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Social Isolation-Induced Decreases in Both the Abundance of Neuroactive Steroids and $GABA_A$ Receptor Function in Rat Brain

Mariangela Serra, Maria Giuseppina Pisu, Martino Littera, Giacomo Papi, Enrico Sanna, Francesca Tuveri, Luca Usala, *Robert H. Purdy, and Giovanni Biggio

*Department of Experimental Biology, University of Cagliari, Cagliari, Italy; and *Department of Psychiatry, University of California, San Diego, California, U.S.A.*

Abstract: The effects of social isolation on behavior, neuroactive steroid concentrations, and $GABA_A$ receptor function were investigated in rats. Animals isolated for 30 days immediately after weaning exhibited an anxiety-like behavioral profile in the elevated plus-maze and Vogel conflict tests. This behavior was associated with marked decreases in the cerebrocortical, hippocampal, and plasma concentrations of pregnenolone, progesterone, allopregnanolone, and allotetrahydrodeoxycorticosterone compared with those apparent for group-housed rats; in contrast, the plasma concentration of corticosterone was increased in the isolated animals. Acute foot-shock stress induced greater percentage increases in the cortical concentrations of neuroactive steroids in isolated rats than in group-housed rats. Social isolation also reduced brain $GABA_A$ receptor function, as evaluated by measuring both GABA-evoked Cl^- currents in *Xenopus* oocytes expressing the rat receptors and *tert*-[^{35}S]butylbicyclophosphorothionate ([^{35}S]TBPS) binding to rat brain membranes. Whereas the amplitude of GABA-induced Cl^- currents did not differ significantly between group-housed and isolated animals, the potentiation of these currents by diazepam was reduced at cortical or hippocampal $GABA_A$ receptors from isolated rats compared with that apparent at receptors from group-housed animals. Moreover, the inhibitory effect of ethyl-β-carboline-3-carboxylate, a negative allosteric modulator of $GABA_A$ receptors, on these currents was greater at cortical $GABA_A$ receptors from socially isolated animals than at those from group-housed rats. Finally, social isolation increased the extent of [^{35}S]TBPS binding to both cortical and hippocampal membranes. The results further suggest a psychological role for neurosteroids and $GABA_A$ receptors in the modulation of emotional behavior and mood. **Key Words:** Social isolation—Anxiety—Neurosteroids—$GABA_A$ receptor.
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Long-term social isolation after weaning markedly affects the behavior of rats. Isolated animals are aggressive and neophobic as well as show an anxiety-like profile in the elevated plus-maze test and increased locomotor activity (Parker and Morinan, 1986; Hilakivi et al., 1989; Wongwitdecha and Marsden, 1996). These characteristics support the notion (Hatch et al., 1963) that isolation is stressful for these normally gregarious animals and that the abnormal behavior of rats so reared is the product of prolonged stress.

Several paradigms of acute stress (handling maneuvers that precede killing, mild foot shock, forced swimming, CO_2 inhalation) induce rapid increases in the brain concentrations of neuroactive steroids such as pregnenolone, progesterone, 3α-hydroxy-5α-pregnan-20-one (allopregnanolone; AP), and allotetrahydrodeoxycorticosterone (THDOC) (Purdy et al., 1991; Barbaccia et al., 1994, 1996, 1997). The latter two steroids are among the most potent positive allosteric modulators of $GABA_A$ receptor function known (Majewska et al., 1986), and their administration in pharmacological doses to rodents elicits anxiolytic, anticonvulsant, and sedative–hypnotic effects (Majewska, 1992). These observations have suggested that AP and THDOC might contribute to the physiological modulation of neuronal excitability. The increases in the brain and plasma concentrations of AP induced by acute stress (Purdy et al., 1991; Barbaccia et al., 1996) have thus been postulated to represent a homeostatic mechanism to restore the function of $GABA_A$ receptors, which is decreased in rat brain by the same stress paradigms (Drugan et al., 1989; Serra et al., 1989*a*; Biggio et al., 1990; Sanna et al., 1992). Consistent with a such role for neuroactive steroids in the

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Address correspondence and reprint requests to Dr. M. Serra at Department of Experimental Biology, University of Cagliari, 09123 Cagliari, Italy. E-mail: mserra@unica.it

Abbreviations used: ACTH, adrenocorticotropic hormone; AP, allopregnanolone; β-CCE, ethyl-β-carboline-3-carboxylate; CRF, corticotropin-releasing factor; DHEA, dehydroepiandrosterone; HPA, hypothalamic–pituitary–adrenal; THDOC, allotetrahydrodeoxycorticosterone; TBPS, *tert*-butylbicyclophosphorothionate.

physiological modulation of $GABA_A$ receptor function, the physiological and pharmacologically induced fluctuations in the rat brain concentration of AP during pregnancy (Concas et al., 1998, 1999; Follesa et al., 1998) and pseudopregnancy (Smith et al., 1998), respectively, are associated with parallel and selective changes in $GABA_A$ receptor activity, the expression of receptor subunit genes, and emotional behavior.

We have now investigated the effects of social isolation on the brain and plasma concentrations of neuroactive steroids as well as on $GABA_A$ receptor function in the brain.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley CD rats at 30 days of age, immediately after weaning, were housed for up to 30 days either 10 per cage or individually in smaller cages. One group of isolated rats was handled twice a day. The animals were maintained under an artificial 12-h light/12-h dark cycle (light on 0800–2000 h) at a constant temperature of 23 ± 2°C and 65% humidity. Food and water were freely available until the time of the experiment. Animal care and handling throughout the experimental procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Foot shock

Foot shock consisted of a series of electrical impulses delivered in individual boxes with floors made of brass rods, 2 cm apart. Shocks (0.2 mA for 500 ms) were delivered every second over a period of 5 min.

Neurochemical studies

Extraction and assay of steroids. Rats were killed either by guillotine (for measurement of plasma steroids) or by focused microwave irradiation (70 W/cm^2 for 4 s) to the head (for measurement of brain steroids). This latter procedure results in a virtually instantaneous inactivation of brain enzymes (Mao et al., 1974), thus minimizing postmortem steroid metabolism. Brains were rapidly (<1 min) removed from the skull, and the cerebral cortices and hippocampus were dissected and then frozen at −20°C until steroid extraction. Steroids were extracted and purified as previously described (Barbaccia et al., 1996). In brief, steroids present in tissue homogenates [400 mg of protein in 4 ml of phosphate-buffered saline (pH 7.0)] were extracted three times with ethyl acetate, and the combined organic phases were dried under vacuum. The resulting residue was dissolved in 5 ml of *n*-hexane and applied to a SepPak silica cartridge (Waters), and components were eluted with *n*-hexane and 2-propanol (7:3 vol/vol). Steroids were separated and further purified by HPLC on a 5-μm Lichrosorb-diol column (250 × 4 mm; Phenomenex) with a discontinuous gradient of 2-propanol (0–30%) in *n*-hexane. Progesterone, which coelutes with cholesterol, was further purified by washing the corresponding dried HPLC fractions twice with 200 μl of dimethyl sulfoxide and 400 μl of water. Progesterone was then extracted from the aqueous phase twice with 1.5-ml volumes of *n*-hexane. The recovery (70–80%) of steroids through the extraction and purification procedures was monitored by adding a trace amount (6,000–8,000 cpm, 20–80 Ci/mmol) of tritiated standard to the brain homogenate.

Blood was collected from the trunk of killed rats into heparinized tubes and centrifuged at 900 *g* for 20 min at room temperature. The resulting plasma was frozen (−80°C) until assayed for steroids. Steroids were extracted from plasma three times with 1.5 ml of ethyl acetate, and the combined organic phases were dried under vacuum.

Steroids from both brain tissue and plasma were quantified by radioimmunoassay as previously described (Purdy et al., 1990; Barbaccia et al., 1994, 1996) with specific antibodies to pregnenolone, progesterone, dehydroepiandrosterone (DHEA), and corticosterone (ICN, Costa Mesa, CA, U.S.A.). Antibodies to THDOC and to AP were generated in rabbits and sheep and characterized as previously described (Purdy et al., 1990).

tert-[^{35}S]Butylbicyclophosphorothionate ([^{35}S]TBPS) binding. Rats were killed by guillotine, the cerebral cortex and hippocampus were dissected, and [^{35}S]TBPS (New England Nuclear) binding was assayed as previously described (Serra et al., 1989*b*). The fresh brain tissue was homogenized with a Polytron PT 10 (setting 5 for 20 s) in 50 volumes of ice-cold 50 m*M* Tris-citrate (pH 7.4 at 25°C) containing 100 m*M* NaCl. The homogenate was centrifuged at 20,000 *g* for 20 min, and the resulting pellet was reconstituted in 50 volumes of Tris-citrate buffer for the binding assay. Nonspecific binding was defined as binding in the presence of 100 n*M* picrotoxin and represented ~10% of total binding. Protein concentration was measured by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Electrophysiological studies

Isolation of brain synaptosomes. Synaptosomes were purified as described by Gray and Whittaker (1972), with minor modifications. In brief, cerebral cortical or hippocampal tissue was homogenized with a Teflon homogenizer in 10 volumes of an ice-cold solution containing 0.32 *M* sucrose, 10 m*M* HEPES-NaOH (pH 7.5), and 0.1 m*M* EDTA. The homogenate was centrifuged at 1,000 *g* for 10 min at 4°C, and the resulting pellet was washed with an equal volume of homogenization buffer. The pooled supernatant fractions (S1) were centrifuged at 18,000 *g* for 10 min, the resulting pellet (P2) was resuspended in 0.32 *M* sucrose containing 1 m*M* EDTA, and 2-ml portions of the resulting suspension were layered onto discontinuous sucrose density gradients comprising 2 ml each of 1.3, 1.0, and 0.85 *M* sucrose solutions. After centrifugation at 48,000 *g* for 30 min, the synaptosomal fraction (interface of the 0.85 and 1.0 *M* sucrose layers) was diluted with ice-cold 10 m*M* HEPES-NaOH (pH 7.5) containing 1 m*M* EDTA and centrifuged at 18,000 *g* for 30 min. The resulting membrane preparation was stored at −20°C until use.

Isolation and injection of Xenopus *oocytes.* Oocytes at stage V or VI were isolated from sections of *Xenopus laevis* ovaries and exposed to collagenase type IA (Sigma) as described previously (Sanna et al., 1996). A suspension (50–100 nl) of synaptosomes (1.5–2 mg/ml) was injected into the cytoplasm of each oocyte. Injected oocytes were cultured until use in modified Barth's solution [88 m*M* NaCl, 1 m*M* KCl, 2.4 m*M* $NaHCO_3$, 10 m*M* HEPES-NaOH (pH 7.5), 0.82 m*M* $MgSO_4$, 0.33 m*M* $Ca(NO_3)_2$, 0.91 m*M* $CaCl_2$] supplemented with 2 m*M* sodium pyruvate, 10 U/ml penicillin, 10 μg/ml streptomycin, 50 μg/ml gentamicin, and 0.5 m*M* theophylline. Oocytes were usually incubated for up to 4 days, during which time they were transferred to fresh incubation medium each day.

Electrophysiological recording. Electrophysiological recording from oocytes was initiated 12–18 h after synaptosome injection and was performed as described previously (Sanna et al., 1996). In brief, oocytes were placed in a rectangular recording chamber (volume, 100 μl) and continuously perifused with modified Barth's solution at a flow rate of 2 ml/min

at room temperature. They were impaled at the animal pole with two microelectrodes (resistance, 0.5–5 MΩ) filled with filtered 3 *M* KCl and subjected to voltage clamp at −90 mV with an Axoclamp 2-B amplifier (Axon Instruments, Burlingame, CA, U.S.A.). Resting membrane potentials usually ranged between −30 and −50 mV. The oocytes were exposed to GABA in the absence or presence of drugs for 20 s. Intervals of 5 min were allowed between applications of low concentrations of GABA alone and of at least 10 min when GABA was applied at higher concentrations or with other drugs. When testing the action of various modulators on GABA-evoked currents, a GABA concentration (EC_{20}) that produced 20% of the maximal current amplitude evoked by 10 m*M* GABA was used as a control response. This concentration was experimentally determined for each oocyte at the beginning of the recording. Diazepam was purchased from FIS (Vicenza, Italy), and ethyl-β-carboline-3-carboxylate (β-CCE) was kindly provided by Schering AG (Berlin, Germany).

Behavioral studies

Vogel's conflict test. Rats were deprived of water for 24 h before the conflict session. The test was performed as previously described (Corda et al., 1983). In brief, rats were placed in a clear Plexiglas box (20 × 28 × 20 cm) with a stainless-steel grid floor, and the box was enclosed in a sound-attenuated and ventilated chamber (Lafayette Instruments, Lafayette, IN, U.S.A.). Water was provided through a stainless-steel drinking tube that extended 1 cm into the box, 3 cm above the floor. The drinking tube and the grid floor were connected to a constant-current shock generator and a "drinkometer." The shock generator delivered one shock (0.25 mA for 0.5 s) for each cumulative period of 15 licks; such a period of cumulative drinking was termed a "licking period." Before the test, rats were habituated to the test box for 5 min; the drinking tube was then inserted and the animals were allowed to lick for three licking periods (training period) before the onset of punishment.

Elevated plus-maze test. The plus-maze was constructed of black polyvinyl chloride and contained two open and two closed arms (12 × 60 cm) mounted 50 cm above the floor. The arms were connected by a central square (12 × 12 cm; start point). The apparatus was located in a quiet, dimly lit room. Each rat was tested only once. The animal was placed at the start point facing a closed arm, and its behavior was scored over 5 min. The number of entries into open and closed arms and the time spent there 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.

Statistical analysis

Data are presented as means ± SEM. Behavioral data were analyzed by Student's *t* test, and other data were assessed by one- or two-way ANOVA followed by Newman–Keuls test. A *p* value of <0.05 was considered statistically significant.

RESULTS

Effects of social isolation on behavior

Consistent with previous data (Parker and Morinan, 1986), male rats isolated for 1 month immediately after weaning (at 25 days of age) exhibited an anxiety-like profile in the elevated plus-maze. The time spent by isolated animals in the open arms of the maze was thus reduced by ~55% compared with that spent by group-housed rats (Fig. 1A). The numbers of entries into the closed arms of the maze did not differ significantly between the two groups of animals (data not shown). Moreover, social isolation significantly reduced the punished consumption of water in Vogel's conflict test; the number of licking periods during punishment for isolated rats was thus reduced by ~50% compared with that for group-housed animals (Fig. 1B).

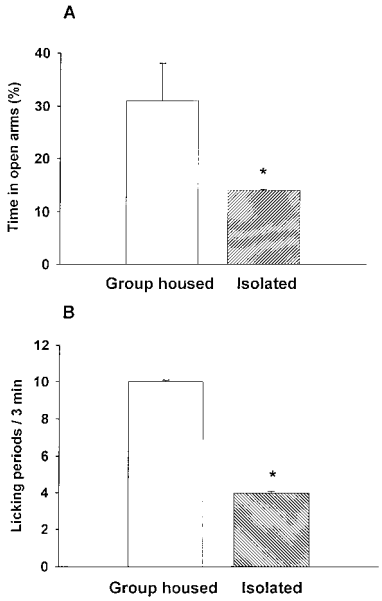

FIG. 1. Effects of social isolation for 30 days on rat behavior. **A:** Effect of social isolation on the percentage of the 5-min test time spent by rats in the open arms of an elevated plus-maze. Data are means ± SEM of values from 10–15 rats. *$p < 0.01$ vs. group-housed animals. **B:** Effect of social isolation on the number of licking periods per 3 min for rats subjected to Vogel's conflict test. Data are means ± SEM of values from 20 rats. *$p < 0.01$ vs. group-housed animals.

Effects of social isolation on steroid concentrations

Basal steroid concentrations. Social isolation for 30 days without any additional stressor induced significant decreases in the basal cerebral cortical and hippocampal concentrations of pregnenolone (−32 and −46%, respectively), progesterone (−35 and −31%, respectively), AP (−45 and −59%, respectively), and THDOC (−35 and −29%, respectively) compared with the corresponding values for group-housed animals. The concentrations of DHEA, which is formed as a result of side chain cleavage of pregnenolone, in the same two brain regions were not affected by social isolation (4.4 ± 0.5 vs. 4.8 ± 0.6 and 4.8 ± 1.1 vs. 4.4 ± 0.9 ng/g of protein in the cerebral cortex and hippocampus, respectively). Whereas social isolation also reduced the plasma concentrations of all neuroactive steroids measured, it increased the plasma corticosterone concentration significantly (184 ± 21 vs. 139 ± 16; $p < 0.05$).

The effects of social isolation on the cortical concentrations of neuroactive steroids were time dependent (Fig. 2). Whereas the cortical concentrations of these steroids remained unchanged during the first 48 h of

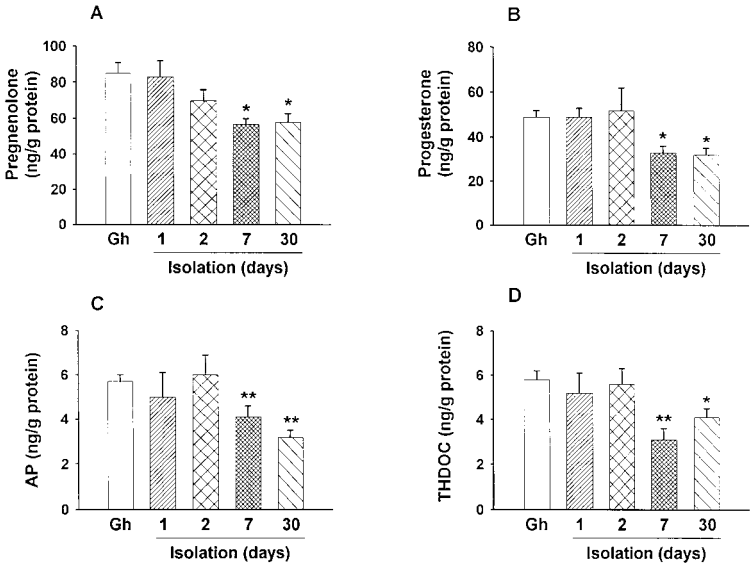

FIG. 2. Time courses of the effects of social isolation on the concentrations of neuroactive steroids in the cerebral cortex of rats. Animals were either group housed (Gh) for 30 days or isolated for the indicated times, after which they were killed directly from the home cage, and the cortical concentrations of pregnenolone (**A**), progesterone (**B**), AP (**C**), and THDOC (**D**) were determined. Data are expressed as nanograms of steroid per gram of protein and are means ± SEM of values from at least eight rats (each sample assayed in duplicate). $^*p < 0.05$, $^{**}p < 0.01$ vs. group-housed animals.

isolation, they were significantly decreased after 7 days (pregnenolone, −33%; progesterone, −33%; AP, −28%; THDOC, −46%) to values similar to those apparent after 30 days of isolation. These low basal concentrations then remained unchanged for at least an additional 40 days (total ≥70 days) of isolation (data not shown). The effects of social isolation for 30 days on the cortical basal concentrations of pregnenolone, progesterone, AP, and THDOC were reversed by twice-daily handling of the animals; the steroid concentrations thus did not differ significantly between the handled, isolated animals and those that were group housed (Fig. 3).

Effect of acute novel stress on socially isolated animals. Given that the hypothalamic–pituitary–adrenal (HPA) axis plays an important role in the steroidogenic response to acute stress (Barbaccia et al., 1997), we examined whether the sensitivity of the HPA axis to an acute novel stress (foot shock) was affected by the long-term stress associated with social isolation. Acute foot shock markedly increased the cerebral cortical (Fig. 4) and plasma (data not shown) concentrations of neuroactive steroids in both group-housed and isolated rats. However, the percentage increases in the cortical steroid concentrations induced by foot shock in isolated rats (pregnenolone, +197%; progesterone, +331%; AP, +395%; THDOC, +293%) were significantly ($p < 0.01$) greater than those induced in group-housed rats (pregnenolone, +75%; progesterone, +81%; AP, +78%; THDOC, +107%).

Effects of social isolation on $GABA_A$ receptor function

GABA-induced currents in oocytes injected with rat brain synaptosomes. To investigate the effects of social isolation on the function of $GABA_A$ receptors in the

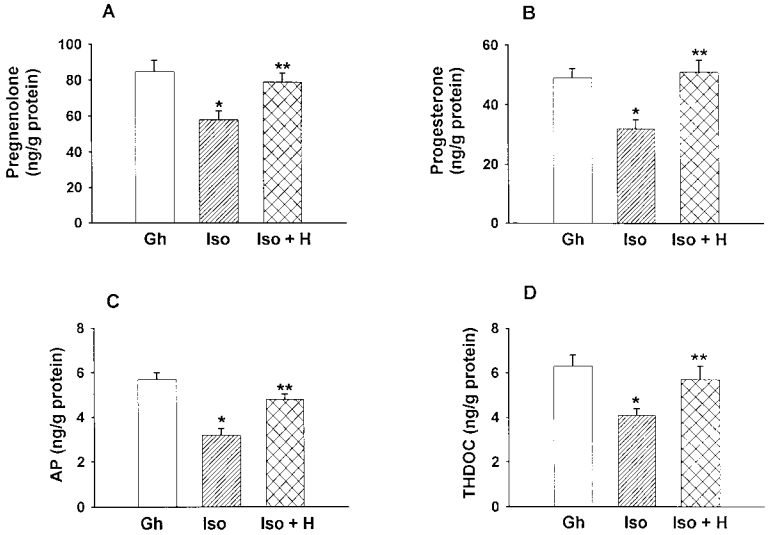

FIG. 3. Effect of handling on the social isolation-induced decreases in the cerebral cortical concentrations of neuroactive steroids in rats. Rats were housed either in groups (Gh) or in isolation for 30 days; the isolated animals were either subjected (Iso + H) or not (Iso) to handling twice daily. The animals were then killed directly from the home cage, and the cortical concentrations of pregnenolone (**A**), progesterone (**B**), AP (**C**), and THDOC (**D**) were measured. Data are means ± SEM of values from at least eight rats (each sample assayed in duplicate). $^*p < 0.05$ vs. group-housed animals; $^{**}p < 0.05$ vs. isolated animals.

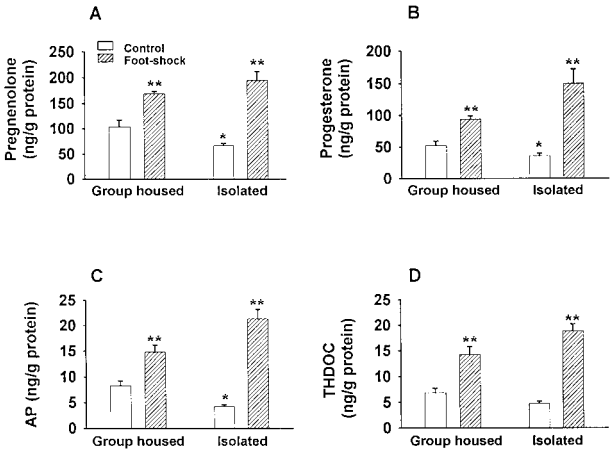

FIG. 4. Effects of social isolation on the acute stress-induced increases in the concentrations of neuroactive steroids in the cerebral cortex of rats. Animals were housed in groups or in isolation for 30 days, after which they were exposed (hatched columns) or not (open columns) to foot-shock stress for 5 min. The animals were killed 30 min thereafter, and the cortical concentrations of pregnenolone (**A**), progesterone (**B**), AP (**C**), and THDOC (**D**) were measured. Data are means ± SEM of values from 8–10 rats (each sample assayed in duplicate). $^{*}p < 0.05$ vs. nonshocked group-housed animals; $^{**}p < 0.01$ vs. respective nonshocked control animals.

brain, we injected synaptosomes isolated from the cerebral cortex or hippocampus of isolated or group-housed rats into *Xenopus* oocytes and characterized the functional properties of the "transplanted" $GABA_A$ receptors with the voltage-clamp technique (Marsal et al., 1995). We have previously shown (Sanna et al., 1996) that this procedure results in the incorporation of preformed $GABA_A$ receptors into the oocyte membrane, likely as a result of fusion of the injected synaptosomes with the oocyte membrane.

Twelve to 18 h after injection of oocytes with synaptosomes, GABA induced an inward Cl^- current in these cells, the peak amplitude of which was dependent on the concentration of the neurotransmitter; maximal current amplitudes induced by 10 m*M* GABA usually averaged ~500 nA. Comparison of the concentration–response curves for oocytes expressing cortical $GABA_A$ receptors revealed that the EC_{50} values for GABA did not differ significantly between group-housed (54.2 μM) and isolated (58.1 μM) rats; similar results were obtained for hippocampal $GABA_A$ receptors (data not shown).

To determine whether social isolation affected the sensitivity of $GABA_A$ receptors to positive and negative allosteric modulators, we tested the effects of diazepam and the β-carboline derivative β-CCE on GABA-evoked Cl^- currents. As expected (MacDonald and Olsen, 1994), diazepam markedly potentiated GABA-evoked Cl^- currents in oocytes expressing cortical $GABA_A$ receptors from group-housed rats (Fig. 5A); this effect was concentration dependent, with a maximal potentiation of 89 ± 9% apparent at 1 μM diazepam and an EC_{50} value of 220 n*M*. In oocytes injected with synaptosomes derived from isolated rats, the potentiating effect of diazepam was less pronounced (57 ± 5% at 1 μM), although the EC_{50} value (210 n*M*) was not significantly altered. Similar results were obtained with oocytes expressing hippocampal $GABA_A$ receptors (Fig. 5C). Thus, social isolation reduced the potentiating effect of 1 or 3 μM diazepam on GABA-evoked currents by ~40%; again, the potency of diazepam was not significantly affected by social isolation (EC_{50} values of 230 and 290 n*M* for group-housed and isolated rats, respectively).

β-CCE inhibited GABA-evoked Cl^- currents in oocytes injected with cortical synaptosomes from group-housed rats by 15 ± 3 and 7 ± 3% at concentrations of 1 and 3 μM, respectively (Fig. 5B). In oocytes expressing cortical $GABA_A$ receptors from socially isolated

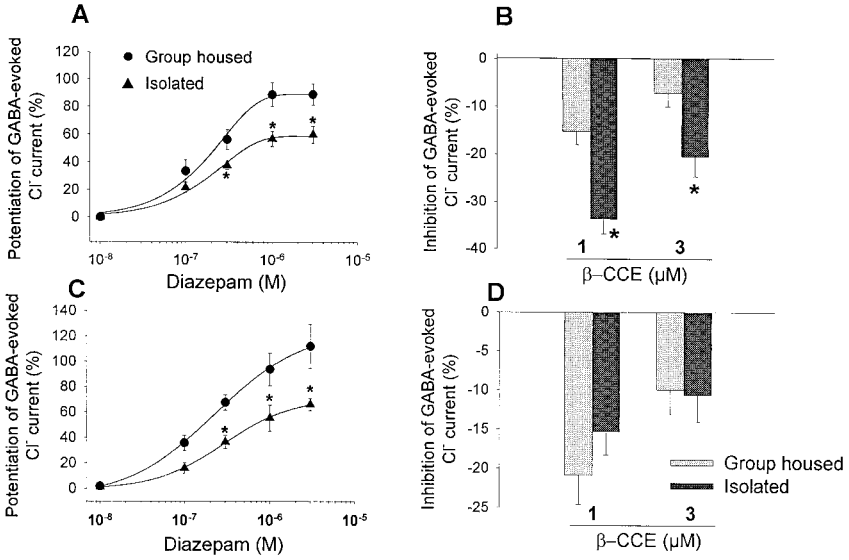

FIG. 5. Effects of social isolation on the modulation of $GABA_A$ receptor function by diazepam and β-CCE. *Xenopus* oocytes were injected with synaptosomes purified from the cerebral cortex (**A** and **B**) or hippocampus (**C** and **D**) of rats that were housed in groups or in isolation for 30 days. Oocytes were voltage-clamped at −90 mV, and the effects of the indicated concentrations of diazepam (A and C) or β-CCE (B and D) on the amplitude of GABA-evoked Cl^- currents were measured. Data are expressed as the percentage potentiation (diazepam) or inhibition (β-CCE) of the GABA response and are means ± SEM of values from six to eight different oocytes injected with synaptosomes from five rats for each experiment. $^{*}p < 0.05$ vs. respective value for group-housed animals.

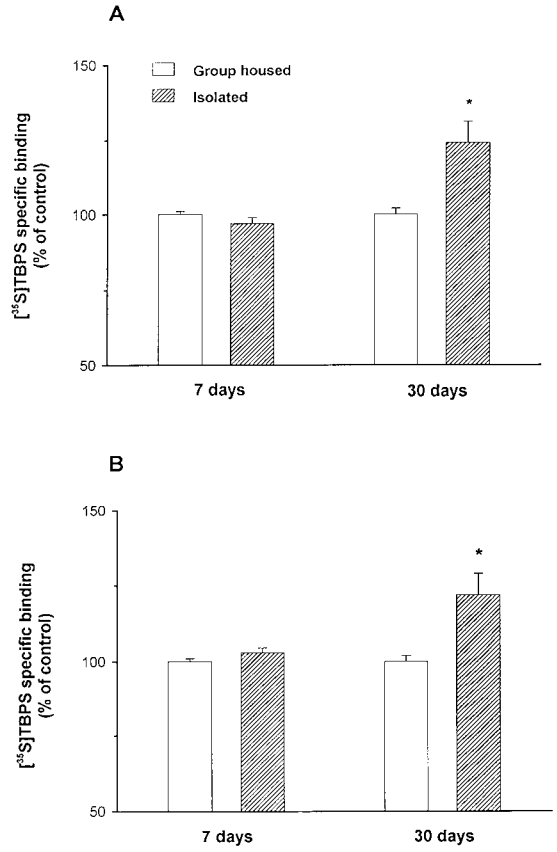

FIG. 6. Effects of social isolation of rats on [^{35}S]TBPS binding to cerebral cortical (**A**) and hippocampal (**B**) membranes. Animals were housed in groups (open columns) or in isolation (hatched columns) for 7 or 30 days, after which they were killed, and [^{35}S]TBPS binding to brain membranes was measured. Data are expressed as a percentage of control binding and are means ± SEM of values from 15 animals (each sample assayed in duplicate). *$p < 0.01$ vs. respective group-housed animals.

animals, β-CCE exhibited a markedly greater inhibitory effect (−34 ± 3 and −20 ± 4% at 1 and 3 μ*M*, respectively). In contrast, the inhibitory action of β-CCE on the GABA responses of oocytes injected with hippocampal synaptosomes was not significantly affected by social isolation (Fig. 5D).

[^{35}S]TBPS binding. Finally, we examined the effect of social isolation on brain $GABA_A$ receptor function by measuring [^{35}S]TBPS binding to cortical and hippocampal membranes. The extent of [^{35}S]TBPS binding to cortical or hippocampal membranes derived from rats subjected to social isolation for 30 days was significantly increased (+24 and +22%, respectively) compared with the corresponding values for group-housed rats; [^{35}S]TBPS binding to membranes from either brain region was not affected by social isolation for only 7 days (Fig. 6). The effect of social isolation for 30 days on [^{35}S]TBPS binding to cortical or hippocampal membranes was completely antagonized by twice-daily handling of the animals (data not shown).

DISCUSSION

We have shown that social isolation results in decreases in the brain and plasma concentrations of neuroactive steroids in rats. These effects are opposite to those elicited by acute stress (Purdy et al., 1991; Barbaccia et al., 1996), which induces large, rapid, and transient increases in the amounts of neuroactive steroids in the brain. Consistent with the results of previous studies showing that chronic or intermittent exposure to stressful conditions, including social isolation, results in a persistent increase in the plasma corticosterone concentration (Kant et al., 1983; Rivier and Vale, 1987; Greco et al., 1989; Hauger et al., 1990), the basal plasma concentration of this steroid was significantly increased in socially isolated rats. This observation is also consistent with the anxiety-like behavior demonstrated by these animals in the Vogel conflict and elevated plus-maze tests. Our findings are opposite to studies showing that isolation either decreased (Miachon et al., 1993; Sanchez et al., 1998) or left unchanged (Haller and Halàsz, 1999) the plasma corticosterone levels. However, the modest increase (+32%) we have found in isolated animals indicates that such experimental condition is a relatively mild stressor.

Social isolation of mice for 6–10 weeks was previously shown to induce a decrease in the cerebral cortical content of AP (Matsumoto et al., 1999). However, in contrast with our data, this previous study also showed that the effect of social isolation was selective for AP, with the brain concentrations of pregnenolone and progesterone remaining unchanged.

The molecular mechanisms that underlie the persistent decrease in the abundance of neuroactive steroids induced by social isolation in the rat remain unclear. Given that the concentrations of all steroids examined, with the exception of that of DHEA, were reduced in both the brain and the plasma of socially isolated animals, an effect of social isolation on the activity or expression of specific enzymes involved in the synthesis of selective neuroactive steroids, as suggested by Matsumoto et al. (1999), appears unlikely.

The observations that adrenalectomy markedly reduces the brain content of neuroactive steroids (Purdy et al., 1991) and abolishes the increases in the plasma and brain concentrations of these steroids induced by acute stress (Barbaccia et al., 1997) suggest that adrenal steroidogenesis plays an important role in maintaining the abundance of neuroactive steroids in both plasma and brain. Thus, a reduced activity of the HPA axis might contribute to the reduction in the amounts of neuroactive steroids apparent in isolated animals.

Neurons that release corticotropin-releasing factor (CRF) are activated by various types of chronic stress (Rivier and Vale, 1987; de Goeij et al., 1991), including that associated with social isolation (Ojima et al., 1995). Moreover, various chronic stress paradigms induce a relative increase in the amount of CRF mRNA in rat brain (Makino et al., 1995; Albeck et al., 1997), and the

intracerebroventricular administration of CRF in rats induces anxiety-like behavior similar to the conflict behavior detected in our socially isolated animals (Britton et al., 1986; Dunn and File, 1987). Given that long-term exposure of cultured pituitary cells to CRF induces desensitization to the effect of this factor on adrenocorticotropic hormone (ACTH) release (Hoffman et al., 1985), a partial reduction in pituitary responsiveness to high concentrations of CRF associated with chronic stress would result in a decrease in the extent of ACTH secretion and in turn a decreased stimulation of the synthesis of neuroactive steroids in the adrenal. Consistent with this scenario, the ACTH response of rat to chronic foot-shock or immobilization stress is reduced compared with that to acute stress (Rivier and Vale, 1987; Hauger et al., 1988).

The reduced basal concentrations of neuroactive steroids associated with social isolation may result from altered regulation of the HPA axis rather than from a decrease in secretory capability per se. Thus, a novel acute stress (foot shock) induced greater percentage increases in the brain and plasma (data not shown) concentrations of neuroactive steroids in socially isolated rats than in group-housed animals. These observations are consistent with the concept that a "facilitatory trace," characterized by hyperresponsiveness of the HPA axis to new stimuli, may develop during chronic stress (Akana et al., 1992).

Physiological and pharmacologically induced fluctuations in the brain concentrations of neuroactive steroids, such as those that occur during pregnancy and pseudopregnancy, result in the selective modulation of the function and expression of $GABA_A$ receptors in the rat cerebral cortex and hippocampus (Fénelon and Herbison, 1996; Concas et al., 1998, 1999; Follesa et al., 1998; Smith et al., 1998). Consistent with these observations, the persistent decreases in the concentrations of neuroactive steroids in the brains of socially isolated rats were associated with a decrease in the function of brain $GABA_A$ receptors. Prolonged social isolation affected the functional coupling between the recognition site for GABA and those for allosteric modulators such as benzodiazepines and anxiogenic β-carbolines. Thus, the efficacy, but not the potency, of diazepam in potentiating GABA-evoked Cl^- currents was markedly decreased in both the cerebral cortex and the hippocampus of isolated rats. In contrast, social isolation potentiated the inhibitory action of the benzodiazepine receptor inverse agonist β-CCE at $GABA_A$ receptors from the cerebral cortex but had no effect on the action of β-CCE at receptors derived from the hippocampus. We also showed that social isolation increased the extent of [^{35}S]TBPS binding to both cerebrocortical and hippocampal membranes. Given that acute stress or administration of negative modulators of GABAergic transmission increases [^{35}S]TBPS binding to brain membranes and induces experimental anxiety in animals or humans (Ninan et al., 1982; Corda et al., 1983; Dorow et al., 1983; Concas et al., 1988; Serra et al., 1989*b*; Biggio et al., 1990), the increase in [^{35}S]TBPS binding to both cortical and hippocampal membranes of socially isolated animals further supports the conclusion that social isolation results in a reduction in $GABA_A$ receptor function. These observations are thus consistent with the present and previous results showing that social isolation increases the level of anxiety in experimental animals, reduces the extent of radioligand binding to brain benzodiazepine receptors (Insel, 1989; Miachon et al., 1990), impairs the anxiolytic effect of diazepam in rats subjected to a social interaction test (Wongwitdecha and Marsden, 1996), and reduces the ability of GABA to stimulate $^{36}Cl^-$ uptake into mouse synaptoneurosomes (Ojima et al., 1997).

In conclusion, our data indicate that long-term social isolation, which alters the emotional behavior of animals, induces persistent decreases in both the plasma and the brain concentrations of neuroactive steroids and in brain $GABA_A$ receptor function. Given that (1) neuroactive steroids exert anxiolytic (Bitran et al., 1991) and antistress (Purdy et al., 1991; Barbaccia et al., 1994, 1996, 1997) effects, (2) the abundance of these steroids is reduced in individuals with depression (Romeo et al., 1998; Uzunova et al., 1998), (3) episodes of depression are often preceded by stressful events, and (4) patients with major depression exhibit signs of CRF hypersecretion (Owens and Nemeroff, 1991), it is possible that neuroactive steroids play a physiological role in the modulation of emotional behavior and mood.

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