* SG $p < 0.05$, respectively for frequency and duration).

3.4. Forced Swim Test — FST

SD mice were overall characterised by a higher *floating* duration (effect of social condition: $F_{(2,21)} = 4.014$; $p = 0.0334$, *post hoc* SD *vs.* SS $p < 0.01$, see Fig. 2C) associated to a lower *struggling* frequency (effect of social condition: ($F_{(2,21)} = 4.461$; $p = 0.0243$, *post hoc* SD *vs.* SG $p < 0.05$). No difference was found among the three groups as for *floating* frequency ($F_{(2,21)} = 2.659$; $p = 0.935$), *struggling* duration ($F_{(2,21)} = 0.717$; $p = 0.4996$) and frequency and duration of *swimming* (effect of social condition: $F_{(2,21)} = 2.106$; 1.665; $p = 0.1468$; 0.2131, respectively for frequency and duration).

3.5. Social Interaction Test — SIT

Despite the prolonged social deprivation (3 weeks) SD mice did not show any signs of aggressiveness while were characterised by a higher frequency and duration of *social investigation* (main effect of social condition: $F_{(2,21)} = 3.755$; 3.206; $p = 0.0403$; 0.0510, respectively for frequency and duration, *post hoc* SD *vs.* SG $p < 0.05$, both for frequency and duration, Fig. 3A) and spent a lower amount of time exploring the environment when compared to both SG and SS (effect of social condition on duration: $F_{(2,21)} = 6.467$; $p = 0.0065$, *post hoc* $p < 0.05$, see Fig. 3B). Frequency of *environmental exploration* did not differ among the social conditions ($F_{(2,21)} = 1.075$; $p = 0.3594$). SS mice were characterised by a lower frequency and duration of *displacement behaviours* (effect of social condition: $F_{(2,21)} = 8.592$; 4.067; $p = 0.0019$; 0.0321, respectively for frequency and duration, *post hoc* SS *vs.* SD and SG $p < 0.05$, see Fig. 3C).

No difference was found as for frequency and duration of *affiliative behaviours* ($F_{(2,21)} = 2.462$; 1.890; $p = 0.1095$; 0.1759, respectively for frequency and duration).

3.6. Corticosterone

Basal CORT levels did not differ as a result of social condition ($F_{(2,20)} = 0.442$; $p = 0.6487$). The Social Interaction Test was effective in inducing the activation of the HPA axis in all the social conditions (effect of repeated measures: $F_{(1,20)} = 295.963$; $p < 0.0001$, *post hoc* basal *vs.* stress $p < 0.01$),
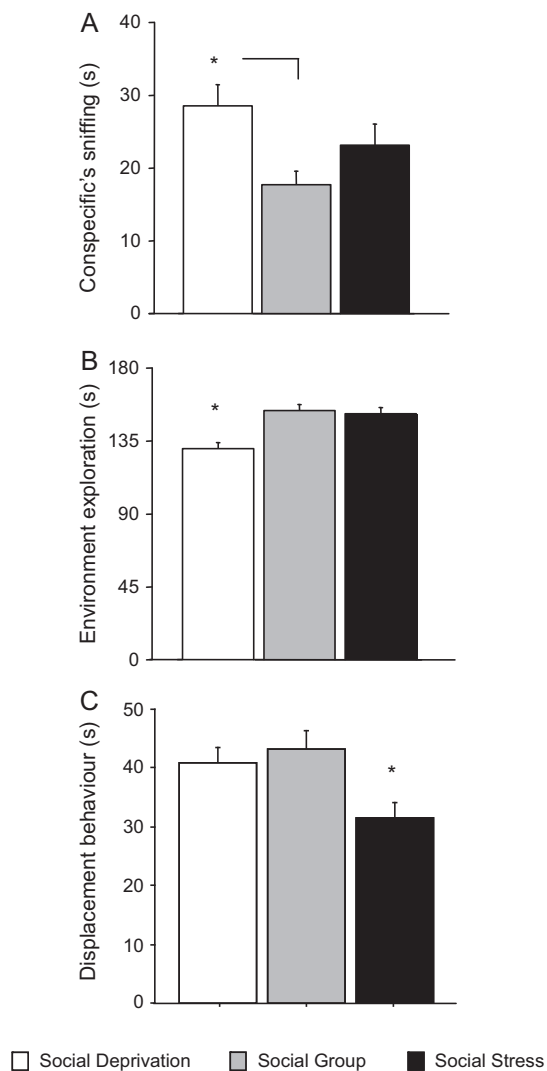
Figure 3 Social anxiety. Results from the Social Interaction Test allowed a fine discrimination of the effects produced by chronic exposure to the different social contexts on social behaviour. Socially deprived (SD) mice spent more time investigating an unknown conspecific partner (A) than the new environment (duration of environmental exploration) (B). The social stress (SS) group spent less time in displacement behaviours than socially deprived and social group (SG) mice (C). Data show mean + S.E.M. (n = 8 for each experimental group). *$p < 0.05$.
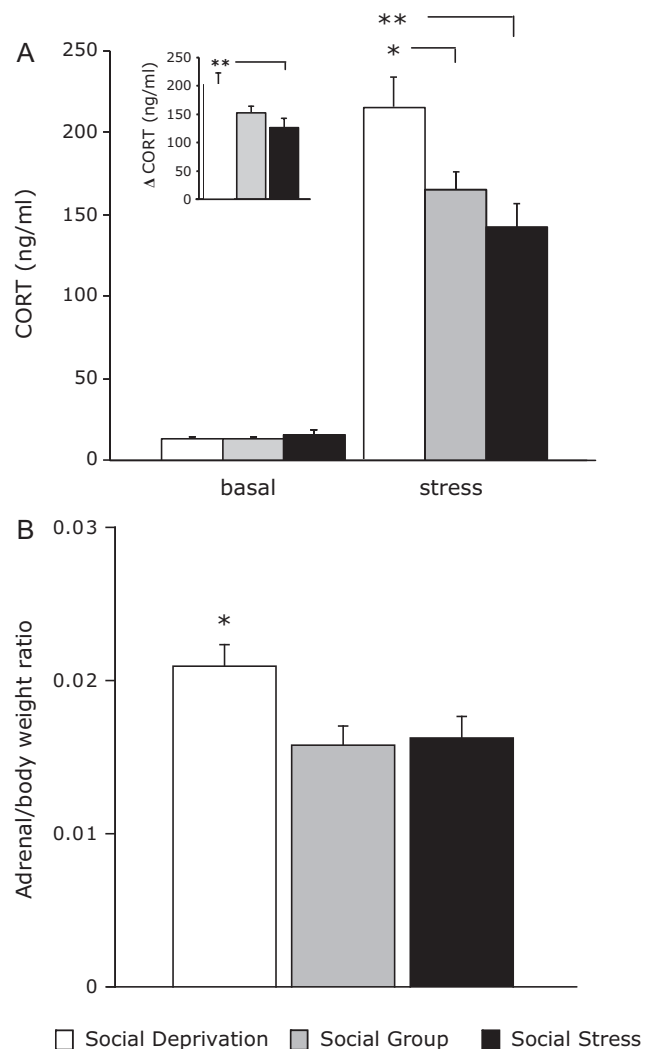

Figure 4 Neuroendocrine activation following a social challenge. Although all subjects showed an activation of the HPA axis following the social challenge, socially deprived (SD) mice were characterised by a greater increase in corticosterone levels. In the inset the difference between basal and stress corticosterone levels (delta) (A). (SD and social group — SG, n = 8; SS, n = 7). In addition, the social deprivation group showed the highest ratio adrenal/body weight, suggesting a chronic stress-induced hypertrophy of the glands (B). Data show mean + S.E.M. (n = 8 for each experimental group). *$p < 0.05$, **$p < 0.01$.

however CORT levels showed a higher increase in the SD group as a result of the social challenge (interaction between social condition and repeated measures: $F_{(2,20)}$ = 5.569; p = 0.0120, post hoc SD vs. SG $p < 0.05$ and SD vs. SS $p < 0.01$, Fig. 4A).

3.7. Adrenals/body weight ratio

Despite no difference in body weight among groups ($F_{(2,21)}$ = 0.385; p = 0.6849), SD mice showed the highest values for adrenals/body weight ratio ($F_{(2,21)}$ = 7.103; p = 0.0044, post hoc SD vs. SG and SS $p < 0.05$, Fig. 4B).

3.8. BDNF

Social condition was able to affect BDNF levels as measured in the frontal cortex, hippocampus, hypothalamus and midbrain ($F_{(2,21)}$ = 16.091; 12.667; 12.764; 4.937; p = 0.0001; 0.0002; 0.0002; 0.0175, respectively for each area, see Fig. 5A—D). In particular the SD group was characterised always by lower levels of this neurotrophin (post hoc SD vs. SG $p < 0.05$, for frontal cortex and hippocampus; SD vs. SG and SS $p < 0.05$, for hypothalamus and midbrain). SD mice were also characterised by a tendency to reduced BDNF levels in the striatum when compared to SG subjects (effect of social
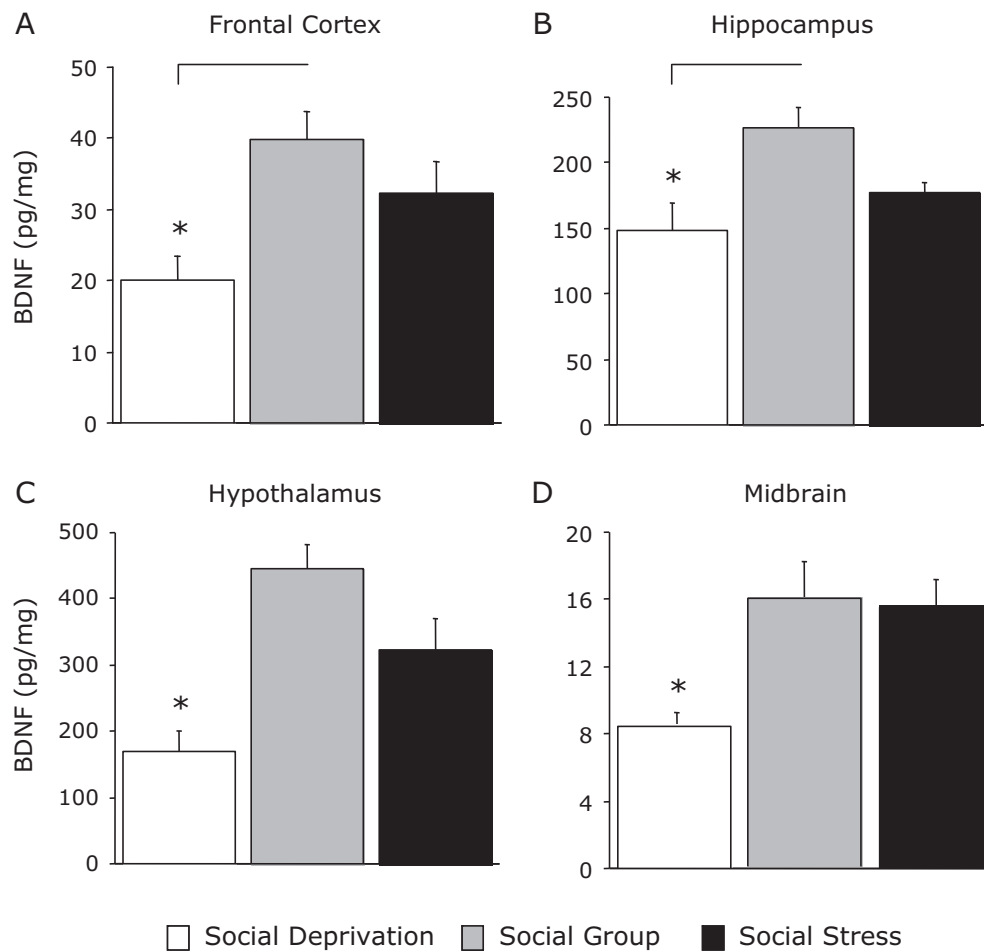
Figure 5 Brain levels of BDNF. The social deprivation (SD) group was characterised by lower BDNF levels when compared only to the social group (SG) in the frontal cortex (A) and hippocampus (B); by contrast, *post hoc* comparisons showed decreased BDNF levels in the hypothalamus (C) and midbrain (D) of SD mice when compared to both the group-housing conditions (SS and SG). Data show mean + S.E.M. ($n = 8$ for each experimental group). *$p < 0.05$.

condition: $F_{(2,21)} = 2.822$; $p = 0.0821$). No difference was found among social conditions when adrenals levels of BDNF were analysed ($F_{(2,21)} = 0.942$; $p = 0.4056$).

4. Discussion

Data from this study show that social deprivation, rather than social stress, leads to increased anxiety and depressive-like behaviours, accompanied by higher levels of corticosterone and reduced brain BDNF levels, all traits indicative of a disrupted emotional state. The results here reported also indicate that mice exposed to different social housing conditions use different behavioural strategies to cope with external challenges.

When emotional behaviours were assessed, SD mice were indeed characterised by the most anxious phenotype, compared to SG and SS mice, as they spent more time in the periphery and showed a higher *self-grooming* frequency in the Open Field. As for the Elevated Plus Maze test, SD mice spent more time in the closed arms of the maze and were characterised by reduced locomotor activity and by a lower latency and higher frequency of *stretched-attend posture*, a behaviour indicative of risk assessment. In addition, in the Forced Swim Test, SD subjects showed the highest *floating* duration and the lowest *struggling* frequency, commonly interpreted as measures of depressive-like behaviour (Porsolt et al., 1977).

In agreement with these behavioural data, when we investigated the effects of a social challenge on HPA axis activity, we found SD mice to be characterised by the highest CORT levels confirming an increased emotional profile in response to social stimuli (Gavrilovic and Dronjak, 2005). This result finds further support in data showing a higher adrenal/body weight ratio in SD subjects, a measure which is often associated with a hyperactive HPA axis (Ulrich-Lai et al., 2006). Worth noticing, adrenal enlargement, in association with increased glucocorticoids levels, have been found in many patients suffering from major depression (Rubin et al., 1987; Nemeroff et al., 1992).

Data from the Social Interaction Test confirm and enlarge the profile previously described. In addition, observations from this test allow a fine discrimination of the effects produced by chronic exposure to different social contexts on the social behaviour of the two group-housed conditions (SG and SS), so far characterised by similar behavioural (Open Field and Elevated Plus Maze) and neuroendocrine (CORT levels) profiles. In fact, although SG and SS groups reacted

similarly to the environmental novelty (clean cage), SS mice were characterised by a lower arousal (*displacement behaviours*) than the SG and by an overall social profile intermediate between SG and SD subjects. Socially deprived mice, in fact, showed a greater social alertness, possibly due to the prolonged deprivation, as suggested by the association between increased exploration of the unknown conspecific and the high amount of *displacement behaviours* (*digging* and *self-grooming*). By contrast, it is possible to hypothesise that SS mice, which were exposed to a highly variable and unpredictable social context, were better prepared than SG and SD to cope with a social challenge (Pardon et al., 2004). These data also seem to suggest that for laboratory rodents, changes in an established social structure, at least as assessed in the C57BL/6J strain, which is characterised by relatively low aggressiveness, might represent a form of social enrichment acting as boredom breaking, rather than a source of stress (Siegfried et al., 1981; Jones and Brain, 1987; Parmigiani et al., 1999).

A growing body of evidence shows that stress decreases the expression of BDNF, a neurotrophin involved in the neuronal plasticity of brain structures underlying mood circuitry, contributing to the atrophy of these areas, and that antidepressant treatment reverses or blocks these effects (Duman and Monteggia, 2006; Castren et al., 2007). Our data show that social deprivation lowers BDNF in a number of limbic regions, including the hippocampus and frontal cortex in line with previous data indicating lower BDNF levels as a result of chronic stress (Tsankova et al., 2006). The hypothalamus, in particular, was characterised by the largest difference in BDNF levels between socially deprived and group-housed mice. This piece of data is in agreement with previous reports suggesting a specific role of hypothalamic BDNF in regulating neuroendocrine responses to stress (Tapia-Arancibia et al., 2004). As for the midbrain, this area is central to antidepressant action as it is characterised by high levels of 5-HT, a neurotransmitter involved in mood disorders, which is directly related to BDNF function (Thoenen et al., 1991; Lindholm et al., 1994).

In our study, disruption of the social structure was used as a chronic stressor capable to induce anxiety- and/or depressive-like symptoms as is often reported in stress-precipitated major depression (Cryan and Holmes, 2005; Nutt and Stein, 2006). At the end of the stressful procedure, SS mice developed a condition of anhedonia while SD mice did not change their preference for the 4% sucrose solution. Interestingly, the SG group showed a decrease in sucrose preference similar, and even stronger, to that observed in the SS group. On the days anhedonia was assessed (day 0 — following 1 week of social deprivation, 7, 14 and 21), SG and SS mice underwent an acute social deprivation, being exposed to single-housing condition for 5 h, in order to score individual sucrose consumption. By contrast, SD mice did not change their social condition. Several studies have suggested that social experiences are intrinsically valuable, even when there is no clear opportunity for the approaching individual to benefit (Panksepp and Lahvis, 2007; Trezza et al., 2010). For instance, subordinate mice express a strong preference for contact with a familiar dominant as long as a barrier prevents fighting behaviour (Van Loo et al., 2001). By contrast, many studies report a higher response to stimuli associated with reward in isolated rodents (Jones et al., 1990; Consorti et al., 1992; Coudereau et al., 1999). In line with these data, our results show that only subjects which underwent the Sucrose Preference Test, following separation from the social group, developed anhedonia suggesting that an acute social deprivation (5 h) is able to affect this behaviour in mice (D'Andrea et al., 2010). These same considerations apply to the fact that, despite most of the studies assess baseline preference for sucrose consumption in individually housed mice, this could represent a bias and should be compared with baseline preference in group-housed mice (see for example Haenisch et al., 2009).

It is important to emphasise that anxiety and depression co-exist and that anxiety symptoms typically precedes depressive disorders (Ballenger, 1999; Ninan, 1999; Nutt and Stein, 2006). Thus, we cannot exclude that, with a longer social deprivation period, changes in anhedonia might ensue also in SD mice, while anxiety might become less evident.

Taken together, our findings indicate that mice exposed to different social housing conditions use different behavioural strategies to cope with external challenges. In addition they suggest that chronic social deprivation — rather than social instability — might trigger the emergence of anxiety, depression-like behaviours and neuroendocrine activation in association to reduced central levels of BDNF, at least as assessed in C57BL/6J adult male mice. Social isolation and lack of social support have deleterious effects on health, being regarded as one of the most relevant causes of diseases in human and other mammalian species. Overall, our data are in line with previous findings suggesting social deprivation as valid model for isolation-induced psychopathologies in human beings (Bartolomucci et al., 2003). By contrast, mice living in social groups (SG and SS) displayed higher levels of neural plasticity markers, such as BDNF, underlying the pivotal role of a stimulating social context for the prevention of psychopathology and giving further support to the neurotrophic theory of depression (Wood and Rebec, 2009; Cirulli et al., 2010a).

Finally, results from this study indicate that anhedonia is a complex behavioural trait, which can be influenced by a number of variables, including the social context. Indeed, since social experiences are able to shape the sensitivity to rewards, a note of caution has to be raised when using the sucrose preference protocols used in many animal models of depression.

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Conflict of interest

None declared.

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