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Effects of chronic diazepam treatment on pre- and postsynaptic 5-HT_{1A} receptors in the rat brain

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Abstract

Biochemical and electrophysiological approaches were used to assess possible changes in 5-HT_{1A} receptors in the rat brain after long-term treatment with an anxiolytic benzodiazepine. Rats were treated with diazepam (2 mg/kg i.p. daily) during 14 days and then untreated for 1 day (protocol A) or 5 days (protocol C) until they were killed for in vitro investigations on 5-HT_{1A} receptors. In addition, other rats (protocol B) received the same 14-day treatment with diazepam, followed by 1 mg/kg of the drug on days 15 and 16, and 0.5 mg/kg on days 17 and 18, and were killed 24 h after the last injection. In vitro binding and quantitative autoradiographic experiments with [^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralin ([^{3}H]8-OH-DPAT) showed that the characteristics of 5-HT_{1A} receptor binding sites in the hippocampus and the dorsal raphe nucleus were not significantly altered by the administration of diazepam under the treatment protocols A, B and C. Furthermore, in vitro electrophysiological recordings of serotoninergic neurons in the dorsal raphe nucleus of brain stem slices revealed no modification in the sensitivity of somatodendritic 5-HT_{1A} autoreceptors in rats treated with diazepam according to the protocols A and B. However, under the conditions of protocol C, the potency of 8-OH-DPAT to depress the firing rate of serotoninergic neurons was significantly enhanced, as expected of a hypersensitivity of somatodendritic 5-HT_{1A} autoreceptors. These data support the hypothesis that some functional changes in these receptors could occur during benzodiazepine withdrawal. However, they do not support the idea of a reduced anxiolytic efficacy of 5-HT_{1A} receptor agonists as a result of prior treatment with a benzodiazepine.

Keywords: 5-HT (5-hydroxytryptamine, serotonin); Diazepam; Withdrawal; 5-HT_{1A} receptor; Raphe nucleus, dorsal; Electrophysiological recording

1. Introduction

In contrast with benzodiazepines, which can induce physical dependence resulting in withdrawal problems (rebound anxiety, tremor, and, occasionally, convulsions) upon cessation of treatment (File, 1990), the novel anxiolytics of the azapirone series, buspirone, gepirone, ipsapirone, do not produce such phenomena (New, 1990; Keppel Hesselink, 1992). The latter drugs have therefore been proposed as an alternative to the benzodiazepines for the treatment of anxiety disorders, especially because the azapirones do not cause the well-known side effects of diazepam and derivatives: sedation, myorelaxation, mnesic deficits (New, 1990; Keppel Hesselink, 1992). However, attempts to substitute buspirone for a benzodiazepine have generally been disappointing because several authors (Schweizer et al., 1986; Napoliello and Domantay, 1988; Ashton et al., 1990), but not all (Pecknold et al., 1985; Delle Chiaie et al., 1995), reported a reduced efficacy of the azapirone to alleviate anxiety in patients who recently received benzodiazepine treatment. Animal studies confirmed this observation as buspirone, gepirone and ipsapirone were occasionally found to be unable to prevent the rebound anxiety-like behaviour in rats which had been treated with a benzodiazepine (File and Andrews, 1991).

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Indeed, some authors even reported that the combined treatment of a benzodiazepine plus buspirone, gepirone or ipsapirone aggravates the withdrawal signs upon cessation of treatment (Goudie and Leathley, 1991a,b; Mizoguchi et al., 1993; Goudie et al., 1994; but see Korkmaz et al., 1994 and Vivian et al., 1994).

Studies on the neurobiological mechanisms of such pharmacodynamic interactions between benzodiazepines and azapirones are still in their infancy, and little is known to date regarding this question. Central serotoninergic neurotransmission might play a crucial role because anxiety is thought to be associated with an increased serotoninergic tone (Iversen, 1984; Andrews and File, 1993; Hamon et al., 1993), whereas, in contrast, anxiolytic drugs are known to reduce the electrical and metabolic activity of serotoninergic neurons (Nakamura and Fukushima, 1977; Trulson et al., 1982; Laurent et al., 1983; Soubrié et al., 1983; De Vry et al., 1992) In the case of azapirones, these effects are due to their direct agonist action at the somatodendritic 5-HT_{1A} autoreceptors (Gozlan et al., 1983; Eison et al., 1986; Jolas et al., 1995), which triggers a negative feedback control of serotoninergic neurons in the dorsal raphe nucleus (Sprouse and Aghajanian, 1987; Haj-Dahmane et al., 1991). Indeed, the local microinfusion of these drugs and other 5-HT_{1A} receptor agonists directly into the dorsal raphe nucleus to stimulate somatodendritic 5-HT_{1A} autoreceptors reproduces both the neurobiological and behavioural (i.e., anxiolytic) effects observed upon their systemic administration (Higgins et al., 1988; Schreiber and De Vry, 1993; Hogg et al., 1994; Jolas et al., 1995; Maurel Remy et al., 1996). Therefore, 5-HT_{1A} autoreceptors in the dorsal raphe nucleus are very probably a key target for the azapirones to exert their anxiolytic action. In addition, postsynaptic 5-HT_{1A} receptors located in limbic forebrain areas might also be involved as some groups also reported a reduction in anxiety-driven behaviour in rats which received 5-HT_{1A} receptor agonists directly into the hippocampus (Kostowski et al., 1989; Kataoka et al., 1991; Carli et al., 1993; Przegalinski et al., 1994, but see Hogg et al., 1994).

Accordingly, it can be hypothesized that some alterations in central 5-HT_{1A} receptors account for the reduced efficacy of azapirones to exert their anxiolytic effects after chronic treatment with a benzodiazepine. We directly addressed this question by looking for possible changes in 5-HT_{1A} receptors both in the dorsal raphe nucleus and the hippocampus of rats after a 14-day treatment with diazepam. Membrane binding assays and quantitative autoradiography labeling of brain sections were used for the determination of the characteristics of 5-HT_{1A} receptor binding sites specifically labelled by [^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralin ([^{3}H]8-OH-DPAT) in these two brain regions (Hall et al., 1985). In addition, electrophysiological recordings in brain stem slices were used for assessing the functional properties of somatodendritic 5-HT_{1A} autoreceptors on serotoninergic neurons within the dorsal raphe nucleus (Haj-Dahmane et al., 1991).

2. Materials and methods

2.1. Animals

Experiments were performed on male Sprague-Dawley rats (Centre d'Elevage R. Janvier, Le Genest-St-Isle, France) weighing 200–250 g at the time of death. Animals were housed in groups of six under standard laboratory conditions (22 ± 1°C, 60% relative humidity, 12 h-12 h light-dark cycle, food and water ad libitum) for at least 1 week prior to the treatments. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (Council Directive No. 87-848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permissions No. 0299 to M.H. and No. 6269 to L.L.).

2.2. Treatments

Rats were injected daily with diazepam (2 mg/kg i.p. at 9:00–9:30 a.m.) or its vehicle (arabic gum in saline, 0.5 ml/injection) for 14 days, and then divided into three different groups. Animals in groups A (diazepam-treated and vehicle-treated paired controls) were killed by decapitation on day 15, 24 h after the last injection; those in groups B were further injected with the vehicle or diazepam at the dose of 1 mg/kg i.p. on days 15 and 16, and of 0.5 mg/kg i.p. on days 17 and 18, and were decapitated on day 19, 24 h after the last injection; rats in groups C remained untreated for 5 days before they were killed (on day 19).

2.3. Membrane binding assays

Brains were removed from the skull immediately after death, and dissected at 4°C (Glowinski and Iversen, 1966). The left and right hippocampi from each rat were pooled, weighed and homogenized in 20 vols. (v/w) of ice-cold 50 mM Tris-HCl (pH 7.4 at 23°C) using a Polytron disrupter (type PT10 OD). Homogenates were centrifuged at $40\,000 \times g$ for 20 min at 4°C. The supernatant was discarded and the pellet was washed twice by resuspension in 20 vols. Tris-HCl buffer followed by centrifugation. The sedimented material was then gently homogenized in 20 vols. Tris-HCl and incubated at 37°C for 10 min to remove endogenous 5-HT (Nelson et al., 1978). Membranes were collected by centrifugation and washed three times by 'resuspension-centrifugation' as before. The final pellet was suspended in 10 vols. of 50 mM Tris-HCl, pH 7.4, and aliquots of the resulting suspensions were kept at −80°C before being used for binding assays.

Binding assays with [^{3}H]8-OH-DPAT were as described in detail elsewhere (Hall et al., 1985). In brief, aliquots of membrane suspensions (50 μl, corresponding

to ≃0.25 mg membrane proteins) were mixed with 50 mM Tris-HCl, pH 7.4, containing [^{3}H]8-OH-DPAT (0.1–4.0 nM) with or without buspirone (1 nM–10 μM) or 8-OH-DPAT (0.1 nM–1 μM), in a total volume of 0.5 ml. Other samples were supplemented with 1 μM 5-HT for the determination of non-specific binding. Incubation proceeded for 30 min at 25°C, and was stopped by rapid filtration through Whatman GF/B filters using a Brandel cell harvester. Filters were rinsed with 3 × 5 ml of ice-cold Tris-HCl, dried, and entrapped radioactivity was measured by liquid scintillation counting.

All assays were performed in triplicate. Saturation and inhibition curves were analyzed by computer-assisted non-linear regression analysis using the EBDA program for the determination of K_d and B_{max}, and the GraphPad program for the calculation of IC_{50} values. The equation of Cheng and Prusoff (1973) was used for the calculation of K_i values from IC_{50} values.

2.4. Quantitative autoradiography

The brain was removed immediately after death, frozen in isopentane cooled with dry ice at −30°C, and stored at −80°C for 1 week. Coronal sections (20 μm thick) were cut using a cryostat microtome at −20°C, at the level of the dorsal raphe nucleus and the dorsal hippocampus (plates 48–49 and 32–33, respectively, of the stereotaxic atlas of Paxinos and Watson, 1986). Sections were mounted on gelatin-coated glass slides (2.5 × 7.5 cm), and stored at −20°C for less than 2 weeks. For the labeling procedure, sections were first equilibrated at room temperature and then preincubated at 20°C for 30 min in 0.17 M Tris-HCl, pH 7.6 (Vergé et al., 1986). Incubation was performed at 20°C for 60 min in the same (fresh) buffer supplemented with 2 nM [^{3}H]8-OH-DPAT, and increasing concentrations of buspirone (10 nM–10 μM). Labeled sections were then rinsed (2 × 5 min) at 4°C with 0.17 M Tris-HCl, pH 7.6, quickly dipped in ice-cold water (3–5 s) and finally dried under a stream of cold air. Non-specific binding was determined on adjacent sections incubated under the same conditions as above, except that 1 μM 5-HT was added to the incubation medium. Sections were finally apposed to [^{3}H]Hyperfilm (Amersham) in X-ray cassettes for 1 month at 4°C. Autoradiograms were developed in Kodak Microdol (10 min at 20°C). Optical densities were measured using a Biocom image analyzer, and converted to fmol [^{3}H]8-OH-DPAT specifically bound per mg tissue with reference to tritium standards (Amersham).

2.5. Electrophysiological experiments

Immediately after the decapitation, the brain was removed and placed in an ice-cold Krebs' solution continuously bubbled with an O_2/CO_2 mixture (95:5%). A block of tissue containing the dorsal raphe nucleus was cut into frontal sections (0.3 mm thick, using a Lancer vibratome) while immersed in Krebs' buffer at 4°C. The entire procedure took 6–10 min. After sectioning, the slices were allowed to recover for 1 h at room temperature in an artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl 126, KCl 3.5, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $CaCl_2$ 2.0, glucose 11, $NaHCO_3$ 25, adjusted to pH 7.3 by continuous bubbling with O_2/CO_2.

For each experiment, a single slice was transferred to a recording chamber (Haj-Dahmane et al., 1991) through which ACSF flowed (2 ml/min) at 35°C. Extracellular recordings were then made with a single-barrel micropipette (15 MΩ) filled with 2 M NaCl. The micropipette was implanted into the dorsal raphe nucleus area which could be easily located in the midline of the slice, between the medial longitudinal fasciculi extending dorsally towards the aqueduct. In all cases, otherwise silent serotoninergic neurons in the brain slices were induced to fire by adding 3 μM phenylephrine (α_1-adrenoceptor agonist) to the ACSF throughout the superfusion experiments (see VanderMaelen and Aghajanian, 1983).

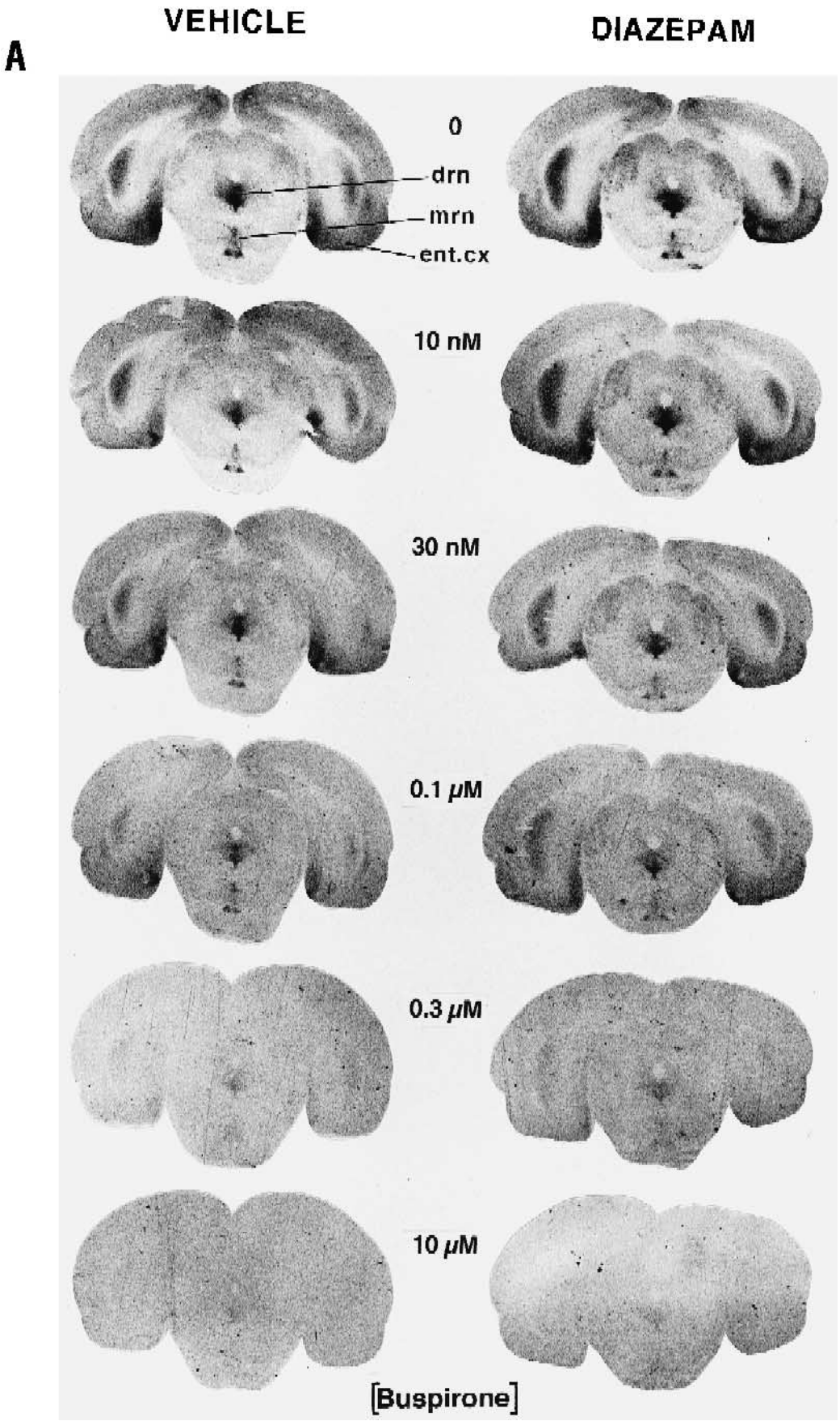
When a cell was recorded, it was identified on line as serotoninergic using the following criteria: biphasic action potentials of 2–3 ms duration and a slow (0.5–2.0 spikes/s) and regular pattern of discharge (VanderMaelen and Aghajanian, 1983). Baseline activity was recorded for 10 min before the infusion of 8-OH-DPAT into the chamber via a three-way tap system. Complete exchange of fluids occurred within 2 min of the arrival of a new solution in the chamber, and we observed that a 2.5 min infusion of 8-OH-DPAT was enough to induce maximal effects. The electric signals were fed into a high-input impedance amplifier, an oscilloscope and an electronic ratemeter triggered by individual neuronal spikes. The integrated firing rate was computed and recorded graphically as consecutive 10 s samples. The effects of each concentration of 8-OH-DPAT (1–30 nM) were evaluated by comparing the mean discharge frequency during 2 min prior to its addition to the superfusing ACSF, and 2–3 min after the end of the drug infusion, when the resulting changes in firing frequency reached their maximal amplitude.

2.6. Statistical calculations

Data were analyzed by one-way ANOVA and, in case of significance ($P < 0.05$), the *F*-test for significant treatment effects was followed by a two-tailed *t*-test to compare the experimental groups with their controls (Snedecor and Cochran, 1967).

2.7. Chemicals

[^{3}H]8-OH-DPAT (110 Ci/mmol) was from the Service des Molécules Marquées (CEA, Gif-sur-Yvette, France). Other drugs were: 5-HT creatinine sulphate (Merck, Darmstadt, Germany), buspirone (Bristol-Myers Squibb,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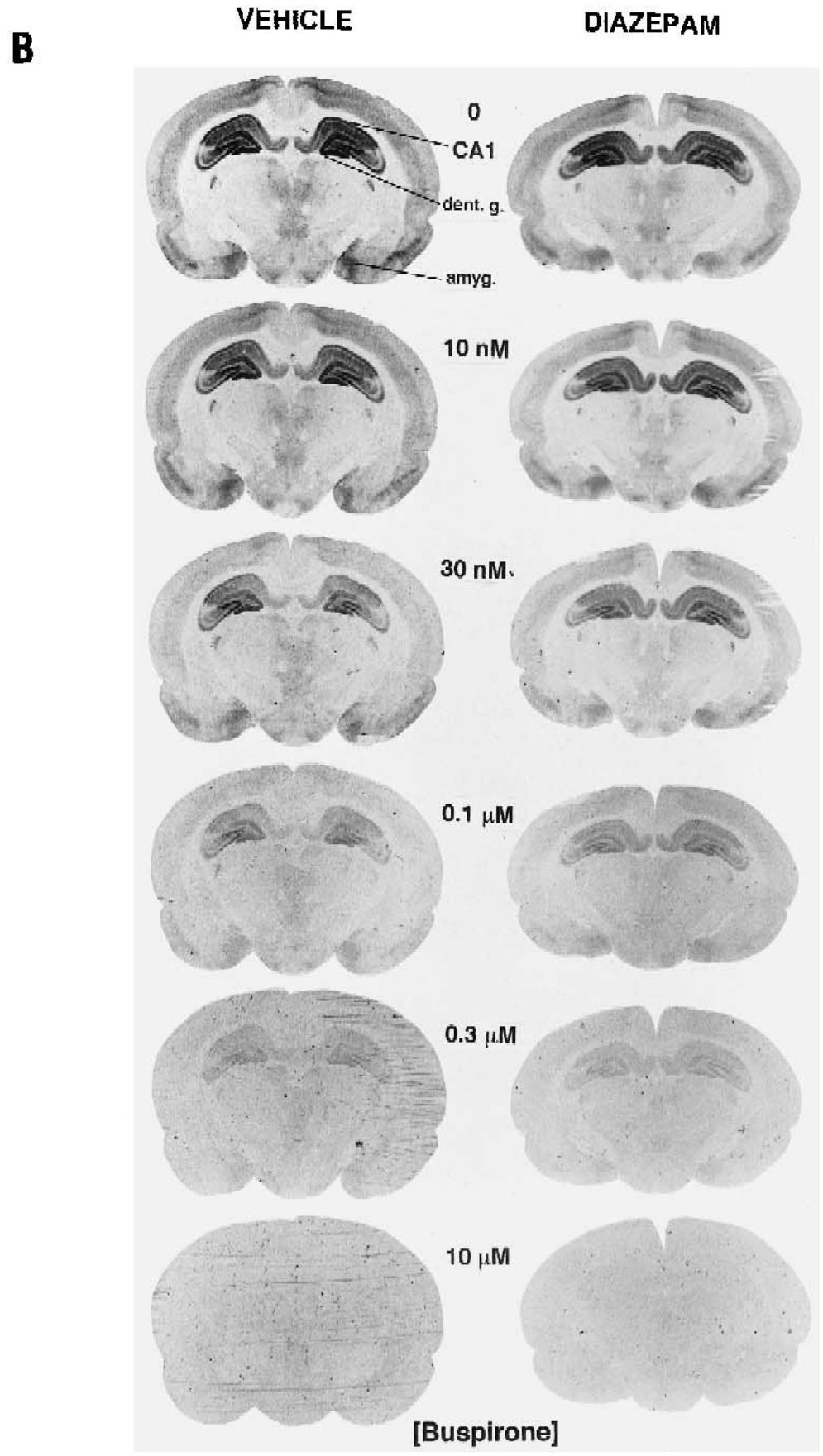
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Wallingford, CT, USA), diazepam (Hoffmann-La Roche, Basel, Switzerland), 8-OH-DPAT (Research Biochemicals International, Natick, MA, USA).

3. Results

3.1. Characteristics of 5-HT_{1A} receptor binding sites in the hippocampus of rats treated for 2 weeks with diazepam

Saturation studies with increasing concentrations of [^{3}H]8-OH-DPAT showed that the labeling of hippocampal 5-HT_{1A} receptor binding sites by this agonist radioligand was not significantly different in control and diazepam-treated rats whatever the treatment protocol, A, B or C, used. In all cases, the K_d was close to 1.5 nM and the B_{max} around 200 fmol of [^{3}H]8-OH-DPAT specifically bound per mg hippocampal membrane protein (Table 1).

Inhibition studies indicated that increasing concentrations of 8-OH-DPAT and buspirone prevented the specific binding of [^{3}H]8-OH-DPAT to hippocampal membranes prepared from diazepam-treated rats and paired controls, in the three treatment protocols A, B and C, with n_H values close to unity. No differences in K_i values of these two drugs ($\sim$1.8 nM for 8-OH-DPAT, $\sim$28 nM for buspirone) were noted among the various treatment groups included in the study (Table 2).

Table 2
K_i values of 8-OH-DPAT and buspirone vs [^{3}H]8-OH-DPAT specific binding to hippocampal membranes from control and diazepam-treated rats

Treatment protocol	K_i (nM)			
	8-OH-DPAT		Buspirone	
	Vehicle	Diazepam	Vehicle	Diazepam
A	1.62 ± 0.25	2.10 ± 0.36	26.6 ± 3.4	31.8 ± 4.1
B	1.96 ± 0.22	1.83 ± 0.26	31.0 ± 4.5	29.7 ± 4.2
C	1.71 ± 0.30	1.59 ± 0.31	29.1 ± 3.4	24.2 ± 3.8

Rats were treated with diazepam or its vehicle according to treatment protocol A, B or C (see Section 2). Hippocampal membranes were incubated with 0.5 nM [^{3}H]8-OH-DPAT in the absence or the presence of 10 different concentrations of 8-OH-DPAT (0.1 nM–1 μM) or buspirone (1 nM–10 μM). K_i values were calculated from IC_{50} values according to the equation of Cheng and Prusoff (1973). Each value is the mean ± S.E.M. of four independent determinations. Whatever the treatment protocol, A, B or C, no significant differences were noted between diazepam-treated rats and their paired, vehicle-treated, controls.

3.2. Characteristics of the inhibition by buspirone of [^{3}H]8-OH-DPAT specific binding in the dorsal raphe nucleus and the hippocampus (dentate gyrus) of rats treated for 2 weeks with diazepam

Autoradiograms in Fig. 1A and B show that increasing concentrations of buspirone (10 nM–10 μM) progressively reduced the labeling of brain sections by [^{3}H]8-OH-DPAT. Quantitative analysis of the concentration-dependent inhibition of [^{3}H]8-OH-DPAT specific binding in the dorsal raphe nucleus (Fig. 1A) and the dentate gyrus of the hippocampus (Fig. 1B) yielded an apparent Hill coefficient not significantly different from 1.0 and similar K_i values, close to 25 nM, for the azapirone in diazepam-treated rats and their paired, vehicle-treated, controls for the three treatment protocols tested (Table 3). Furthermore, absolute values of [^{3}H]8-OH-DPAT specifically bound in the dorsal raphe nucleus (range: 148–166 fmol/mg tissue) and the dentate gyrus (range: 208–231 fmol/mg tissue) did not significantly differ in diazepam-treated rats and their paired controls in groups A, B and C.

Table 1
Effects of 2-week treatments with diazepam on the characteristics of [^{3}H]8-OH-DPAT specific binding to hippocampal 5-HT_{1A} sites

Treatment protocol	Vehicle		Diazepam	
	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)
A	1.48 ± 0.27	196 ± 22	1.39 ± 0.22	200 ± 24
B	1.56 ± 0.26	196 ± 27	1.51 ± 0.31	211 ± 28
C	1.46 ± 0.30	194 ± 31	1.34 ± 0.24	207 ± 26

Binding assays were carried out with eight different concentrations of [^{3}H]8-OH-DPAT ranging between 0.1 and 4.0 nM. K_d and B_{max} values (means ± S.E.M. of 4–6 independent determinations) were calculated from saturation curves using the EBDA program. Whatever the treatment protocol, A, B, or C (see Section 2), neither the K_d values nor the B_{max} values were significantly different between diazepam-treated rats and their paired, vehicle-treated, controls.

◄ Fig. 1. Autoradiograms of serial coronal brain sections labeled by [^{3}H]8-OH-DPAT at the level of the dorsal raphe nucleus (A) or the dorsal hippocampus (B). Comparison of concentration-dependent inhibition by buspirone in diazepam-treated rats and paired, vehicle-treated, controls. Adjacent sections (20 μm thick) were incubated with 2 nM [^{3}H]8-OH-DPAT in the absence or the presence of five different concentrations (10 nM–10 μM) of buspirone, and autoradiograms were generated as described in Section 2. VEHICLE and DIAZEPAM corresponded to a vehicle-treated rat and a diazepam-treated rat in the treatment protocol C, respectively. Abbreviations: (A) drn, dorsal raphe nucleus; ent.cx, entorhinal cortex; mrn, median raphe nucleus. (B) amyg., amygdala; CA1, CA1 area of Ammon's horn; dent.g., dentate gyrus. Magnification: ×3.9 (A, vehicle and diazepam; B, vehicle); ×3.7 (B, diazepam).

Table 3
K_i values of buspirone vs. [^{3}H]8-OH-DPAT specific binding in the dorsal raphe nucleus and the dentate gyrus of brain sections from control and diazepam-treated rats

Treatment protocol	K_i (nM)			
	Dorsal raphe nucleus		Dentate gyrus	
	Vehicle	Diazepam	Vehicle	Diazepam
A	22.9 ± 3.0	23.1 ± 4.3	24.5 ± 3.6	25.7 ± 4.1
B	22.1 ± 3.5	24.5 ± 3.2	27.6 ± 2.8	25.7 ± 5.0
C	28.4 ± 3.3	24.2 ± 4.3	30.8 ± 3.6	24.5 ± 4.4

Rats were treated with diazepam or its vehicle according to treatment protocol A, B or C (see Section 2). Coronal brain sections were labeled by 2 nM [^{3}H]8-OH-DPAT in the absence or the presence of five different concentrations of buspirone (10 nM–10 μM) as illustrated in Fig. 1A and B. Densitometric measurements at the levels of the dorsal raphe nucleus and the dentate gyrus on autoradiograms allowed the calculations of IC_{50} values which were converted to K_i values according to Cheng and Prusoff (1973). Each value is the mean ± S.E.M. of four independent determinations (in four rats). Whatever the treatment protocol, A, B or C, no significant differences were noted between the K_i values of buspirone in diazepam-treated rats and their paired, vehicle-treated, controls.

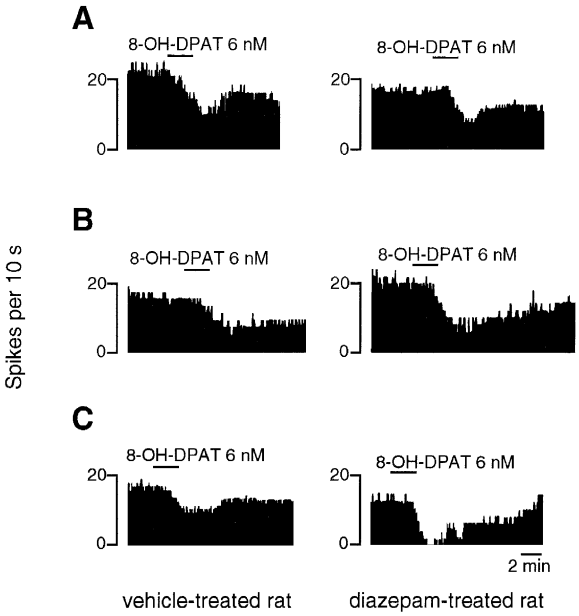

Fig. 2. Integrated firing rate histograms (in spikes per 10 s) of serotoninergic neurons in the dorsal raphe nucleus of brain stem slices superfused with 6 nM 8-OH-DPAT. Comparison between control and diazepam-treated rats. Rats were treated with diazepam or vehicle according to the protocol A, B or C, and in vitro recording experiments were performed as described in Section 2. 8-OH-DPAT (6 nM) was applied for 2.5 min. Records on the left represent typical responses of 5-HT neurons from vehicle-treated rats, and records on the right are those of 5-HT neurons from diazepam-treated rats. The inhibition of firing due to 8-OH-DPAT was regularly larger in rats treated with diazepam under the conditions of protocol C, as compared to paired-controls as well as both vehicle-treated and diazepam-treated animals in groups A and B.

3.3. 8-OH-DPAT-induced inhibition of the electrical activity of serotoninergic neurons in the dorsal raphe nucleus of rats treated for 2 weeks with diazepam

No significant differences between diazepam-treated and vehicle-treated rats were noted in the in vitro baseline firing rate of serotoninergic neurons in the dorsal raphe nucleus. In the three treatment protocols, A, B and C, of the present study, the discharge rate of these neurons ranged between 12 and 25 spikes/10 s in diazepam-treated animals as well as in their paired controls. This range corresponded to that usually found in experiments performed on brain stem slices from untreated rats (Lanfumey et al., 1993).

Like that previously noted in untreated animals (Lanfumey et al., 1993), the addition of 8-OH-DPAT to the ACSF superfusing brain stem slices from vehicle-treated rats resulted in a concentration-dependent reduction in the firing rate of serotoninergic neurons (Figs. 2 and 3). This effect was at its maximum within 2–3 min after a 2.5 min application of 8-OH-DPAT (1–30 nM) and lasted approximately 5 min. Complete recovery of the baseline

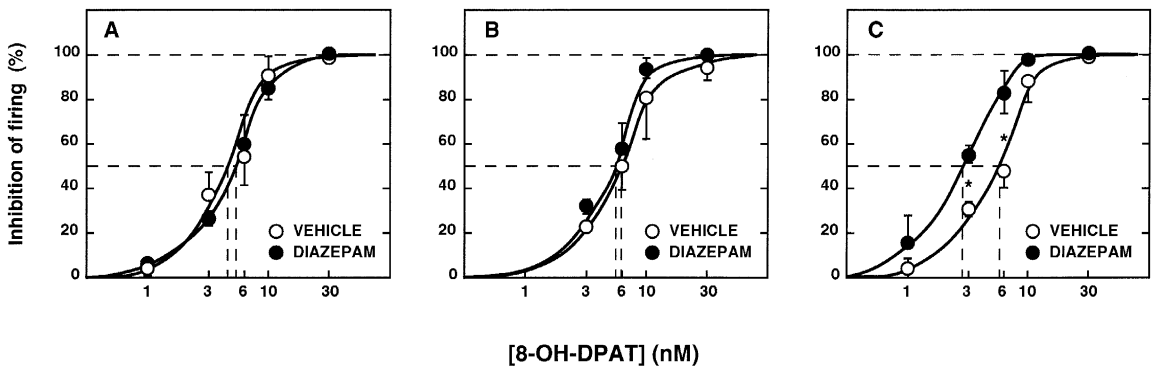

Fig. 3. Concentration-dependent inhibition by 8-OH-DPAT of the firing of serotoninergic neurons in the dorsal raphe nucleus after the administration of diazepam or its vehicle under protocol conditions A, B or C. Experiments were performed as described in the legend to Fig. 2, except that 8-OH-DPAT was used at various concentrations (1, 3, 6, 10 and 30 nM – abscissa). Inhibition, expressed as a percentage of the baseline firing rate, was calculated as described in Section 2. Each point is the mean ± S.E.M. of data obtained in 4–6 cells. The asterisk (*) denotes a significant difference ($P < 0.05$) between 8-OH-DPAT-induced inhibition in the two groups. Under conditions of the treatment protocol C, the IC_{50} value of 8-OH-DPAT (dotted lines) was twice as high in vehicle-treated rats as in diazepam-treated animals (5.7 nM and 2.8 nM, respectively).

firing rate was usually observed 15–30 min after removal of 8-OH-DPAT from the superfusing ACSF (Fig. 2). These time-course changes (Fig. 2) and the potency (Fig. 3) of 8-OH-DPAT were similar in the three control groups of the treatment protocols A, B and C. In all cases, the IC_{50} value of the 5-HT_{1A} receptor agonist was close to 5 nM (Fig. 3).

Parallel experiments performed with brain stem slices from diazepam-treated rats indicated that 8-OH-DPAT affected the firing rate of serotoninergic neurons as in paired controls in the case of the treatment protocols A and B (Figs. 2 and 3). Indeed, the IC_{50} values of 8-OH-DPAT were also close to 5 nM in diazepam-treated rats which were killed 24 h after the 2-week treatment with the benzodiazepine (protocol A) or after a further 4-day treatment with progressively decreasing doses of the drug (protocol B).

In contrast, in rats of treatment protocol C which received no drug for 5 days prior to death, a 2-week treatment with diazepam resulted in an increased potency of 8-OH-DPAT to inhibit the firing of serotoninergic neurons in the dorsal raphe nucleus. As illustrated in Fig. 2C, addition of 6 nM 8-OH-DPAT to the superfusing ACSF produced a much larger reduction in the firing rate of these neurons in diazepam-treated rats than in paired, vehicle-treated, controls. The concentration-response curve of the 5-HT_{1A} receptor agonist was shifted to the left in diazepam-treated rats as compared to paired controls, with IC_{50} values of 2.8 nM and 5.7 nM, respectively (Fig. 3).

4. Discussion

The present study investigated whether changes in central 5-HT_{1A} receptors could account for the reduced anxiolytic effects of buspirone after a chronic pretreatment with a benzodiazepine, diazepam (Schweizer et al., 1986; Ashton et al., 1990). The characteristics of 5-HT_{1A} receptors were examined in the dorsal raphe nucleus, where these receptors act as autoreceptors located on the soma and dendrites of serotoninergic neurons (Sotelo et al., 1990), and the hippocampus, where they correspond to postsynaptic receptors located on the targets of serotoninergic projections (Vergé et al., 1986). Microinjection studies showed that 5-HT_{1A} receptors in these two regions are very probably responsible for the anxiolytic action of buspirone and other azapirones (Higgins et al., 1988; Kostowski et al., 1989; Carli et al., 1993; Jolas et al., 1995; Maurel Remy et al., 1996). In addition, three different treatment protocols were used so as to produce marked differences in the intensity of the withdrawal syndrome after the chronic administration of diazepam (File, 1990). In the treatment protocol A, rats were killed only 24 h after a 2-week treatment with diazepam, i.e., before the appearance of the major signs of withdrawal behaviour (File, 1990). In the treatment protocol B, rats received progressively decreasing doses of the benzodiazepine for the 5-day interval between cessation of the 2-week treatment with the drug and death. Under this condition, the withdrawal syndrome should have been largely prevented (File, 1990). Finally, in the treatment protocol C, rats remained untreated for 5 days after the 2-week administration of diazepam, which allows the development of the withdrawal syndrome (File, 1990).

The present data showed that the molecular targets of buspirone, i.e., 5-HT_{1A} receptors (Gozlan et al., 1983; Stahl et al., 1992), were essentially unchanged in rats treated for 2 weeks with diazepam at the daily dose of 2 mg/kg i.p. In the three different treatment protocols, A, B and C, binding studies with hippocampal membranes revealed no difference in the B_{max} of [^{3}H]8-OH-DPAT specific binding sites as well as in their affinity for 8-OH-DPAT and buspirone. Furthermore, within the dorsal raphe nucleus, the concentration-dependent inhibition of [^{3}H]8-OH-DPAT binding by buspirone was also unaltered by a prior 2-week treatment with diazepam indicating that the affinity of somatodendritic 5-HT_{1A} autoreceptors for the azapirone was the same after this treatment as in controls.

These data are at variance with previous investigations since Lima et al. (1995) noted a large decrease (> 50%) in the B_{max} of [^{3}H]8-OH-DPAT specific binding sites in hippocampal membranes 24 h after the last injection of a 10 day-treatment with clonazepam at 1 or 10 mg/kg i.p. per day. In addition, Rump and Jakowicz (1995) observed a very high increase (+132–186% over the control value) in the B_{max} of [^{3}H]8-OH-DPAT specific binding sites in the anterior raphe area of rats killed 1–7 days after a 2-week treatment with diazepam at the dose of 5 mg/kg s.c. per day. These data are in fact surprising because previous investigations by Wagner et al. (1985) failed to detect any change in 5-HT_1 receptor binding sites labelled by [^{3}H]5-HT in the brain stem and frontal cortex of rats 2 days after a 10-day treatment with diazepam up to the dose of 30 mg/kg i.p. per day. Furthermore, the latter authors also noted that clonazepam at 1, 2.5 or 5 mg/kg i.p. per day for 10 days failed to alter the characteristics of 5-HT_1 receptor binding sites in the brain stem of rats (Wagner et al., 1985). Because 5-HT_{1A} receptors account for a large proportion of 5-HT_1 receptor binding sites in the brain stem (Pazos et al., 1988; Radja et al., 1991), such huge changes in the density of [^{3}H]8-OH-DPAT-labeled 5-HT_{1A} sites as those noted by Rump and Jakowicz (1995) should have been detected, at least in part, by Wagner et al. (1985) using [^{3}H]5-HT as radioligand. Anyhow, our data do not support these previous observations by Rump and Jakowicz (1995) and Lima et al. (1995). Differences in the treatment protocols (dose and/or nature of the injected benzodiazepine) and the rat strains (Wistar versus Sprague-Dawley) used in these various studies might account, at least partly, for such discrepancies. Further experiments with the recently developed antagonist radioligand, [^{3}H]WAY 100,635 ([^{3}H]*N*-[2-[4-(2-methoxyphenyl)-1-

piperazinyl]ethyl]-*N*-(2-pyridinyl) cyclohexane carboxamide, Gozlan et al., 1995), to label both G-protein-coupled high-affinity 5-HT_{1A} receptors and uncoupled 5-HT_{1A} receptor binding sites, have to be performed in order to reach a definitive conclusion as to whether chronic benzodiazepine treatment affects, or does not affect, the binding characteristics of 5-HT_{1A} receptors in the rat brain.

As expected of the lack of changes in the binding characteristics of somatodendritic 5-HT_{1A} autoreceptors in the dorsal raphe nucleus, investigations on the response of serotoninergic neurons to the activation of these receptors by 8-OH-DPAT revealed no alteration after a 2-week treatment with diazepam under the conditions of protocols A and B to avoid the full development of the withdrawal syndrome. Thus, the concentration-dependent inhibition of the firing of serotoninergic neurons by the 5-HT_{1A} receptor agonist (Sprouse and Aghajanian, 1987; Lanfumey et al., 1993) was similar in rats treated with the benzodiazepine or its vehicle under these conditions. In contrast, under conditions of the treatment protocol C where withdrawal behaviour can develop (File, 1990), a significant change was noted in the potency of 8-OH-DPAT to inhibit the electrical activity of serotoninergic neurons. The shift to the left of the 8-OH-DPAT inhibition curve suggests that somatodendritic 5-HT_{1A} autoreceptors were hypersensitive in diazepam-treated rats as compared to controls under these particular conditions. Changes in the functional properties of somatodendritic 5-HT_{1A} autoreceptors in the absence of any significant modification of the characteristics of corresponding binding sites are not unique to the present study as previous investigations with direct 5-HT_{1A} receptor agonists (Schechter et al., 1990) and selective 5-HT reuptake inhibitors (Jolas et al., 1994; Le Poul et al., 1995) showed that chronic treatments with these drugs also produced functional alterations but changed neither the K_d nor the B_{max} of 5-HT_{1A} autoreceptors. Possible changes in the coupling between the 5-HT_{1A} receptor binding subunit and its associated G protein and/or the effector of this receptor complex, i.e., 4-aminopyridine-sensitive K^+ channels (Haj-Dahmane et al., 1991), might account for such functional alterations of somatodendritic 5-HT_{1A} autoreceptors (see Le Poul et al., 1995). Further investigations are needed to unravel which mechanism(s) account(s) for the effects of chronic diazepam under the treatment protocol C.

The chronic administration of both direct 5-HT_{1A} receptor agonists and selective 5-HT reuptake inhibitors produced a functional desensitization of somatodendritic 5-HT_{1A} autoreceptors (Schechter et al., 1990; Jolas et al., 1994; Le Poul et al., 1995; Maudhuit et al., 1996a), whereas, in contrast, chronic treatment with diazepam under conditions C induced a functional hypersensitivity of these receptors, similar to that previously noted in rats which were rendered 'helpless' by exposure to unescapable footshocks (Maudhuit et al., 1996b). Behavioural studies have shown that both direct 5-HT_{1A} receptor agonists and selective 5-HT reuptake inhibitors exert anxiolytic effects under chronic treatment conditions (Stahl et al., 1992; Griebel et al., 1994), whereas anxiety-driven behaviour is observed in helpless rats (Martin and Puech, 1990) and during withdrawal from diazepam (File, 1990). Such a parallelism between electrophysiological and behavioural observations further supports the idea that somatodendritic 5-HT_{1A} autoreceptors may be critically involved in the expression of anxiety-driven behaviour. Indeed, as already emphasized in Section 1, several groups recently demonstrated that somatodendritic 5-HT_{1A} autoreceptors in the dorsal raphe nucleus, much more than postsynaptic 5-HT_{1A} receptors in various forebrain areas, are the key targets from which the azapirones and other 5-HT_{1A} receptor agonists exert their anxiolytic action (Schreiber and De Vry, 1993; Hogg et al., 1994; Jolas et al., 1995; Maurel Remy et al., 1996). In any case, recording of serotoninergic neurons in the dorsal raphe nucleus of rats with intact afferent inputs (especially the GABAergic inhibitory input on which diazepam acts, see Gallager, 1978) has to be performed in order to verify that the same adaptive changes of 5-HT_{1A} autoreceptors as those presently observed in vitro actually occur in vivo after diazepam treatment.

The receptor binding as well as the electrophysiological data of the present study do not support the idea that the inefficiency of buspirone and other azapirones to alleviate withdrawal anxiety after a chronic benzodiazepine treatment (Goudie and Leathley, 1991a,b; Mizoguchi et al., 1993; but see Korkmaz et al., 1994; Vivian et al., 1994) can be due to a functional desensitization of somatodendritic 5-HT_{1A} autoreceptors. In contrast, even a supersensitivity of these receptors was observed under the treatment conditions C to allow the expression of withdrawal-driven behaviour. In line with the key role of these receptors in the anxiolytic action of buspirone, Andrews and File (1993) reported that in the social interaction test, this drug exerted anxiolytic-like effects at lower doses in rats suffering from the diazepam withdrawal syndrome than in naive rats. In contrast, there were no significant changes in the anxiolytic-like potency of buspirone in rats which did not experience withdrawal after chronic diazepam treatment (File and Andrews, 1994), in agreement with the presently observed unchanged functional properties of somatodendritic 5-HT_{1A} autoreceptors under such conditions (protocols A and B). This parallelism between the functional status of somatodendritic 5-HT_{1A} autoreceptors and the anxiolytic action of buspirone further supports that these receptors are the key target of this drug (see Jolas et al., 1995).

In any case, the either unaltered or enhanced sensitivity of somatodendritic 5-HT_{1A} autoreceptors after chronic exposure to diazepam cannot explain why a decreased anxiolytic action of buspirone may occur in some (Schweizer et al., 1986; Ashton et al., 1990), but not all (Pecknold et al., 1985; Delle Chiaie et al., 1995), patients after benzodi-

azepine treatment. Because 5-HT_{1A} receptors in limbic areas, notably in the hippocampus (Kostowski et al., 1989; Kataoka et al., 1991; Carli et al., 1993; Przegalinski et al., 1994), might also possibly contribute to the anxiolytic action of buspirone (but to a minor part as compared to 5-HT_{1A} autoreceptors in the dorsal raphe nucleus; see Jolas et al., 1995), it can be hypothesized that, in contrast, these receptors desensitize after chronic benzodiazepine treatment. Indeed, recent studies showed that 5-HT_{1A} autoreceptors in the dorsal raphe nucleus and postsynaptic 5-HT_{1A} receptors in the hippocampus exhibit different functional and regulatory properties (Corradetti et al., 1996). However, functional desensitization should occur with no change in the density of the latter receptors nor in their affinity for buspirone (Tables 2 and 3). Alternatively, possible neurobiological alterations associated with resistance to the anxiolytic action of buspirone might concern neuronal process(es) beyond 5-HT_{1A} receptors. In particular, recent studies showed that 5-HT_{1A} receptor activation exerts a negative influence on the increased activity of cholecystokinin-containing neurons in the frontal cortex of 'anxious' rats (Becker et al., 1996), and a possible alteration in this mechanism might occur in benzodiazepine-pretreated animals. Finally, it is well known that buspirone and the other azapirones are metabolized giving rise to 1-(2-pyrimidinyl)-piperazine (1-PP) whose α_2-adrenoceptor antagonistic properties can counteract the anxiolytic action of the parent compounds (Engberg, 1989). Whether this metabolic pathway could be activated after the chronic administration of a benzodiazepine also offers another possible explanation to the poor anxiolytic efficiency of buspirone in at least some patients (Schweizer et al., 1986; Ashton et al., 1990). All these working hypotheses are currently tested in our laboratory.

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