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Effects of stress on the functional properties of pre- and postsynaptic 5-HT_{1B} receptors in the rat brain

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Abstract

Numerous studies have clearly shown that the turnover and release of serotonin (5-hydroxytryptamine, 5-HT) are increased under acute stressful conditions. Inasmuch as this latter process is under the control of a feedback mechanism involving the stimulation of presynaptic 5-HT_{1B} autoreceptors, we have investigated the possible effects of acute restraint (40 min) on the functional properties of 5-HT_{1B} receptors. The efficacy of the selective 5-HT_{1B} receptor agonist 3-[1,2,5,6-tetrahydropyrid-4-yl]pyrrolo-[3,2-*b*]pyrid-5-one (CP-93,129) in inhibiting in vitro the K^+-evoked release of [^{3}H]5-HT, was significantly reduced in stressed rats as compared to naive animals. Similarly, the responsiveness of 5-HT_{1B} receptors inhibiting the release of [^{3}H]acetylcholine (presynaptic 5-HT_{1B} heteroreceptors), was reduced by restraint. These effects were observed in the hippocampus, but using the inhibitory effect of CP-93,129 on forskolin-stimulated adenylyl cyclase activity as an index of 5-HT_{1B} receptor function, it could be shown that the 5-HT_{1B} receptors located in the substantia nigra are also desensitized by stress. The number as well as the apparent affinity constant of 5-HT_{1B} binding sites labelled by [^{125}I]iodocyanopindolol, as measured by quantitative autoradiography and membrane binding, were similar in naive and restraint-stressed rats suggesting that the stress-induced desensitization of 5-HT_{1B} receptors is not due to a reduced number of 5-HT_{1B} binding sites. As stress is thought to be a causal factor for the etiology of anxiety and depression, these results support the potential involvement of 5-HT_{1B} receptor dysfunction in the development of these neurological disorders.

Keywords: Stress; 5-HT (5-hydroxytryptamine, serotonin); 5-HT_{1B} receptor; 5-HT release

1. Introduction

Numerous studies have pointed out the involvement of the central serotonergic system in the cerebral reactions to stress. In particular, since the pioneering work of Thierry et al. (1968), many laboratories have consistently shown that a variety of acute stressful stimuli increase the synthesis and catabolism of serotonin (5-hydroxytryptamine, 5-HT, see Chaouloff, 1993 for review). These metabolic changes are thought to result from the increased availability of tryptophan, the precursor of 5-HT, to the brain (Kennett and Joseph, 1981; Joseph and Kennett, 1983). However, the observation that the activity of tryptophan hydroxylase, the initial and rate limiting enzyme in the synthesis of 5-HT, is enhanced in the midbrain and cortex of rats exposed acutely to sound stress (Boadle-Biber et al., 1989), indicates that an increased tryptophan hydroxylase activity might also contribute to elevate the brain synthesis of 5-HT under stressful conditions.

The increased synthesis and turnover of 5-HT in response to stress, might be occurring to avoid release-induced depletion of 5-HT from serotonergic neurons (Joseph and Kennett, 1983; Chaouloff, 1993). Indeed, the content of 5-hydroxyindoleacetic acid (5-HIAA), the main catabolite of 5-HT, has been shown to be increased in the brain of rats exposed to stress while the levels of 5-HT itself either decrease (Adell et al., 1988; Dunn, 1988) or remain unchanged (Morgan et al., 1975; Kennett and Joseph, 1981; Joseph and Kennett, 1983). This is thought to reflect an increase in the

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release of 5-HT. Even if these studies should be taken with caution, since a rise in the extracellular levels of 5-HIAA is not a reliable index of the release of 5-HT (Kuhn et al., 1986; Kalen et al., 1988), more recent work using intracerebral microdialysis has nevertheless confirmed that the release of 5-HT in the brain is enhanced by different stressful stimuli including immobilization (Shimizu et al., 1992; Kawahara et al., 1993; Vahabzadeh and Fillenz, 1994).

The release of 5-HT is under the control of a feed back mechanism involving the stimulation of presynaptic 5-HT_{1A} and 5-HT_{1B} autoreceptors. The stimulation of the former receptors, which are located on the dendrites and/or soma of the dorsal raphe 5-HT cells (Vergé et al., 1985), inhibits the firing of serotonergic neurons and, consequently, reduces the synthesis and release of 5-HT (Hamon et al., 1988; Sharp et al., 1989). The release of 5-HT is also inhibited by the stimulation of presynaptic 5-HT_{1B} autoreceptors which are located on the synaptic terminals of serotonergic neurons (Engel et al., 1986; Maura et al., 1986). In a recent study, it was observed that the functional activity associated to the stimulation of presynaptic 5-HT_{1A} receptors was not affected in rats exposed to cold stress (Zamfir et al., 1992). However, it is not known to what extend the functional properties of presynaptic 5-HT_{1B} receptors are affected by stress.

In order to determine the possible involvement of presynaptic 5-HT_{1B} autoreceptors in the alterations of the serotonergic system induced by stress, we measured in the present study the efficacy of a selective 5-HT_{1B} receptor agonist to inhibit the K^+-evoked release of [^{3}H]5-HT in hippocampal synaptosomes from naive or restraint-stressed rats. In addition, since 5-HT_{1B} receptors are also located postsynaptically on cholinergic terminals (5-HT_{1B} presynaptic heteroreceptors) where they exert a negative control of acetylcholine release (Maura and Raiteri, 1986; Bolaños and Fillion, 1989), the effects of stress on the functional activity of these receptors were also examined. Finally, 5-HT_{1B} receptors being negatively coupled to adenylyl cyclase activity (Bouhelal et al., 1988), we also investigated the possible changes in this effect after acute exposure to immobilization stress.

2. Materials and methods

2.1. Animals and stress procedure

All the experiments were performed in male Wistar rats (Iffa Credo, l'Arbresle, France), weighing 200–250 g at the time of the experiment. After arrival to the laboratory, the animals were housed in groups of five animals per cage and kept under controlled conditions: 20 ± 1°C temperature, 12 h night-dark cycle (lights on at 6 a.m.), with ad libitum access to water and food, during at least one week before any experimental manipulation.

Rats were subjected to restraint stress by keeping them immobilized into glass cylinders (6 cm diameter $\times$ 17 cm long) during 40 min. Immediately after the restraint stress session, the animals were killed by decapitation and the hippocampus and substantia nigra dissected on the cold. These experimental manipulations were performed between 9–11 a.m. to prevent any interference with the circadian variations in the plasmatic levels of corticosterone.

2.2. Release experiments

The release of [^{3}H]5-HT from hippocampal synaptosomes was performed according to the method described by Bolaños-Jiménez et al. (1994). Immediately after dissection, the hippocampus from naive or stressed rats was homogenized in 40 volumes (v/w) of ice-cold sucrose (0.32 M) buffered at pH 7.4 with phosphate. The homogenate was centrifuged at 1000 $\times$ g during 10 min at 4°C, the sedimented material was discarted and a crude synaptosomal fraction was obtained by the centrifugation of the supernatant at $12\,000 \times g$ during 20 min. The crude synaptosomal pellet was then resuspended in 2 ml of Krebs medium (pH = 7.4), pre-warmed to 37°C under a constant atmosphere of O_2/CO_2 (95/5%) and supplemented with 10 μM pargyline and 0.57 mM ascorbic acid. After addition of [^{3}H]5-HT (at a final concentration of 60 nM), the synaptosomes were incubated during 15 min at 37°C.

The composition of the Krebs medium (in mM), was as follows: NaCl 125, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, NaH_2PO_4 1.0, $NaHCO_3$ 22, glucose 10.

At the end of the incubation period, aliquots (200 μl) of the synaptosomal suspensions were distributed on Whatman glass fiber filters (GF/F) under light vacuum. The filters were then placed at the bottom of a superfusion apparatus and perfused at a rate of 0.5 ml/min with Krebs medium, warmed at 37°C under a constant atmosphere of O_2/CO_2. After a stabilisation period of 30 min, 6 min fractions were collected until the end of the experiment. 2 min periods of depolarization (20 mM KCl substituting for an equimolar concentration of NaCl) were applied at 36 and 54 min after the beginning of the superfusion (S_1 and S_2, respectively) to evoke the release of [^{3}H]5-HT. 12 min before the second depolarization, the synaptosomes were perfused with Krebs buffer containing different concentrations of the selective 5-HT_{1B} receptor agonist 3-(1,2,5,6-tetrahydropyrid-4-yl)-pyrrolo[3,2-*b*]pyrid-5-one (CP-93,129, Macor et al., 1991; Hoyer et al., 1994), which remained in the superfusion medium through the rest of the experiment.

The tritium content of both, the superfusate samples and the filters recovered at the end of the experiment, was determined by scintillation counting using Biodegradable Counting Scintillant (BCS, Amersham).

The amount of tritium released per 6 min sample, was expressed as a percentage of the total synaptosomal tritium content at the onset of the respective collection period. The K^+-evoked release of [^{3}H]5-HT (S_1 and S_2), was calculated by substracting the percentage of radioactivity present in the fraction collected just before the depolarization from the percentage of the fraction collected after depolarization. The effects of CP-93,129 on the K^+-evoked release of [^{3}H]5-HT were determined by comparison of the S_2/S_1 ratio obtained in the presence of this drug with that obtained by the perfusion of Krebs medium without drug.

The release of [^{3}H]acetylcholine from perfused hippocampal synaptosomes was measured according to the protocol described above with the following minor modifications: the Krebs medium was devoid of pargyline and ascorbic acid and the synaptosomes were labelled with [^{3}H]choline at a final concentration of 0.1 μM.

It has been previously shown that under these experimental conditions, when Ca^{2+} is ommitted from the superfusion medium, the basal release of tritium is not affected but the K^+-evoked release of [^{3}H]5-HT or [^{3}H]acetylcholine is totally abolished (Maura et al., 1986; Bolaños and Fillion, 1989).

2.3. Adenylyl cyclase assays

The activity of adenylyl cyclase was estimated by the conversion of [α-^{32}P]ATP into [α-^{32}P]cAMP. Freshly dissected substantia nigra from control or stressed rats were homogenized in 1 ml of ice-cold Tris maleate buffer (pH = 7.4), supplemented with ethylene glycol bis (2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, 2 mM) and sucrose (0.3 M). The homogenates were centrifuged at 1000 × *g* for 10 min at 4°C and the supernatant was used for the assay. 50 μl of the supernatant were incubated during 20 min at 30°C in a reaction medium made of (in mM): Tris-HCl (50), theophylline (10), ATP (0.1), GTP (0.01), $MgSO_4$ (1), phosphocreatine (20), forskolin (0.01), NaCl (100), 40 μg of creatine-kinase, 1 μCi of [α-^{32}P]ATP and ~ 40 000 cpm of [^{3}H]cAMP to quantify recovery. Samples also contained varying concentrations of CP-93,129. The reaction was stopped by the addition of 200 μl of a solution of sodium dodecyl sulfate (SDS, 1% w/v) containing 5 mM cAMP, 5 mM ATP and Tris-HCl 50 mM. The amount of [α-^{32}P]cAMP formed was separated by sequential chromatography on dowex and alumina columns.

2.4. Quantitative autoradiography studies

As a correlation to the results of functional studies, receptor binding using brain slices (quantitative autoradiography) or hippocampal membranes was performed to look for possible changes in the number of 5-HT_{1B} receptors. For the quantitative autoradiography studies, naive or stressed rats were decapitated and their brains rapidly removed and frozen using dry ice. Brain sections were cut in 20 μm slices with a microtome-cryostat, thaw-mounted onto gelatin-coated slides and stored at −20°C until use. For the labelling, the sections were preincubated twice during 15 min in Tris-HCl buffer (170 mM, pH 7.4) containing NaCl 150 mM. The slides were then incubated during 2 h at room temperature in the above (but fresh) buffer supplemented with 20 pM [^{125}I]iodocyanopindolol and 20 μM isoproterenol (to mask β-adrenoceptors). Nonspecific binding was determined by incubating adjacent sections in incubation buffer containing 1 μM of 5-methoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1*H*-indole (RU 24969). After the incubation period, the slides were washed by a brief dipping in ice-cold preincubation buffer, devoid of NaCl, followed by two 15 min washes in the same buffer. Finally, the sections were dried under a stream of cold air.

Autoradiograms were generated by apposing the labelled tissues to [^{3}H]hyperfilms along with [^{125}I]-iodinated standards available from Amersham. After 48 h of exposure, the films were developed and the autoradiograms were quantified with a computer image-analysis system.

B_{max} values were obtained by correcting the number of receptors bound per milligram of protein (B) for full occupancy of the receptor according to the following equation: $B_{max} = B[1 + K_d/L]$, where K_d = affinity constant and L = concentration of radioactive ligand.

2.5. Membrane binding experiments

Hippocampal tissues from naive or stressed rats were homogenized in 20 volumes of 50 mM Tris-HCl, pH 7.4 and centrifuged at 40 000 × *g* for 20 min at 4°C. The resulting pellet was suspended in 40 volumes of the same buffer and recentrifuged twice. The sedimented material was then resuspended in 40 volumes of Tris-HCl buffer for an incubation at 37°C during 10 min to eliminate endogenous 5-HT. After centrifugation, the final pellet was suspended in 50 volumes of 50 mM Tris-HCl pH 7.4.

Aliquots (50 μl corresponding to 40–50 μg protein), of thawed membrane suspensions were mixed with incubation buffer (10 mM Tris-HCl and 150 mM NaCl, pH 7.7 at room temperature), containing 15–400 pM [^{125}I]iodocyanopindolol and 20 μM isoproterenol in a

final volume of 0.25 ml. The samples were incubated at 37°C during 10 min and then filtered through Whatman GF/B filters using a Brandel Cell harvester. The filters were subsequently washed with 3×5 ml of ice-cold Tris buffer, dried and transfered to polypropylene tubes for radioactivity counting in a Beckman gamma counter at a counting efficiency of 80%. The specific binding was defined as the difference between total binding and that which persisted in the presence of 10 μM 5-HT.

Proteins were measured according to Lowry et al. (1951), with serum albumin (BSA) as the standard.

2.6. Corticosterone assay

In some experimental series, immediately following killing, trunk blood samples were collected in chilled tubes. The samples were centrifuged at $1000 \times g$ for 15 min and the supernatant removed and frozen at −20°C until assayed for corticosterone. The serum levels of corticosterone, after its extraction with ethylacetate, were measured by radioimmunoassay (RIA) using a commercial kit provided by Sigma and [1,2,6,7-^{3}H]corticosterone as radioligand.

2.7. Data analysis

All results are expressed as means ± S.E.M. The concentration-response curves of CP-93,129 in inhibiting 5-HT release and forskolin-stimulated adenylyl cyclase activity were submitted to non-linear regression analysis (Prism, GraphPad), to calculate the concentration producing half the maximal inhibitory effect (EC_{50}). Statistical analysis of changes between experimental groups and their controls, were determined by analysis of variance followed by two-tailed Student's t-test.

2.8. Drugs

[^{3}H]5-HT (59 Ci/mmol), [^{3}H]choline (75–85 Ci/mmol) and [^{125}I]iodocyanopindolol (2000 Ci/mmol) were obtained from Amersham, [^{3}H]corticosterone (87 Ci/mmol) from NEN and CGS 12066B was purchased from Research Biochem. (Natick, MA, USA). CP-93-129 was a generous gift from Pfizer (Groton, CT, USA).

3. Results

3.1. Effects of stress on the functional properties of presynaptic 5-HT$_{1B}$ autoreceptors

In hippocampal synaptosomes from naive rats, the basal release of [^{3}H]5-HT, expressed as percentage of total tritium content before the first depolarization (b_1), was 4.36 ± 0.32%. The efflux of radioactivity amounted to 8.73 ± 0.39% with the first K^+ stimulus (S_1). A similar pattern of release was observed with a second depolarization (S_2) performed 18 min later although there was a 20% decrease in the amount of tritium released by the second stimulation. Actually, the ratio of [^{3}H]5-HT released during the two depolarizations (S_2/S_1) corresponded to 0.79 ± 0.04.

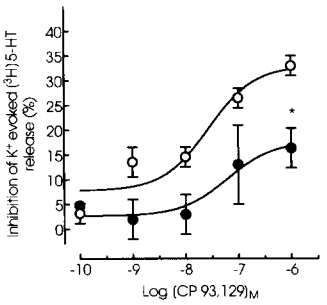

Fig. 1. Effects of restraint stress on presynaptic 5-HT$_{1B}$ autoreceptor sensitivity. Curves represent the concentration-dependent inhibitory effect of CP-93,129 on K^+-evoked [^{3}H]5-HT release in hippocampal synaptosomes from naive (open symbols) or restraint-stressed (solid symbols) rats. Aliquots of crude synaptosomal fractions loaded with [^{3}H]5-HT were superfused with Krebs medium at a rate of 0.5 ml/min as described in Materials and methods. Two peaks of depolarization (S_1 and S_2) were produced with 20 mM KCl. The selective 5-HT$_{1B}$ receptor agonist CP-93,129 was added 12 min before the second depolarization and remained in the medium until the end of the experiment. Data are expressed as percentage of K^+-evoked [^{3}H]5-HT release (S_2/S_1 ratio) in the absence of agonist. Each point in the curves corresponds to the mean ± S.E.M. of at least six independent experiments performed in triplicate. * $P < 0.01$ when compared to the respective value in naive animals (two tailed Student's t-test).

The selective 5-HT$_{1B}$ receptor agonist CP-93,129 (Macor et al., 1991; Hoyer et al., 1994), added 12 min before the second depolarization, reduced in a concentration-dependent manner the K^+-evoked release of [^{3}H]5-HT without altering the basal efflux of tritium (Fig. 1). The maximal inhibitory effect amounted to −33% and the non-linear regression analysis of the concentration-response curve indicated an EC_{50} value of 27.4 nM.

The basal as well as the K^+-evoked release of [^{3}H]5-HT in hippocampal synaptosomes from restraint-stressed rats, exhibited nearly the same characteristics as those observed in synaptosomes from naive animals ($b_1 = 4.67 \pm 0.32\%$ and $S_1 = 7.66 \pm 0.43\%$). However, the efficacy of CP-93,129 in inhibiting the depolarization-induced release of [^{3}H]5-HT was significantly reduced. Indeed, restraint-stress produced a shift to the right of the concentration-response curve of CP-93,129 (Fig. 1). As a consequence, the EC_{50} value increased by a factor of two (61.7 nM). Concomitantly, there was half a decrease in the maximal inhibitory effect of CP-93,129 (Fig. 1).

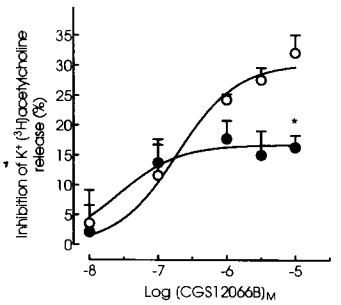

Fig. 2. Effects of restraint stress on presynaptic 5-HT_{1B} heteroreceptor sensitivity. Curves represent the concentration-dependent inhibitory effect of CGS 12066B on K^+-evoked [^{3}H]acetylcholine release in hippocampal synaptosomes from naive (open symbols) or restraint-stressed (solid symbols) rats. Experimental details as in the legend of Fig. 1. Data are expressed as percentage of K^+-evoked [^{3}H]acetylcholine release (S_2/S_1 ratio) in the absence of agonist. Each point in the curves corresponds to the mean ± S.E.M. of at least six independent experiments performed in triplicate. * $P < 0.01$ when compared to the respective value in naive animals (two-tailed Student's t-test).

3.2. Effects of stress on presynaptic 5-HT_{1B} heteroreceptors

The K^+-evoked release of [^{3}H]acetylcholine in hippocampal synaptosomes from naive rats, was smaller than that of [^{3}H]5-HT ($S_1 = 3.73 \pm 0.10\%$). However, the ratio of [^{3}H]acetylcholine and [^{3}H]5-HT released during the two K^+ depolarizations (S_1/S_2), was similar (0.86 ± 0.01 and 0.79 ± 0.04 for the release of [^{3}H]acetylcholine and [^{3}H]5-HT, respectively).

The perfusion of CP-93,129 reduced the K^+-evoked release of [^{3}H]acetylcholine (data not shown). However, under our experimental conditions, the efficacy of CP-93,129 in inhibiting the release of [^{3}H]acetylcholine was lower than that of 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)-pyrrolo[1,2-a]quinoxaline (CGS 12066B), another 5-HT_{1B} receptor agonist (Bolaños-Jiménez et al., 1994; Hoyer et al., 1994). The latter compound was therefore used for the determination of the changes induced by stress on the functional properties of 5-HT_{1B} presynaptic heteroreceptors.

In agreement with previous results, CGS 12066B reduced in a concentration-dependent manner the K^+-evoked release of [^{3}H]acetylcholine without affecting the basal release of tritium (Bolaños-Jiménez et al., 1994). In hippocampal synaptosomes from naive rats, the maximal inhibitory effect of CGS 12066B amounted to 32% and the EC_{50} value corresponded to 0.29 μM.

Restraint stress affected neither the basal nor the release of [^{3}H]acetylcholine induced by K^+ depolarization (data not shown). However, as illustrated in Fig. 2, the maximal inhibitory effect of CGS 12066B on this presynaptic process was significantly attenuated by stress. Indeed, whereas 10 μM CP-93,129 produced a 30% inhibition of K^+-evoked [^{3}H]acetylcholine release in naive rats, the same concentration of agonist reduced only 15% of the depolarization-induced release of [^{3}H]acetylcholine in hippocampal synaptosomes from stressed animals (Fig. 2). Thus, both auto- and heteropresynaptic 5-HT_{1B} receptors are desensitized by restraint stress. However, whereas restraint stress seems to produce both a shift to the right of the concentration-response curve and a reduction in the maximal inhibitory effect of CP-93,129 on [^{3}H]5-HT release, only a reduction in the maximal inhibitory effect of [^{3}H]acetylcholine release could be observed after the stimulation of 5-HT_{1B} presynaptic heteroreceptors by CGS 12066B in stressed rats.

3.3. Effects of stress on 5-HT_{1B} receptors negatively coupled to adenylyl cyclase

The basal convertion ratio of [α-^{32}P]ATP into [α-^{32}P]cAMP in substantia nigra homogenates from naive rats, was 62.32 ± 3.86 pmol/mg protein per 20 min. In the presence of 10 μM forskolin, the convertion ratio of [α-^{32}P]ATP, expressed in percentage above basal levels, increased to $755.88 \pm 54.21\%$. Exposure to increasing concentrations of CP-93,129 reduced the accumulation of [α-^{32}P]cAMP induced by forskolin (Fig. 3). The maximal inhibitory effect amounted to $23 \pm 1.74\%$ and the concentration producing half the maximum inhibitory effect, determined by non-linear regression

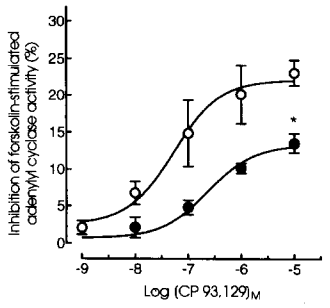

Fig. 3. Comparison of the concentration-dependent inhibitory effect of CP-93,129 on forskolin-stimulated adenylyl cyclase activity in substantia nigra homogenates from naive (open symbols) or restraint-stressed (solid symbols) rats. Fresh substantia nigra from naive or restraint-stressed rats was homogenized in ice-cold tris-maleate buffer (2 mM) containing 2 mM EGTA and 0.3 M sucrose. The homogenates were centrifuged at 1000 × g at 4°C during 10 min and the supernatant was used for the assay. The enzymatic activity of adenylyl cyclase was determined by the conversion of [α-^{132}P]ATP into [α-^{132}P]cAMP as described in Materials and methods. Data were analyzed by non-linear regression analysis (Prism, GraphPad) and are expressed as percentage of forskolin-stimulated adenylyl cyclase activity in the absence of CP-93,129. Each point in the curves represents the mean ± S.E.M. of triplicate determinations from at least five independent experiments. * $P < 0.01$ when compared to the respective value in naive animals (two-tailed Student's t-test).

analysis, was 53.4 nM. These data are equivalent to previous values concerning the maximal inhibition produced by 5-HT or CP-93,129 on forskolin-stimulated adenylyl cyclase activity via the specific stimulation of 5-HT_{1B} receptors (Bouhelal et al., 1988; Macor et al., 1991).

The functional activity associated to the stimulation of 5-HT_{1B} receptors located in the substantia nigra, was also reduced by restraint stress. Thus, the concentration-response curve of CP-93,129 in homogenates from stressed rats, was shift to the right in relation to that of controls (Fig. 3) and the EC_{50} value of CP-93,129 was increased to 237 nM. Restraint stress produced also a 40% attenuation of the maximal response. It is conceivable that these biochemical changes result from a reduction of the sensitivity of 5-HT_{1B} receptors since neither the basal nor the forskolin-stimulated accumulation of [α-^{32}P]cAMP were affected by stress (data not shown).

3.4. Effects of stress on the serum content of corticosterone

The plasmatic levels of corticosterone in naive animals were slightly higher than those usually observed under basal conditions (Fig. 4), which might indicate that handling induces some degree of stress. As expected from a stressful stimulus, the serum content of corticosterone was significantly enhanced by restraint. Actually, there is an increase of 400% in the serum levels of corticosterone in stressed rats in relation to naive animals (Fig. 4).

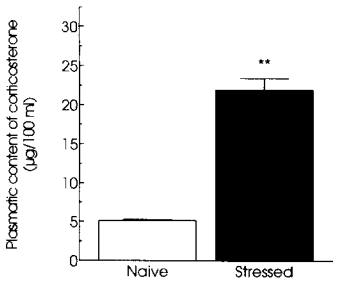

Fig. 4. Effects of restraint stress on the serum levels of corticosterone. Corticosterone levels were determined in trunk blood samples collected from male rats immediately after their immobilization into glass cylinders for 40 min. ** $P < 0.001$ as determined by two-tailed Student's t-test for independent groups ($n = 6$).

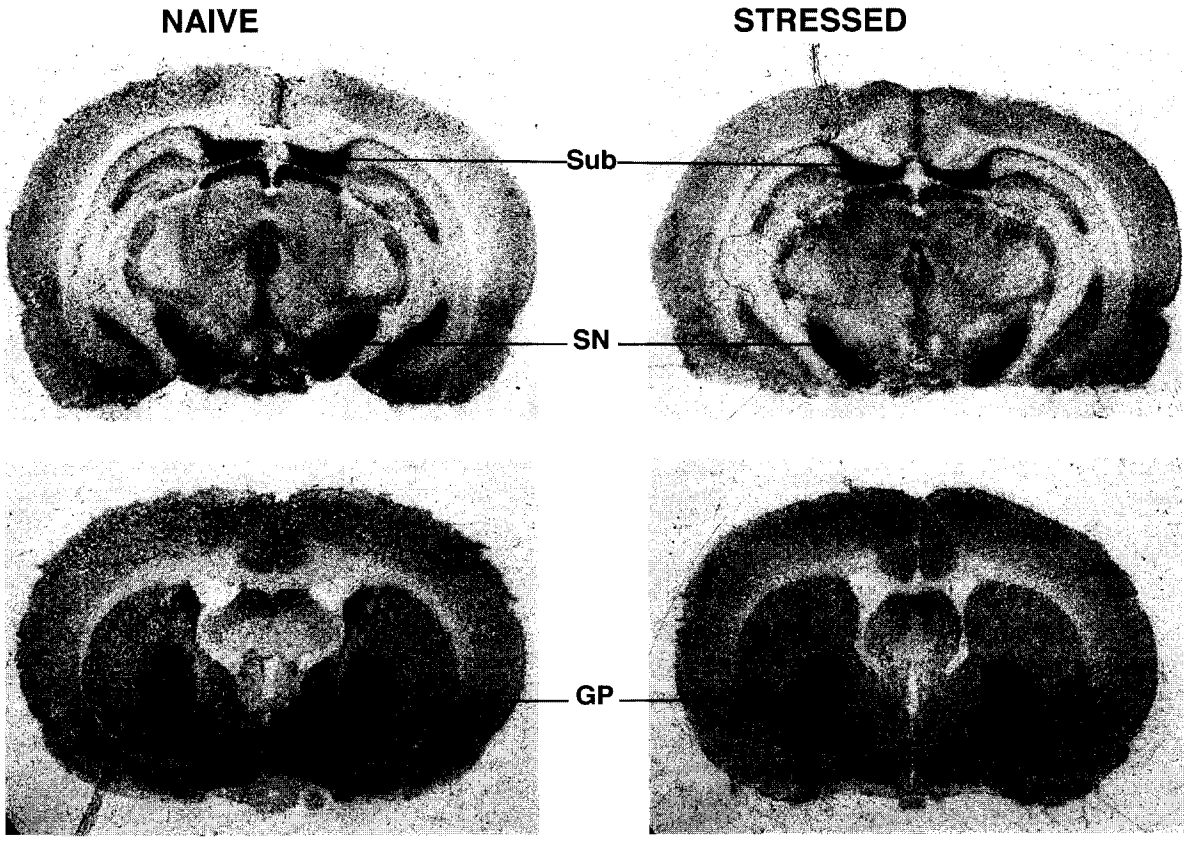

Fig. 5. Autoradiograms illustrating the anatomical distribution of [^{125}I]iodocyanopindolol specific binding to 5-HT_{1B} receptors in the brain of naive or restraint-stressed rats. Sections were incubated in Tris-HCl buffer (170 mM + 150 mM NaCl, pH 7.4) containing 20 pM [^{125}I]iodocyanopindolol and 20 μM isoproterenol (to mask β-adrenoceptors) during 2 h at room temperature. Non-specific binding was determined by incubating adjacent sections in incubation buffer supplemented with 1 μM RU 24969. GP: globus pallidus; Sub.: subiculum; SN: substantia nigra.

Table 1
Densitometric analysis of [^{125}I]iodocyanopindolol specific binding to 5-HT$_{1B}$ receptors in several brain regions of naive or restraint-stressed rats

Brain region	Specific binding (fmol/mg * protein)	
	Naive	Stressed
Globus pallidus	531.4 ± 150.6	446.5 ± 108.9
Substantia nigra	414.3 ± 2.5	331.5 ± 41.8
Hippocampus		
Dorsal subiculum	388.8 ± 16.4	417.2 ± 24.9
CA_3 field	739.9 ± 118.6	769.3 ± 90.1

Sections were labelled with 20 pM [^{125}I]iodocyanopindolol in the absence or in the presence (non-specific binding) of 1 μM RU 24969. Optical density was measured in corresponding autoradiograms and transformed to fmol of radioligand bound per mg of tissue through use of curves generated from coexposure to film of [^{125}I]iodinated standards available from Amersham. Values represent means of specific binding ± S.E.M. made in four to six sections from six rats. No statistically significant differences were found between naive and restraint-stressed rats.

3.5. Effects of stress on the specific binding of [^{125}I]iodocyanopindolol to 5-HT$_{1B}$ receptors

As a first attemp to determine the cellular mechanism underlying the desensitization of 5-HT$_{1B}$ receptors induced by stress, quantitative autoradiography analysis and saturation studies of the specific binding of [^{125}I]iodocyanopindolol to 5-HT$_{1B}$ receptors were performed. Our analyses were focussed to those regions in which the functional activity of 5-HT$_{1B}$ receptors could be measured, i.e. hippocampus and substantia nigra.

In agreement with the regional distribution of 5-HT$_{1B}$ receptors in the rat brain (Pazos and Palacios, 1985; Bruinvels et al., 1993), intense [^{125}I]iodocyanopindolol labelling was observed in globus pallidus, substantia nigra and the dorsal subiculum of the hippocampus (Fig. 5). The maximal density of 5-HT$_{1B}$ binding sites in these brain regions under our experimental conditions, ranged between 331 and 769 fmol/mg * protein. No significant differences in the number of 5-HT$_{1B}$ binding sites were found between naive and restraint-stressed rats in any of the brain regions examined (Table 1). Only a small (−16%) but not statistically significant reduction of the labelling of [^{125}I]iodocyanopindolol was observed in the globus pallidus of restraint-stressed rats as compared to controls.

In agreement with these results, the Scatchard analysis of the saturation curves of the specific binding of [^{125}I]iodocyanopindolol to hippocampal 5-HT$_{1B}$ receptors, indicated that there are no significant differences between naive and restraint animals in either the maximal number (B_{max}) or apparent affinity constant (K_d) of 5-HT$_{1B}$ binding sites (Table 2).

Table 2
Effects of restraint stress on the characteristics of the specific binding of [^{125}I]iodocyanopindolol to hippocampal 5-HT$_{1B}$ receptors in the rat brain

	B_{max} (fmol/mg * protein)	K_d (nM)
Naive	117.1 ± 17.3	0.16 ± 0.03
Stressed	132.9 ± 10.6	0.18 ± 0.02

Binding assays were performed using eight different concentrations of [^{125}I]iodocyanopindolol. Each value is the mean ± S.E.M. of four independent saturation binding experiments. Each saturation experiment was performed on tissue from one rat. No statistically significant differences were found between naive and restraint-stressed rats.

4. Discussion

The results of this study show that both auto- and hetero-presynaptic 5-HT$_{1B}$ receptors are desensitized by stress. Actually, the efficacy of two selective 5-HT$_{1B}$ receptor agonists to inhibit the release of 5-HT (CP-93,129) or acetylcholine (CGS 12066B), is significantly reduced in rats subjected to acute restraint stress. These effects were observed in the hippocampus, but using the inhibitory effect of CP-93,129 on forskolin-stimulated adenylyl cyclase activity as an index 5-HT$_{1B}$ receptor function, it could be shown that the 5-HT$_{1B}$ receptors located in the substantia nigra are also desensitized by stress.

Neither the basal nor the K^+-evoked release of [^{3}H]5-HT or [^{3}H]acetylcholine were affected by stress, indicating that the reduced efficacy of CP-93,129 and CGS 12066B to inhibit the release of [^{3}H]5-HT or [^{3}H]acetylcholine, is rather due to a decreased sensitivity of 5-HT$_{1B}$ receptors than to a modification of the release process of these neurotransmitters. Similarly, the fact that the basal as well as the forskolin-stimulated levels of cAMP in naive and stressed rats were the same, strongly suggest that the stress-induced desensitization of 5-HT$_{1B}$ receptors does not result from the modification of the enzymatic activity of adenylyl cyclase.

Our data are in agreement with a previous study showing no change in the basal release of [^{3}H]5-HT in perfused hippocampal synaptosomes from noise-stressed rats (Mennini et al., 1993). In this latter study, a reduced efficacy of the uptake process of 5-HT in response to stress was also pointed out indicating that the extracellular content of 5-HT is enhanced in acutely stressed animals. In fact, several groups have shown that the in vivo release of 5-HT in the hypothalamus and hippocampus of the rat brain, as measured by microdialysis, is increased to 200–250% above basal levels in rats subjected to psychological or restraint stress (Shimizu et al., 1992; Kawahara et al., 1993; Vahabzadeh and Fillenz, 1994). It could therefore be argued that the reduced efficacy of the 5-HT$_{1B}$ recep-

tor agonists to inhibit the release of 5-HT or acetylcholine, results from their competition at 5-HT_{1B} receptors with the endogenous 5-HT released during stress. However, the fact that under our experimental conditions the accumulation of 5-HT in the synaptic cleft is minimal, as well as the observation that the release of [^{3}H]5-HT and [^{3}H]acetylcholine evoked by the first K^+ depolarization (S_1) is similar in naive and stressed rats, are not in favour with this interpretation. Indeed, if the endogenous 5-HT released during stress had any incidence on the effects of CP-93,129 or CGS 12066B, we would expect a reduced S_1 value in stressed rats as compared to that of naive animals.

The quantitative autoradiography analysis of 5-HT_{1B} binding sites labelled by [^{125}I]iodocyanopindolol in several regions of the rat brain, indicated that the number of 5-HT_{1B} receptors was similar in naive and stressed rats. Moreover, no significant differences were observed between naive and stressed rats in either the total density (B_{max}) or apparent affinity constant (K_d) of hippocampal 5-HT_{1B} binding sites. Thus, the decreased responsiveness of 5-HT_{1B} receptors cannot be explained by a reduction of their synthesis. Indeed, there is a lack of temporary correspondence between the process of down-regulation, that usually takes several hours to develop, and the effects of stress on the function of 5-HT_{1B} receptors which are already observed after 40 min of restraint. These data further support the idea that the stress-induced changes in the sensitivity of 5-HT_{1B} receptors are due to the specific modification of their functional properties.

The mechanism by which restraint stress reduces the sensitivity of 5-HT_{1B} receptors is unclear. The desensitization of both auto- and hetero-presynaptic 5-HT_{1B} receptors has been reported to occur also after the chronic administration of antidepressants (Blier et al., 1988; Moret and Briley, 1990; Bolaños-Jiménez et al., 1994). Since most of the drugs used in these studies are potent and selective 5-HT uptake blockers, it was proposed that these neurochemical modifications result from the enhancement in the extracellular content of 5-HT. In support of the idea that the overstimulation of 5-HT_{1B} receptors by 5-HT might led to their desensitization, two independent studies have shown that pre-exposure to 5-HT reduced the number as well as the functional activity associated to the stimulation of 5-HT_{1B} receptors in opossum kidney cells (Pleus and Bylund, 1992; Unsworth and Molinoff, 1992). The desensitization of 5-HT_{1B} receptors reported in the present study, might therefore be occurring in response to stress-induced increases in serotonergic activity. However, exposure of kidney cells to 5-HT during a period of at least 3 h is necessary to observe any change in the number or sensitivity of 5-HT_{1B} receptors (Pleus and Bylund, 1992; Unsworth and Molinoff, 1992). This observation is again incompatible with the rapid effects of stress on 5-HT_{1B} receptor function. Moreover, the long term enhancement of extracellular 5-HT, through 5-HT uptake blockade, does not induce systematically the desensitization of 5-HT_{1B} receptors (Moret and Briley, 1990). Thus, the stress-induced increase in the extracellular content of 5-HT might not be the sole factor involved in the desensitization of 5-HT_{1B} receptors observed immediately after restraint.

The specific binding of [^{125}I]iodocyanopindolol to 5-HT_{1B} receptors in the dentate gyrus has been shown to be increased by adrenalectomy (Mendelson and McEwen, 1992). In addition, the number of hippocampal and cortical 5-HT_{1B} receptors has been shown to be decreased in rats chronically exposed to high concentrations of corticosterone (Mendelson and McEwen, 1992). These data might indicate, as already suggested for 5-HT_{1A} receptors (Mendelson and McEwen, 1992; Chalmers et al., 1993), that circulating corticosteroids exert an inhibitory effect on the functional expression of 5-HT_{1B} receptors. Since the serum levels of corticosterone increase importantly during stress (see Fig. 4), one possible explanation for the restraint-induced desensitization of 5-HT_{1B} receptors might be that corticosterone interferes with the transduction mechanism of 5-HT_{1B} receptors. Indeed, even if the effects induced by corticosteroids have generally a slow onset and involve alterations in protein synthesis, corticosteroids can also induce rapid effects in the brain (see McEwen, 1991 for review). In particular, it has been shown that cortisol or corticosterone decrease rapidly the excitability of hippocampal pyramidal cells in vitro through the inhibition of voltage-gated Ca^{2+} channels currents (ffrench-Mullen, 1995). Interestingly, this effect seems to be mediated by the activation of a G-protein-coupled mechanism (ffrench-Mullen, 1995). The possible role played by corticosteroids in the desensitization of 5-HT_{1B} receptors induced by stress, remains to be determined.

The cell bodies of the 5-HT neurons innervating the forebrain, are mainly localized within the dorsal and median raphe nuclei (Dahlström and Fuxe, 1964). The ascending 5-HT containing fibers arising from these two systems are morphologically dissimilar and preferentially projected to different brain structures (Azmitia and Segal, 1978; Törk, 1990). In addition to these anatomical differences, it has been suggested that the median and dorsal raphe nuclei are affected differentially by several pharmacological and behavioural manipulations including stress (Lee et al., 1987; O'Hearn et al., 1988; Blier et al., 1990). In the present study, the effects of restraint stress on the functional activity associated to the stimulation of 5-HT_{1B} receptors in the hippocampus, which contains 5-HT axons arising predominantly from the median raphe, and the substantia nigra, which appears to receive 5-HT projections mainly from the dorsal raphe, were examined. In

both regions, restraint decreased the sensitivity of 5-HT_{1B} receptors indicating that the median and dorsal raphe serotonergic systems are affected in the same way by this type of stress or, alternatively, that there are no regional variations in the responsiveness of 5-HT_{1B} receptors to restraint stress.

Stress is thought to be a causal factor for the etiology of anxiety and depressive disorders (Eison, 1990, Deakin and Graeff, 1991; Anisan and Zacharko, 1992), two pathological conditions related to increased (anxiety) or decreased (depression) central serotonergic activity. In this context, the herein reported results might suggest that some of the neurobiological disturbances of the serotonergic system observed in anxiety and depression could be due, at least partially, to 5-HT_{1B} receptor dysfunction. Indeed, the potential involvement of 5-HT_{1B} receptors in the mechanism of action of antidepressants and the etiology of depression has been documented by several studies. Thus, both auto- and hetero-presynaptic 5-HT_{1B} receptors have been shown to be desensitized after the long term treatment with antidepressants (Blier et al., 1988; Moret and Briley, 1990; Bolaños-Jiménez et al., 1994). Moreover, 5-HT_{1B} receptor stimulation blocks the antidepressant-like effects of selective 5-HT uptake inhibitors, such as citalopram and fluoxetine, in two animals models of depression: the forced swimming test (Cervo et al., 1989) and the learned helplessness paradigm (Martin and Puech, 1991). Interestingly, the specific binding of [^{125}I]iodocyanopindolol to 5-HT_{1B} sites has been shown to be increased in the cortex, hippocampus and septum of learned helplessness rats (Edwards et al., 1991). This suggests that the development of learned helplessness, which might be considered as a depressive-like state, is associated with enhanced 5-HT_{1B} receptor sensitivity. The increased basal and K^+-evoked release of cortical 5-HT in learned helplessness rats, as determined in vivo by microdialysis (Petty et al., 1992), is also in favour with the idea that 5-HT_{1B} receptors are hypersensibilized in learned helplessness animals.

In summary, it was shown that restraint stress induces a significant desensitization of presynaptic 5-HT_{1B} autoreceptors as indicated by the reduced efficacy of the 5-HT_{1B} receptor agonist CP-93,129 to inhibit the K^+-evoked release of [^{3}H]5-HT. It is tempting to propose that this desensitization of presynaptic 5-HT_{1B} autoreceptors contributes to the enhancement of 5-HT release induced by acute stressful stimuli. However, it cannot be defined at the present time, if the desensitization of 5-HT_{1B} presynaptic autoreceptors in restraint-stressed animals results from, or leads to, the increased release of 5-HT. The functional efficacy of 5-HT_{1B} receptors inhibiting the hippocampal release of acetylcholine (presynaptic 5-HT_{1B} heteroreceptors) or forskolin-stimulated adenylyl cyclase activity (in the substantia nigra), was also reduced by restraint. These data further suggest that no regional differences exist in the responsiveness of 5-HT_{1B} receptors to stress. As stress has been suggested to play a role in the etiology of anxiety and depression (Chopin et al., 1994), these results support the potential involvement of 5-HT_{1B} receptor dysfunction in the development of these neurological disorders.

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