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Effects of voluntary ethanol consumption on emotional state and stress responsiveness in socially isolated rats

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Abstract

Social isolation of rats immediately after weaning is thought to represent an animal model of anxiety-like disorders. This mildly stressful condition reduces the cerebrocortical and plasma concentrations of 3 α -hydroxy-5 α -pregnan-20-one (3 α ,5 α -TH PROG) as well as increases the sensitivity of rats to the effects of acute ethanol administration on the concentrations of this neuroactive steroid. We further investigated the effects of voluntary consumption of ethanol at concentrations increasing from 2.5 to 10% over 4 weeks of isolation. Isolated rats showed a reduced ethanol preference compared with group-housed animals. Ethanol consumption did not affect the isolation-induced down-regulation of BDNF or Arc, but it attenuated the increase in the cerebrocortical concentration of 3 α ,5 α -TH PROG induced by foot shock stress in both isolated and group-housed animals as well as increased the percentage of number of entries made by socially isolated rats into the open arms in the elevated plus-maze test. Ethanol consumption did not affect expression of the α_4 subunit of the GABA_A receptor in the hippocampus of group-housed or isolated rats, whereas it up-regulated the δ subunit throughout the hippocampus under both conditions. The results suggest that low consumption of ethanol may ameliorate some negative effects of social isolation on stress sensitivity and behaviour.

Keywords

social isolation; ethanol; 3 α -hydroxy-5 α -pregnan-20-one; GABA_A receptor; BDNF; Arc

1. Introduction

Adverse life experiences (death or illness of a family member, divorce, financial crises), family influences, and alcohol accessibility are the most common environmental factors

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implicated in increased risk for alcohol abuse in humans (Prescott and Kendler, 1999; Averna and Hesselbrock, 2001). Animals have been used to model the effects of adverse life experiences on the development of drinking behavior. Repeated maternal separation stress during early development (Cruz et al., 2008) or repeated episodes of social defeat stress in adulthood (Croft et al., 2005; Funk et al., 2005) have been shown to increase alcohol abuse. Moreover, separation of rats from their peers during adolescence or adulthood increases voluntary ethanol consumption (Schenk et al., 1990; Wolffgramm, 1990; Juárez and Vázquez-Cortés, 2003; Thorsell et al., 2005). Rats deprived of social contact with other rats at a young age experience a form of prolonged stress that leads to long-lasting alterations in their behavioral profiles (Fone and Porkess, 2008). We previously showed that rats subjected to social isolation at weaning exhibit reduced brain levels of neuroactive steroids and manifest anxiety-like behavior as adults (Serra et al., 2000). Furthermore, such animals are more sensitive to the positive effects of acute ethanol administration on brain concentrations of the neuroactive steroid 3 α -hydroxy-5 α -pregnan-20-one (3 α ,5 α -TH PROG) (Serra et al., 2003). These findings, together with the observations that social isolation enhances the stimulatory effect of acute ethanol administration on brain steroidogenesis (Sanna et al., 2004), GABA_A receptor structure and function and associated behavior (Serra et al., 2006), suggest that this chronic social stress may induce plastic adaptations of neuronal systems that contribute to vulnerability to ethanol abuse.

Compelling evidence demonstrates a role for the brain-derived neurotrophic factor (BDNF) in regulating ethanol intake in rodents. Specifically, transgenic mice with decreased BDNF levels show elevated ethanol intake (Hensler et al. 2003). In addition, rat strains selected to drink high levels of ethanol show reduced BDNF expression when compared with their lower-drinking controls (Yan et al. 2005). The involvement of BDNF in the regulation of mood disorders and antidepressant effects has been established (Castrén, 2004, 2005; Duman and Monteggia, 2006; Castrén et al., 2007). According with the first observation that antidepressants increase the brain synthesis of BDNF in the rat (Nibuya et al., 1995), several studies have shown that serum BDNF levels are decreased in depressed patients and can be normalized by antidepressant treatment (Sen et al., 2008). BDNF is also implicated in the pathophysiology of anxiety disorders (Martinowich et al., 2007). Preclinical studies have shown that chronic stress down-regulates the expression of BDNF in the hippocampus (Duman and Monteggia, 2006) and in mice carrying the BDNF Val66Met polymorphism, a reduced secretion of BDNF from neurons was associated to lower hippocampal volumes, less dendritic arbors and more anxiety-like behaviour (Chen et al., 2006).

BDNF induces the expression of many genes in hippocampal cells, with one of the most prominently affected genes being that for activity-regulated cytoskeletal associated protein, or Arc (Alder et al., 2003). Arc is the product of an immediate-early gene and contributes to activity-dependent neural plasticity in corticolimbic brain regions. It has also been implicated in modulation of cellular functions that are perturbed in depressive states. Given that its expression in the dendrites of neurons is modulated by synaptic activity, Arc has been proposed as a neuronal marker (Tzingounis and Nicoll, 2006).

Aim of our study was to examine whether social isolation stress affects alcohol consumption, and whether voluntary ethanol consumption modifies the effects of isolation on neuroactive steroid concentrations, anxiety-like behavior, and GABA_A receptor gene expression. Moreover, we investigated the effects of voluntary ethanol consumption on the expression of BDNF and Arc in the brain of isolated rats.

2. Experimental procedures

2.1 Animals

Male Sprague-Dawley CD rats were bred in house and, at 25 days of age, immediately after weaning, were housed for 30 days either in groups of three per cage or individually in smaller cages unless indicated otherwise. They were maintained under an artificial 12-h-light, 12-h-dark cycle at a constant temperature of $23^{\circ} \pm 2^{\circ}\text{C}$ and 65% humidity. Food and water were freely available at all times. Separate groups of rats were used in each of the experiments. Animal care and handling throughout the experimental procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2 Voluntary ethanol consumption

Ethanol (Sigma-Aldrich S.r.l., Milan, Italy) was diluted in tap water. Liquid loss or ethanol evaporation were prevented by equipping bottles with dual ball-bearing caps (Tecniplast, Buguggiate, Italy). Rats at weaning were housed individually (isolated) or three per cage (group-housed) and were randomly assigned to either the control condition, in which the animals were given 24-h access to two bottles containing water, or to the ethanol condition, in which they were given 24-h access to one bottle containing water and one bottle containing ethanol in water. The concentration of ethanol was increased every 7 days, with the animals being exposed to 2.5, 5.0, 7.5, and 10% (v/v) solutions over the course of the experiment (4 weeks). The positions of the two bottles were changed every 2 days to control for position preference. At the end of the 4 weeks of treatment animals were then decapitated, blood (~5 ml) was collected from the body trunk, and plasma was prepared and assayed for ethanol concentration with the use of a Quantichrom ethanol assay kit (DIET-500, Gentaur, Belgium). Because of the small amount of ethanol consumed each day, fluid intake was measured once a week throughout the isolation period and the mean daily intake per animal was calculated.

The intake of ethanol/water in control animals (group-housed, 3 per cage) was calculated as the total intake divided by the number of animals. Ethanol preference was calculated as: $100\% \times \text{ethanol consumption (g)}/\text{total fluid consumption (g)}$.

2.3 Elevated plus-maze test

The test was performed at the end of the treatment (18 animals per experimental group). The plus-maze was constructed of black polyvinyl chloride, consisted of two open and two closed arms (each 12 by 60 by 3 cm) connected by a central square (12 by 12 by 3 cm), and was mounted 50 cm above the floor of a quiet, dimly lit room. Each rat was tested only once. The animal was placed in the central square facing a closed arm, and its behavior was scored over 5 min. The number of entries into and time spent in open and closed arms were recorded; arm entry was defined as the presence of all four feet of the animal in the arm. The maze was cleaned after each trial.

2.4 Foot shock

Animals were exposed to foot-shock stress at the end of the treatment (postnatal day 53). Foot shock consisted of a series of electrical impulses delivered in individual boxes with

floors made of brass rods positioned 2 cm apart. Shocks (0.2 mA for 500 ms) were delivered every second over a period of 5 min. Animals were killed 25 min after the end of foot shock for steroids measurement.

2.5 Extraction and assay of steroids

Rats were killed by focused microwave irradiation (70 W/cm² for 4 s) of the head. The cerebral cortices and hippocampi were dissected and then frozen at -20°C until steroid extraction. 3 α ,5 α -TH PROG was extracted from cerebrocortical homogenates and purified as previously described (Serra et al., 2000). The extract residue was dissolved in 5 ml of *n*-hexane and applied to a SepPak silica cartridge (Waters SpA, Milan, Italy), and eluted components were separated and further purified by HPLC on a 5- μ m Lichrosorb-diol column (250 by 4 mm, Phenomenex, Torrance, California, USA) with a discontinuous gradient of 2-propanol (0 to 30%) in *n*-hexane. The recovery (70 to 80%) of 3 α ,5 α -TH PROG through the extraction and purification procedures was monitored by addition of a trace amount (6000 to 8000 cpm, 20 to 80 Ci/mmol) of ³H-labeled standard (Perkin Elmer Italia, Monza (Milan), Italy) to the brain homogenate. 3 α ,5 α -TH PROG was quantified by radioimmunoassay with specific antibodies generated in sheep (Purdy et al., 1990; Serra et al., 2000).

2.6 Immunohistochemistry

At the end of the 4 weeks of the ethanol treatment, rats were anesthetized by intraperitoneal injection of Equithesin (1 g of sodium pentobarbital, 4.251 g of choral hydrate, 2.125 g of MgSO₄, 12 ml of absolute ethanol, and 43.6 ml of propylene glycol, adjusted to a total volume of 100 ml with distilled water) at a dose of 0.3 ml per 100 g of body mass, and they were then perfused through the ascending aorta with 4% paraformaldehyde in phosphate-buffered saline (PBS). The brain was removed, exposed to the same fixative for 1 h, and then transferred to 20% (w/v) sucrose. Sagittal sections (thickness, 50 μ m) were cut with a vibratome and maintained in antifreeze solution (30% ethylene glycol, 20% glycerol, 50% 0.05 M phosphate buffer) at -20°C. Indirect immunohistochemistry was performed with free-floating sections. The sections were washed with PBS, incubated for 30 min at room temperature with 0.1% phenylhydrazine, permeabilized for 1 h with 0.2% Triton X-100 in PBS (PBS-T), and incubated for 1 h with 10% normal donkey serum (Jackson ImmunoResearch) in PBS-T. They were then incubated with goat antibodies to the α_4 or δ subunits of the GABA_A receptor (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) diluted 1:500 in PBS-T containing 10% normal donkey serum. After several washes, the sections were incubated for 2 h with biotinylated donkey antibodies to goat IgG (Jackson ImmunoResearch) diluted 1:1000 in PBS-T. Immune complexes were detected by incubation first for 2 h with horseradish peroxidase-conjugated streptavidin (Jackson ImmunoResearch Europe Ltd) at 2 μ g/ml and then for 6 to 10 min with 0.4 mM 3,3'-diaminobenzidine (Sigma) and 0.01% H₂O₂. After washing, the sections were mounted on gelatin-coated slides, dehydrated, and covered. Control sections not exposed to primary antibodies did not yield positive staining. Sections were examined with an Olympus BX-41 microscope equipped with a Plan 2 $\times$ objective (numerical aperture, 0.05) or a UPlan FI 20 $\times$ objective (numerical aperture, 0.50) and were photographed with an Olympus F-View charge-coupled device camera. Semiquantitative analysis of the images was performed with AnalySIS 3.2 software (Soft Imaging System, Münster, Germany). Different areas of the hippocampus, plates 48 to 50 of the Paxinos atlas (Paxinos and Watson, 2005) were selected for each image, and the intensity of gray values was measured for each region of interest.

2.7 Immunoblot analysis

At the end of the 4 weeks of the ethanol treatment, animals were killed for immunoblot analysis. The hippocampus was homogenized in a solution of lyses buffer containing 2%

sodium dodecyl sulfate. Protein samples (20 µg/20 µl) were incubated for 5 min at 95°C before fractionation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 15% minigels (Mini Protean II, Bio-Rad, Milan, Italy). The separated proteins were transferred to a polyvinylidene difluoride membrane and subjected to immunoblot analysis with rabbit polyclonal antibodies to BDNF (1:200 dilution, Santa Cruz Biotechnology) or with mouse monoclonal antibodies to Arc (1:200 dilution, Santa Cruz Biotechnology). The membrane was incubated with primary antibodies overnight at 4°C, and immune complexes were detected with horseradish peroxidase–conjugated secondary antibodies and chemiluminescence reagents (ECL, Amersham Biosciences). The amounts of BDNF and Arc were quantified by analysis of the corresponding bands on the autoradiogram with a densitometer (GS-700 Bio Rad Hercules, CA, USA). Data were normalized by dividing the optical density of the bands corresponding to BDNF or Arc by that of the band for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, internal standard), which was revealed by reprobing the membrane with a mouse monoclonal antibody to this protein (Chemicon).

The antibodies to BDNF were generated in response to a peptide corresponding to an internal region of the human protein that differs from the corresponding region of the rat protein at several amino acid positions. Immunoblot analysis of a protein sample from rat hippocampus with these antibodies yielded several bands including those corresponding to pro-BDNF (~32 kDa) and mature BDNF (~14 kDa), the latter of which comigrated with recombinant human BDNF (Santa Cruz Biotechnology) (Fig. 4A). None of these bands was detected if the blot was incubated with the antibodies in the presence of the peptide antigen (Santa Cruz Biotechnology), demonstrating antibody specificity (Fig. 4A). The antibodies to Arc were generated in response to a peptide corresponding to residues 1 to 300 of human Arc, which also differs from the corresponding sequence of the rat protein at several amino acid positions. Immunoblot analysis of a protein sample from rat hippocampus with these antibodies yielded a single band of ~55 kDa (Fig. 4B). This band was not detected if the blot was incubated with the antibodies in the presence of the peptide antigen (Santa Cruz Biotechnology), again demonstrating antibody specificity (Fig. 4B).

2.8 Statistical analysis

Quantitative data are presented as means ± SEM and were compared by one-way or multiple-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test (STATISTICA 6.0, StatSoft inc.). A *p* value of <0.05 was considered statistically significant.

3. Results

3.1 Ethanol and water intake in socially isolated rats

Voluntary ethanol consumption by group-housed or isolated rats increased slightly as the concentration of the ethanol solution increased during the first 3 weeks from 2.5 to 5.0 to 7.5%, and it then approximately doubled during the 4th week when the ethanol concentration was 10% (Fig. 1). During the 1st and 2nd weeks, the amount of ethanol drank by isolated animals was smaller ($p = 0.71$; $p = 0.90$, respectively) than that consumed by group-housed rats. ANOVA revealed a significant effect of ethanol treatment [$F(3, 197)=25.786$, $p=0.00000$]; no significant effect of isolation [$F(1, 197)=1.3970$, $p=0.23865$] or interaction between factors [$F(3, 197)=0.86495$, $p=0.46023$] were found.

Although the mean daily intake of ethanol did not differ significantly between the two rearing conditions, isolated rats showed a significant reduction (−31%) in ethanol preference compared with group-housed animals (Table 1). This difference was due to a significant

increase (+21%) in water consumption by isolated rats (Table 1); such an effect was also observed in isolated rats provided with only water, with the isolated animals showing a mean daily water intake of 97.4 ± 3.0 g per kilogram of body mass compared with a value of 79.4 ± 5.7 g/kg ($p < 0.01$) for group-housed animals. Plasma ethanol concentrations resulting from voluntary ethanol consumption were below the limit of detection for rats reared under either condition (data not shown).

3.2 Effects of voluntary ethanol consumption and foot shock on the abundance of 3 α ,5 α -TH PROG in socially isolated rats

Consistent with our previous data (Serra et al., 2000), social isolation for 30 days in the absence of any other stressor induced significant decreases in the cerebrocortical (Fig. 2) and hippocampal (data not shown) concentrations of 3 α ,5 α -TH PROG compared with the corresponding values for group-housed animals. Voluntary ethanol consumption throughout the isolation period tended to reduce the cerebrocortical level of 3 α ,5 α -TH PROG in both socially isolated and group-housed rats, although this effect was not significant (Fig. 2). As expected (Serra et al., 2000), the percentage increase in the cerebrocortical concentration of 3 α ,5 α -TH PROG induced by foot shock, used as a novel acute stressor, was markedly greater in isolated rats than in group-housed animals ($p < 0.05$, Fig. 2). Voluntary ethanol consumption greatly attenuated this effect of foot shock stress in both isolated and group-housed animals. ANOVA revealed a significant effect of isolation [$F(1, 125)=10,348$, $p=0,00165$], ethanol treatment [$F(1, 125)=25,492$, $p=0,00000$], foot-shock stress [$F(1, 125)=30,474$, $p=0,00000$] and a significant interaction between ethanol treatment and foot-shock stress [$F(1, 125)=9,9817$, $p=0,00198$]; no interaction between isolation and ethanol [$F(1, 125)=1,8209$, $p=0,17965$], isolation and foot-shock [$F(1, 125)=0,01284$, $p=0,90995$], isolation, ethanol and foot-shock [$F(1, 125)=0,27513$, $p=0,60084$] were detected.

3.3 Effect of voluntary ethanol consumption on anxiety-like behavior in socially isolated rats

Consistent with our previous finding (Serra et al., 2000), rats isolated for 30 days immediately after weaning exhibited an anxiety-like profile in the elevated plus-maze test, as revealed by significant decreases in both the percentage entries into and time spent in the open arms of the maze compared with the corresponding values for group-housed rats (Fig. 3). Voluntary ethanol consumption throughout the isolation period significantly increased both the percentage of time spent and entries in the open arms of the maze in socially isolated rats (+151% and +173%, respectively, relative to isolated water-drinking rats, $p < 0.05$). However these two parameters were still markedly reduced compared with the corresponding values for group-housed animals with access to ethanol or to water alone. No significant difference in total locomotor activity of rats was detected among the various conditions (data not shown). Data are expressed as the mean $\pm$ SEM of 18 animals per group. Analyzing the percentage of time spent in open arms, ANOVA revealed a significant effect of isolation [$F(1, 68)=59,621$, $p=0,00000$], no significant effect of ethanol treatment [$F(1, 68)=0,01096$, $p=0,91693$] or interaction between factors [$F(1, 68)=0,46221$, $p=0,49890$]. The analysis of the percentage of entries in open arms revealed a significant effect of isolation [$F(1, 68)=39,728$, $p=0,00000$] and interaction between factors [$F(1, 68)=4,2792$, $p=0,04239$]; no significant effect of ethanol treatment [$F(1, 68)=1,0443$, $p=0,31045$] was detected.

3.4 Effects of voluntary ethanol consumption on hippocampal BDNF and Arc levels

We examined the expression of BDNF and Arc in the hippocampus of rats by immunoblot analysis, which revealed a band at 14 kDa corresponding to the mature form of BDNF and a band at 55 kDa corresponding to Arc (Fig. 4). We found that voluntary ethanol consumption did not significantly affect the down-regulation of BDNF (Fig. 5A, B) or Arc (Fig. 5C, D)

expression apparent in the hippocampus of socially isolated rats. Ethanol consumption slightly reduced the hippocampal abundance of these proteins in group-housed animals, but this effect was not statistically significant. ANOVA revealed a significant effect of isolation [$F(1, 60)=16,946$, $p=0,00012$]; no significant effect of ethanol treatment [$F(1, 60)=0,11099$, $p=0,74020$] or interaction between factors [$F(1,60)=0,61592$, $p=0,43571$] were detected.

3.5 Effects of voluntary ethanol consumption on expression of α_4 and δ subunits of the GABA_A receptor in the hippocampus of isolated or group-housed rats

Expression of the α_4 and δ subunits of the GABA_A receptor in the hippocampus of isolated or group-housed rats after voluntary ethanol consumption was examined by immunohistochemistry with specific antibodies generated in response to extracellular epitopes of these proteins. The antibodies recognized single proteins of ~70 and 54 kDa for the α_4 and δ subunits, respectively, in immunoblot analysis of a crude membrane fraction prepared from rat hippocampal neurons, and the immunoreactive bands were not detected when blots were incubated with the antibodies in the presence of the corresponding peptide antigen (Sanna et al., 2003; Follesa et al., 2005). Antibody specificity was also previously confirmed by immunohistochemical analysis (Serra et al., 2006).

Consistent with our previous observations (Serra et al., 2006), the levels of α_4 (Fig. 6A, C) and δ (Fig. 7A, C) subunit immunoreactivity were increased throughout the hippocampus of socially isolated rats compared with those in the hippocampus of group-housed rats. Whereas voluntary ethanol consumption did not affect the expression of the α_4 subunit in the hippocampus of either group-housed or socially isolated animals (Fig. 6B, C), it increased that of the δ subunit in the CA1 and CA3 regions of the hippocampus as well as in the dentate gyrus of animals raised alone or in groups (Fig. 7B, C).

4. Discussion

Our results show that ethanol voluntary consumption abolished the hypersensitivity of isolated animals to acute novel stress (Serra et al., 2000), as demonstrated by the attenuation of the increase in the cerebrocortical concentration of $3\alpha,5\alpha$ -TH PROG induced by foot shock stress and reduced anxiety as shown by the increase in the percentage of number of entries made by socially isolated rats into the open arms in the elevated plus-maze test.

The decrease in ethanol preference apparent in isolated rats compared to group-housed in the present study might have been unexpected given that previous studies have shown ethanol drinking and preference to be increased as a result of innate anxiety level (Spanagel et al., 1995; Hensler et al., 2004) or of stressful conditions including social isolation (Parker and Radow, 1974; Wolffgramm, 1990; Hall et al., 1998; McCool and Chappell, 2009). However, in these previous studies, rats were either different in strains or exposed to ethanol in adulthood, after social isolation for 4 to 8 weeks, whereas in our protocol the animals were isolated at weaning and given continuous access to ethanol during the isolation procedure. Our results are in line with those of a study showing that early weaned social isolated rats manifested a decrease in ethanol consumption and preference when allowed continuous access to the drug (Fahlke et al., 1997). The effects of social isolation on voluntary ethanol consumption or preference thus appear to differ depending on strain of rats and experimental paradigm. Consistent with a previous study (Parker and Morinan, 1986), we also found a decrease, although not significant, in ethanol consumption apparent during the first 2 weeks of social isolation. Moreover, socially isolated rats have been shown to possess low circulating and brain levels of the progesterone metabolite $3\alpha,5\alpha$ -TH PROG (Serra et al., 2000), and systemic and central $3\alpha,5\alpha$ -TH PROG administration was found to increase ethanol intake in rats and mice (Morrow et al., 2001; Sinnott et al., 2002; Ford et al., 2007). It is therefore possible that group-housed rats tend to drink more alcohol than isolated

animals as a result of their higher brain levels of $3\alpha,5\alpha$ -TH PROG. The reduced ethanol preference apparent in isolated rats in the present study is also consistent with the observed reduced preference for sucrose (Pisu et al., submitted), with both findings indicating a reduction in the response to rewarding stimuli.

Voluntary ethanol consumption abolished or significantly reduced the foot shock stress-induced increase in $3\alpha,5\alpha$ -TH PROG concentration in the cerebral cortex of group-housed or isolated animals, respectively. These results are in agreement with the anxiolytic action of ethanol. The animals had 24-h access to ethanol and were tested early in the morning, 1 to 2 h after the end of the dark phase. However, the amount of ethanol consumed is probably not sufficient to completely prevent the effect of foot shock stress in isolated animals because they are more sensitive to this stimulus (Serra et al., 2000). The decrease in the $3\alpha,5\alpha$ -TH PROG response to foot shock stress associated with ethanol consumption in isolated rats having low levels of $3\alpha,5\alpha$ -TH PROG (Serra et al., 2000), is consistent with previous data showing that low doses of ethanol decreased anxiety, the acoustic startle response and in the elevated plus maze during a progesterone withdrawal (Smith et al., 2004), as well as with our present data showing that voluntary ethanol consumption partially reversed social isolation-induced conflict behavior in the elevated plus maze. However, whereas voluntary ethanol consumption throughout the isolation period exerted a significant anxiolytic effect in the elevated plus-maze test in the present study, it did not reverse the down-regulation of BDNF or Arc expression in the hippocampus of isolated rats. This result suggest that an anxiolytic effect induced by long-term drug treatment is not necessarily associated with molecular changes related to hippocampal neuronal plasticity. In contrast with our finding, the anxiolytic effect of acute ethanol exposure was associated with enhancement of the BDNF-induced increase in Arc expression in both the central and medial amygdala of rats (Pandley et al., 2008). Chronic ethanol administration was previously shown to reduce the level of BDNF gene expression in the rat hippocampus (MacLennan et al., 1995; Miller et al., 2002), and BDNF has been shown to increase the expression of Arc at both mRNA and protein levels in the hippocampus via activation of the extracellular signal-regulated kinase (ERK) signaling pathway (Waltereit et al., 2001; Yin et al., 2002). Although we did not observe a significant effect of ethanol on BDNF expression in the hippocampus, ethanol consumption tended to decrease this parameter in group-housed rats, suggesting that the slight decrease in Arc expression in group-housed rats induced by voluntary ethanol consumption might have been secondary to the effect on BDNF expression; alternatively, it might have been due to a direct alteration in the ERK signaling pathway induced by ethanol (Pandley et al., 2008).

Chronic ethanol exposure is associated with marked changes in the expression of GABA_A receptor subunits at both the mRNA and protein levels in various brain regions (Grobin et al., 2000). Such treatment thus results in a decrease in the expression of α_1 and α_2 subunits and a parallel increase in that of α_4 , α_6 , β_1 , β_2 , β_3 , γ_1 , and γ_2 subunits in the cerebral cortex and cerebellum. In addition, chronic intermittent ethanol treatment resulted in down-regulation of α_1 and δ subunit expression and up-regulation of α_4 , γ_1 , and γ_2 subunit expression in the hippocampus (Mahmoudi et al., 1997; Cagetti et al., 2003). We found that voluntary ethanol consumption failed to modify the level of α_4 subunit immunoreactivity in the hippocampus of isolated or group-housed rats, whereas it induced small increases in the amount of δ subunit immunoreactivity throughout the hippocampus of both isolated and group-housed rats. In the forebrain, the δ subunit preferentially assembles with the α_4 subunit (Korpi et al., 2002). GABA_A receptors containing α_4 and δ subunits are restricted to extrasynaptic locations (Nusser et al., 1998) and mediate tonic GABAergic conductance (Nusser and Mody, 2002; Stell and Mody, 2002). The increase in the expression of the δ subunit induced by voluntary ethanol consumption might thus be expected to result in an increase in GABA_A receptor-mediated tonic inhibitory current in granule cells of the

dentate gyrus. Indeed, the increased expression of both α_4 and δ subunits in the hippocampus of isolated rats was paralleled by an increase in the amplitude of GABA_A receptor-mediated tonic inhibitory currents in granule cells of the dentate gyrus measured in hippocampal slices from such animals (Serra et al., 2006). The further increase in the expression of the δ subunit and the likely further increase in tonic current, may be crucial for the positive effects of voluntary ethanol consumption on emotional state and stress responsiveness. Accordingly, the increased expression of $\alpha_4\beta\delta$ subunit isoforms of the GABA_A receptor is expected to have important consequences for the ability of ethanol to modulate GABAergic transmission. In fact, recombinant GABA_A receptors comprising the α_4 and δ subunits were shown to be selectively sensitive to extremely low concentrations of EtOH (Sundstrom-Poromaa et al. 2002; Wallner et al. 2003). Moreover, transgenic mice lacking expression of the δ subunit have reduced responsiveness to ethanol (Mihalek et al., 2001), while, progesterone withdrawal rats, where the expression of $\alpha_4\beta\delta$ subunit isoforms of the GABA_A receptor is increased (Guliniello et al., 2003), demonstrated high sensitivity to the anxiolytic effects very low doses of ethanol (Smith et al., 2004).

In conclusion we found that low voluntary consumption of ethanol may ameliorate some negative effects of social isolation on stress sensitivity and behaviour.

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Fig. 1. Voluntary ethanol consumption in group-housed or socially isolated rats

Rats were isolated or group-housed for 30 days with free access to water and ethanol at concentrations that increased from 2.5 to 5.0 to 7.5 to 10% at weekly intervals. Ethanol consumption (grams per kilogram of body mass per day) was determined at the end of each week. Data are means $\pm$ SEM for about 12 or 39 rats per group (group-housed and isolated, respectively). Data were analyzed by two-way ANOVA followed by Newman-Keuls post-hoc test).

^a $p < 0.05$ vs Group-housed

Fig. 2. Effect of voluntary ethanol consumption on the acute stress-induced increase in the concentration of 3 α ,5 α -TH PROG in the cerebral cortex of group-housed or socially isolated rats Animals were housed in groups or in isolation for 30 days with free access to both increasing concentrations of ethanol and water or to water alone, after which they were exposed (or not) to foot shock stress for 5 min. The animals were killed 25 min thereafter, and the cerebrocortical concentration of 3 α ,5 α -TH PROG was measured. Data are means $\pm$ SEM for about 17 rats per group (14-19 animals/group). ^a p < 0.01 vs. group-housed animals provided with water alone; ^b p < 0.01 vs. isolated animals provided with water alone; ^c p < 0.05 vs. isolated animals provided with ethanol; ^d p < 0.05 vs. group-housed animals (water) exposed to foot-shock; ^e p < 0.05 vs. isolated animals (water) exposed to foot-shock (three-way ANOVA followed by Newman-Keuls post-hoc test)

Fig. 3. Effect of voluntary ethanol consumption on anxiety-like behavior in socially isolated rats Animals were housed in groups or in isolation for 30 days with free access to both increasing concentrations of ethanol and water or to water alone, after which they were subjected to the elevated plus-maze test. The percentage time spent by each animal in the open arms of the maze (**A**) as well as the percentage number of entries into the open arms (**B**) during the 5-min test were determined. Data are means $\pm$ SEM for 12 group-housed and 22 isolated, respectively. ^a $p < 0.01$ vs. group-housed rats provided with water alone, ^b $p < 0.05$ vs. socially isolated rats provided with water alone (Two-way ANOVA followed by Newman-Keuls test).

Fig. 4. Specificity of antibodies to BDNF or to Arc in the rat hippocampus

(A) Immunoblot analysis of a rat hippocampal homogenate (20 μ g of protein) was performed with antibodies to BDNF in the absence (lane a) or presence (lane c) of peptide antigen (1 μ g/ml). Purified recombinant human BDNF (1 μ g) was also probed with the antibodies (lane b). Bands corresponding to pro-BDNF (32 kDa) and mature BDNF (14 kDa) are indicated. (B) Immunoblot analysis of a rat hippocampal homogenate (20 μ g of protein) was performed with antibodies to Arc in the absence (lane d) or presence (lane e) of peptide antigen (1 μ g/ml). The band corresponding to Arc (55 kDa) is indicated.

Fig. 5. Effects of social isolation on BDNF and Arc expression in the hippocampus

(A, C) Rats were isolated or group-housed for 30 days with free access to water alone or to increasing concentrations of ethanol and water, after which hippocampal homogenates were prepared and subjected to immunoblot analysis with antibodies to BDNF (A) or to Arc (C) as well as with those to GAPDH (loading control). Each lane corresponds to an individual animal. (B, D) Immunoblots similar to those in (A) and (C), respectively, were subjected to densitometric analysis, and the abundance of BDNF or Arc was normalized by the corresponding amount of GAPDH. Data are expressed as percentage change relative to the value for group-housed animals provided with water alone (not shown) and are means $\pm$ SEM for 16 rats per condition. ^a $p < 0.05$ vs. group-housed rats provided with water only (Two-way ANOVA followed by Newman-Keuls post-hoc test).

Fig. 6. Lack of effect of voluntary ethanol consumption on expression of the α_4 subunit of the GABA_A receptor in the hippocampus of group-housed or socially isolated rats
 (A, B) Animals were housed in groups (GH) or in isolation (ISO) for 30 days with free access to water alone (A) or to both increasing concentrations of ethanol and water (B), after which hippocampal sections were subjected to immunohistochemical analysis with antibodies to the α_4 subunit of the GABA_A receptor. (C) Images similar to those in (A) and (B) were subjected to quantitative analysis of α_4 subunit expression in the CA1 and CA3 regions of the hippocampus as well as in the dentate gyrus (DG). Data are expressed relative to the corresponding value for group-housed rats provided with water alone and are means $\pm$ SEM for 14 animals for each condition. ^a $p < 0.05$ vs. the corresponding value for group-housed rats provided with water only (Two-way ANOVA followed by Newman-Keuls post-hoc test).

Fig. 7. Effects of voluntary ethanol consumption on expression of the δ subunit of the GABA_A receptor in the hippocampus of group-housed or socially isolated rats
 (A, B) Animals were housed in groups (GH) or in isolation (ISO) for 30 days with free access to water alone (A) or to both increasing concentrations of ethanol and water (B), after which hippocampal sections were subjected to immunohistochemical analysis with antibodies to the δ subunit of the GABA_A receptor. (C) Images similar to those in (A) and (B) were subjected to quantitative analysis of δ subunit expression in the CA1 and CA3 regions of the hippocampus as well as in the dentate gyrus (DG). Data are expressed relative to the corresponding value for group-housed rats provided with water alone and are means $\pm$ SEM for 14 animals for each condition. ^a $p < 0.05$ vs. the corresponding value for group-housed rats provided with water only, ^b $p < 0.05$ vs. the corresponding value for socially isolated rats provided with water only (Two-way ANOVA followed by Newman-Keuls post-hoc test).

Table 1

Effects of social isolation on ethanol and water consumption as well as ethanol preference in rats.

	Daily intake (g of liquid/kg of body mass)			Ethanol preference (%)
	Total fluid	Water	Ethanol	
Group-housed	86.2 ± 5.5	68.9 ± 5.6	17.3 ± 1.6	20.1 ± 2.1
Isolated	97.2 ± 3.2 ^a	83.7 ± 3.3 ^a	13.5 ± 1.1	13.9 ± 1.1 ^a

Rats were isolated or group-housed for 30 days with free access to water and ethanol at concentrations that increased from 2.5 to 5.0 to 7.5 to 10% at weekly intervals. Given that isolated animals differ in body mass from group-housed controls (351.5 ± 5.9 g vs. 325.8 ± 4.2 g, respectively; $p < 0.01$ (One-way ANOVA followed by Newman-Keuls post-hoc test), mean daily intake and ethanol preference during the 4-week period were corrected for body mass. Data are means ± SEM from two experiments, each performed with at least 15 rats per condition.

^a $p < 0.01$ vs. the corresponding value for group-housed rats (One-way ANOVA followed by Newman-Keuls post-hoc test).