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Prevention of diazepam withdrawal syndrome by nifedipine—behavioural and neurochemical studies

S. El Ganouni[a,*], N. Hanoun[b], C. Boni[b], A. Tazi[c], F. Hakkou[c], M. Hamon[b]

[a]*Department of Biology, Faculty of Sciences and Techniques, P.O. Box 577, Route de Casablanca, Settat 2000, Morocco*
[b]*INSERM U288, Faculty of Medicine Pitié-Salpêtrière, 75634 Paris Cedex 13, France*
[c]*Department of Pharmacology, Faculty of Medicine, 19 Rue Tarik Bnou Ziad, Casablanca, Morocco*

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Abstract

Our studies aimed at investigating whether the dihydropyridine calcium antagonist, nifedipine, could prevent anxiogenic-like consequences of diazepam withdrawal in rats. Animals withdrawn from chronic diazepam (2 mg/kg/day i.p. for 2 weeks) drank significantly less water than did control rats in the unfamiliar arm of a Y maze. This anxiogenic-like effect could be prevented by acute administration of nifedipine (at 10 mg/kg i.p., but not at lower doses), which, on its own, did not change water intake in naive rats. Given chronically in combination with diazepam for the second half of a 2-week treatment with this drug, nifedipine (at the daily dose of 5 mg/kg i.p.) also suppressed the reduction of water intake normally observed on diazepam withdrawal. Biochemical measurements showed that acutely, as well as chronically, administered nifedipine increased 5-HT turnover in the hippocampus of diazepam-treated rats, thereby suggesting that the prevention of diazepam withdrawal-induced anxiogenic behaviour by the calcium antagonist might be underlain by serotoninergic mechanisms.

Keywords: Nifedipine; Dihydropyridine calcium antagonist; Rat; Anxiolytic; Novel environment; Diazepam; Withdrawal

1. Introduction

Benzodiazepines are the most commonly prescribed anxiolytic drugs, being effective against a wide spectrum of anxiety disorders. However, addiction, tolerance, and dependence/withdrawal may develop with these drugs, as well as adverse side effects that include sedation, cognitive and psychomotor impairments, and anterograde amnesia. Benzodiazepine withdrawal in human is associated with increased anxiety, insomnia, sensory disturbances, and even seizures (Ladewig, 1984). Similar symptoms have been observed in animals withdrawn from chronic benzodiazepine treatment (for review, see File, 1990). The cellular and molecular mechanisms underlying the withdrawal syndrome are still poorly understood. There is growing evidence that limbic structures and neurotransmitters, such as noradrenaline, serotonin (5-hydroxytryptamine, 5-HT), and GABA, are implicated. In particular, benzodiazepines are well known to act at allosteric sites on GABA A receptors to potentiate GABA-mediated opening of the receptor-chloride channel. Chronic modulation of GABA-benzodiazepine receptor complex plays a major role in central nervous system dysregulation during benzodiazepine abstinence (Malcolm, 2003). It thus appears that withdrawal symptoms stem, in part, from a resulting decrease in GABAergic inhibitory neurotransmission. However, increases in the activity of other neurotransmitters' systems also seem to play a role in benzodiazepine-withdrawal syndrome (Malcolm, 2003). In particular, the activation of the noradrenergic system has been shown to mediate benzodiazepine withdrawal-induced locomotor hyperactivity (Kunchandy and Kulkarni, 1986), but not the associated anxiogenic response (Baldwin et al., 1989). The increased anxiety associated with benzodiaze-

* Corresponding author. Tel.: +212 61 24 14 11; fax: +212 37 66 14 55.
E-mail address: sganouni@hotmail.com (S. El Ganouni).

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pine withdrawal is more probably related to excessive 'rebound' 5-HT activity. Indeed, there is abundant experimental evidence supporting the idea that the median raphe nucleus–dorsal hippocampal 5-HT pathway mediates diazepam withdrawal-induced anxiety (Andrews et al., 1997). Moreover, withdrawal from chronic diazepam treatment has been shown to induce an increase in both [^{3}H]5-HT release from and $^{45}Ca^{2+}$ uptake into hippocampal slices, and these changes could be prevented by in vivo administration of the GABA B agonist, baclofen (Andrews and File, 1993). By acting at terminal GABA B heteroreceptors, baclofen antagonizes the raise in calcium uptake that normally occurs during diazepam withdrawal, thereby preventing Ca^{2+}-dependent elevation in [^{3}H]5-HT release. On this basis, we postulated that calcium channel antagonists (CCAs) might block or reverse the increased anxiety observed during benzodiazepine withdrawal, possibly through some effects on serotoninergic neurotransmission.

Indeed CCAs appear to have some efficacy in the treatment of mood disorders (Post et al., 1998). In animal studies, the administration of CCAs has been reported to exert an anxiolytic-like effect in the four-plate test (Chopin and Briley, 1987), the elevated plus-maze (Pucilowski and Kostowski, 1991), and in the paradigm consisting of measuring water intake by animals placed in a novel, anxiogenous, environment (Tazi et al., 1992). Furthermore, the CCA nitrendipine was shown to dose-dependently reduce the convulsions induced by withdrawal from flurazepam (Dolin et al., 1988), which raises the possibility that CCAs may prevent the benzodiazepine withdrawal syndrome (Hitchcott et al., 1992; Gupta et al., 1996).

In a previous study, we demonstrated that the anxiolytic-like effects of another potent CCA, nifedipine, could be modulated by the 5-HT_{1A} receptor agonist, ipsapirone (El Ganouni et al., 1998). Accordingly, we postulated that the anxiolytic-like effects of CCA may be mediated by the 5-HT system. To further assess this hypothesis, we herein examined whether nifedipine affected 5-HT turnover in selected brain regions under conditions when this drug effectively prevents and/or reverses axiogenic-like behaviour in rats withdrawn from diazepam. In addition, activity of the hypothalamo–pituitary–adrenal (HPA) axis was also assessed in these animals.

2. Materials and methods

2.1. Animals and drugs

Male Wistar rats weighing 250–290 g and housed 3–4 per cage were used. The rat room was lit with dim light from 08:00 to 20:00 h and maintained at 22±1 °C. Food and water were freely available to all animals. The experimental procedures used in this study were conducted in conformity with the institutional guidelines that are in compliance with international laws and policies (the UK Animals 'Scientific Procedures 'Act, 1986, and its associated guidelines).

Diazepam (Roche, Morocco), obtained under solution form (10 mg/2 ml), was diluted in saline (0.9% NaCl) and used at a dose of 2 mg/kg i.p. Nifedipine (kindly supplied by Bayer Laboratories, Germany) was dissolved in Tween 80 (1%) and used at doses of 2.5, 5, and 10 mg/kg i.p. Control animals received saline and/or Tween i.p. injections, as appropriate. Drugs studied were injected in a volume of 5 ml/kg.

2.2. Behavioural test: water consumption in a novel environment

Anxiety-like behaviour was assessed by measuring water intake by rats in a novel environment. For this purpose, a Plexiglass Y maze was used. The three arms (50 cm long, 15 cm wide, 35 cm high) were positioned at 60° to each other. The floor and walls of arms 1 and 2 are black; those of arm 3 are white. A guillotine door could be used to separate arm 3 from the rest of the maze. A bottle could be connected to the end of arm 2 (black) or 3 (white). A light bulb (40 W) was placed 40 cm above arm 3.

2.2.1. Training session

During the week preceding the experiments, the rats were subjected to daily handling, weighing, and vehicle injections. They were water deprived for 36 h before the test session. Training consisted of seven 10-min sessions performed 24 h apart. During this period (day 1–7), the guillotine door was closed to prevent access to arm 3 (white), and the water bottle was placed in arm 2 (black). The rats were placed individually at the end of arm 1 and allowed to run into arm 2 to reach drink water. Water intake was measured by weighing the bottle before and after each session. Previous experiments have shown that water intake exhibited only slight variations after the fifth training day, allowing the quantitative determination of a "plateau" of water consumption from day 5 to 7 of training.

2.2.2. Test session

On the eighth day, rats were divided into groups, matched according to water intake during the last training session (day 7). They were given the drugs under study or their vehicles, 30 min before the test. The guillotine door was removed and the water bottle placed in arm 3 (white). This arm was brightly lit by an additional bulb (60 W), placed 40 cm above the floor. Rats were placed in arm 1 and allowed to run within the Y maze to drink water in arm 3.

This experimental procedure was previously shown to be very sensitive to anxiolytic drugs such as benzodiazepines (Tazi et al., 1992).

2.3. Experimental procedures

2.3.1. Anxiolytic-like effects of nifedipine

Rats were injected with nifedipine at 5 mg/kg i.p. daily for 8 days and subjected to the test session 30 min after the last injection. Other rats received only an acute dose of nifedipine (2.5, 5, or 10 mg/kg i.p.) 30 min prior to the test session. Paired control animals were injected chronically or acutely with the vehicle under the very same time conditions.

2.3.2. Diazepam withdrawal syndrome

Rats were treated with diazepam (2 mg/kg i.p.) once daily (between 16:00 and 17:00 h) for 2 weeks. After the first week of treatment (from day −7 to 0), rats were subjected daily to the training session as described above, for the following 7 days (day 1–7). Twenty four hours later (day 8: testing day), diazepam-dependent rats were randomized on the basis of water consumption on the last day of training session (day 7) and divided into two groups, which were then treated with either an additional dose of diazepam (2 mg/kg i.p) or saline, 30 min before the test. Paired control animals were injected with saline instead of diazepam and subjected to the same procedure.

2.3.3. Effects of nifedipine administration on diazepam-withdrawal syndrome

Rats rendered dependent on diazepam were randomized on the basis of water consumption on the last training day (day 7; procedure as above) and given an additional injection of vehicle, diazepam (2 mg/kg i.p.), or nifedipine (2.5, 5, or 10 mg/kg i.p.), 30 min before the test session. To assess specifically its effects on the *development* of diazepam dependence, nifedipine (5 mg/kg i.p.) was coadministered with the benzodiazepine during the 7 days of training session. On the eighth day, rats were randomized on the basis of water consumption on the last day of training session (day 7) and received an additional injection of either vehicle, diazepam (2 mg/kg i.p.), or nifedipine (5 mg/kg i.p.).

Preliminary experiments showed that the vehicle, Tween 80 (1% in water), exerted no anxiolytic-like effect in the water intake test. Behavioural testings were performed in a soundproof chamber under continuous white noise and were conducted by an observer blind to drug treatment. The apparatus was thoroughly cleaned between two tests.

2.4. Biochemical measurements

Biochemical determinations were performed in other groups of rats, which received the very same treatments as for those subjected to the Y maze paradigm but did not undergo behavioural testings. In all cases, animals were decapitated 30 min after either acute treatments or the last injection of repeated treatments.

2.4.1. Corticosterone assay

Immediately after decapitation, blood from trunk vessels was collected in prechilled tubes for the measurement of serum corticosterone levels. Serum corticosterone was quantified by radioimmunoassay after extraction in ethanol, using [^{3}H]corticosterone (87 Ci/mmol, Amersham-Pharmacia Biotech, Les Ulis, France) as radiotracer, anticorticosterone antibodies (Sigma-Aldrich), and corticosterone (Sigma, St. Quentin Fallavier, France) as standard (see Le Poul et al., 2000).

2.4.2. Quantitative determination of glucocorticoid receptor (GR) mRNA

The method used to measure mRNAs was based on a competitive RT-PCR technique (Siebert and Larrick, 1992), in which mRNAs of analyzed gene are reverse transcribed and amplified in the presence of a homologous deleted internal standard RNA. The quantitative determination of GR mRNA in the brain stem, the hippocampus, and the cerebral cortex was performed as described by Le Poul et al. (2000), using an RT-PCR Access System Kit (Promega, Madison, WI, USA). Reverse transcription (40 min at 60 °C) proceeded with 0.4 μg of total tissue RNA in the presence of standard deleted RNA at increasing dilutions (10^{-6} to 3×10^{-8}). The sequences of upstream and downstream oligonucleotide primers were 5′-ATGGGGAATGACTTGGGCTACC-3′ (nucleotides 337–359) and 5′-CCGCCAAAGGAGAAAGCAAGTT-3′ (nucleotides 709–688), respectively (Danielsen et al., 1986). PCR amplification was performed with 1–2 U of Tfl DNA polymerase, 1 mM $MgSO_4$, and 1 pg/μl of each primer for 30 cycles (1 min at 95 °C, 1 min at 58 °C, and 1 min at 72 °C). After electrophoretic separation in 2% agarose gel stained with 4% ethidium bromide, both standard and tissue RT-PCR products were quantified with a gel analyzer software (NIH 1.6).

2.4.3. Measurements of tissue levels of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA)

After decapitation, the brains were immediately removed at 0 °C. Brain structures (hippocampus and cerebral cortex) were dissected out and homogenized in 250 μl of 5% $HClO_4$ supplemented with 0.05% $Na_2S_2O_5$ and 0.05% disodium EDTA. Homogenates were centrifuged at 30,000×*g* for 15 min at 4 °C, and the supernatants were neutralized with 2 M KH_2PO_4/K_2HPO_4, pH 7.4, and supplemented with ascorbate oxidase (Boehringer Mannheim; final concentration 0.01 mg/ml). After a second centrifugation as above, clear supernatants were saved, and aliquots (10 μl) were injected directly into a high-performance liquid chromatography (HPLC) column (Ultrasphere IP, Beckman, Gagny, France; 25×4.6 cm, C18 reversed phase, particle size 5 μm) protected with a Brownlee precolumn (3 cm, 5 μm). The mobile phase for the elution (at a flow rate of 1 ml/min) consisted of (in mM) the following: KH_2PO_4, 70; triethylamine, 2.1; disodium EDTA, 0.1; octane sulphonate, 1.25;

methanol, 16%; adjusted to pH 3.02 with solid citric acid (Hamon et al., 1988). The electrochemical detection system (ESA 5011, Bedford, USA) comprises an analytical cell with dual coulometric monitoring electrodes (+50 and +350 mV). The generated signal was integrated by a computing integrator (Millenium 32-Waters, Saint Quentin en Yvelines, France). Quantitative determinations of 5-HT and its metabolite 5-HIAA were made with reference to appropriate standards.

2.5. Statistical analyses

Data are presented as means+S.E.M. The Student's *t*-test was used to compare two independent groups. One-way analysis of variance (ANOVA; treatment factor), followed by Newman–Keuls post hoc test, was used to compare the group means. When necessary, unpaired *t*-test was also used to compare two means. A level of probability at $p<0.05$ was accepted as statistically significant.

3. Results

Fig. 1 shows the effects of acute and chronic administration of nifedipine on water consumption by rats subjected to the Y maze paradigm. Vehicle-treated animals exhibited a marked decrease in water intake during the test session (day 8) compared with the last training day (day 7; 2.02±0.99 vs. 10.02±0.27 ml; means±S.E.M., $n=10$ in each group; $p<0.001$). Analysis of variance on mean water consumption during the test session indicated a significant group effect [$F(3,36)=7.25$, $p<0.0001$]. Post hoc analysis showed that acute treatment with nifedipine at 5 and 10 mg/kg i.p. increased water intake as compared with vehicle-treated animals ($p<0.01$) and rats treated with only 2.5 mg/kg of nifedipine ($p<0.01$ and $p<0.001$, respectively). Similarly, water intake was significantly ($p<0.01$) increased in rats that received nifedipine for 8 (7+1) days. Indeed, whereas water drinking was reduced by more than 50% in vehicle-treated rats, those which received subchronic nifedipine (5 mg/kg i.p. daily) drank as much water for the test session (unfamiliar arm) as for the last training session (familiar arm).

Fig. 2 depicts the effects of diazepam withdrawal on water intake in the Y maze paradigm. Control animals exhibited a significant decrease in water consumption during the test session (day 8) compared with the last training day (day 7; 5.35±0.68 vs. 10.32±0.47 ml; means±S.E.M.; $n=10$ in each group; $p<0.001$). ANOVA showed a significant difference between groups [$F(2,31)=17.62$; $p<0.0002$]. Moreover, post hoc analysis of the group means revealed that non-diazepam-withdrawn animals, which were treated with an additional injection of diazepam on the test session, exhibited a significant increase in water intake in arm 3 of the Y maze compared with control animals ($p<0.01$). Fig. 2 also shows that water intake in the diazepam-withdrawn group was significantly lower than that in the vehicle-treated ($p<0.05$) and non-diazepam-withdrawn groups ($p<0.001$). Indeed, on test session, water intake was markedly reduced, to less than 2 ml, in rats withdrawn from chronic diazepam, suggesting an exacerbated anxiogenic-like behaviour associated with diazepam withdrawal. The additional diazepam injection on the test session exerted an anxiolytic-like release of such behavioural suppression, indicating a protective action of diazepam against anxiogenic-like behaviour. This result indicates that the Y maze paradigm is sensitive to withdrawal from chronic benzodiazepine treatment.

Fig. 3A depicts the effects of acute nifedipine administered at various doses on water intake in diazepam-withdrawn rats. Analysis of variance indicated a significant effect of treatment [$F(4,39)=6.98$, $p<0.0001$]. Post hoc comparison of the group means indicated that withdrawal from diazepam induced a marked decrease in water intake

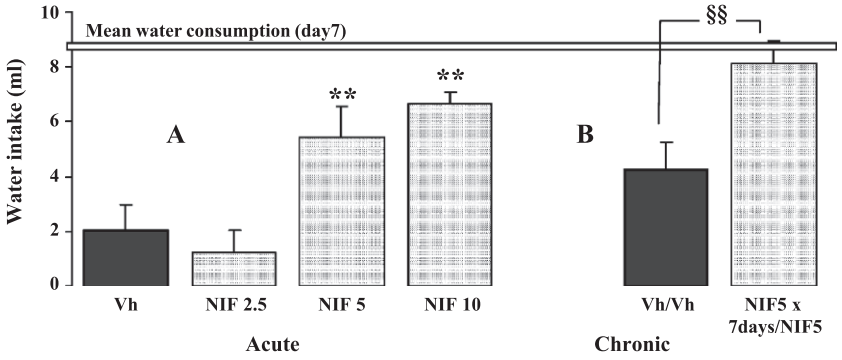

Fig. 1. The effects of acute (A) and chronic (B) nifedipine treatment on water intake (in ml) during the test session in the unfamiliar white arm of the Y maze. (A) Animals were injected i.p. 30 min before the test session with either 1% Tween 80 (Vh) or nifedipine at 2.5 mg/kg (NIF2.5), 5 mg/kg (NIF5) or 10 mg/kg (NIF10). (B) In chronic experiment, nifedipine (5 mg/kg) or 1% Tween 80 (Vh) was injected i.p. for 7 days, and an additional injection of each (Vh in 'Vh/Vh' group, NIF5 in the 'NIF5×7days/NIF5' group) was made 30 min before the test session on day 8. Each bar represents the mean+S.E.M. of water intake (in ml) in groups of 10 rats. The horizontal straight line indicates the mean water intake by all rats during the last training session (day 7). **$p<0.01$ as compared with water intake in rats injected with the vehicle (Vh) or nifedipine at 2.5 mg/kg i.p.; $^{§§}p<0.01$, significant difference between the 'Vh/Vh' and 'NIF5×7 days/NIF5' groups.

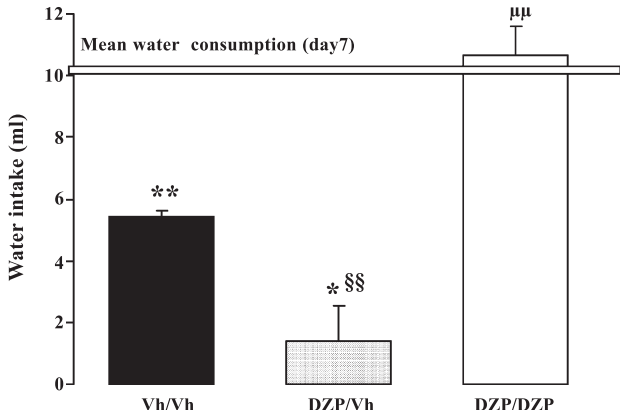

Fig. 2. The effect of withdrawal from chronic diazepam on water intake (in ml) during the test session in the unfamiliar white arm of the Y maze. Animals were injected for 15 consecutive days with either saline (Vh) or diazepam at 2 mg/kg/day (DZP) and were trained for the last 7 days to drink water in the Y maze. On day 8 (test session), animals received, 30 min before the session, either saline (Vh/Vh, DZP/Vh) or diazepam (DZP/DZP). Each bar represents the mean (+S.E.M.) amount of water (in ml) drunk in groups of 10 rats. The horizontal straight line corresponds to the mean water intake by all rats during the last training session (day 7). $^{**}p<0.01$ as compared with water intake on the last training day (day 7); $^{\mu\mu}p<0.01$ as compared with the Vh/Vh group; $^{*}p<0.05$ as compared with the Vh/Vh group; $^{\S\S}p<0.001$ as compared with the DZP/DZP group.

compared with that measured on the last training session (day 7; $p<0.001$). On the other hand, rats treated acutely with either diazepam (2 mg/kg i.p.) or nifedipine (10 mg/kg i.p.) exhibited an important increase in water consumption in comparison with those treated with the vehicle or nifedipine at 2.5 or 5 mg/kg ($p<0.001$). No significant difference in water intake was observed between the last three groups.

Fig. 3B shows the effects of chronic nifedipine treatment on the diazepam withdrawal syndrome. Data analysis showed a significant group effect [$F(2,23)=5.91$, $p<0.0001$]. Post hoc comparison of group means indicated that rats coadministered with nifedipine (5 mg/kg i.p.) and diazepam (2 mg/kg i.p.) for the second half of a 2-week treatment with diazepam exhibited an important increase in water consumption as compared with diazepam-withdrawn rats ($p<0.001$).

Accordingly, the strong suppression of water intake in diazepam-withdrawn rats was reversed by acute treatment with a high dose of nifedipine (10 mg/kg i.p.). Under subchronic treatment conditions, a lower dose (5 mg/kg i.p.) of nifedipine, coadministered with diazepam for the second half of a two-week treatment with the benzodiazepine, effectively prevented the increase in anxiogenic-like behaviour normally associated with diazepam withdrawal. Indeed, the anxiolytic-like effect of chronic nifedipine treatment was as strong as that of an additional injection of diazepam on the test session (Fig. 3B).

Fig. 4 shows serum corticosterone levels in diazepam-withdrawn rats that received an additional acute injection of the vehicle, diazepam (2 mg/kg i.p.), or nifedipine (10 mg/kg i.p.), or a chronic treatment with the latter drug (5 mg/kg/day for 8 days). A significant decrease in serum corticosterone levels was seen 30 min after the additional diazepam injection in diazepam-withdrawn rats compared with the diazepam-withdrawn group injected with the vehicle ($p<0.05$) or with the other two groups of rats treated with

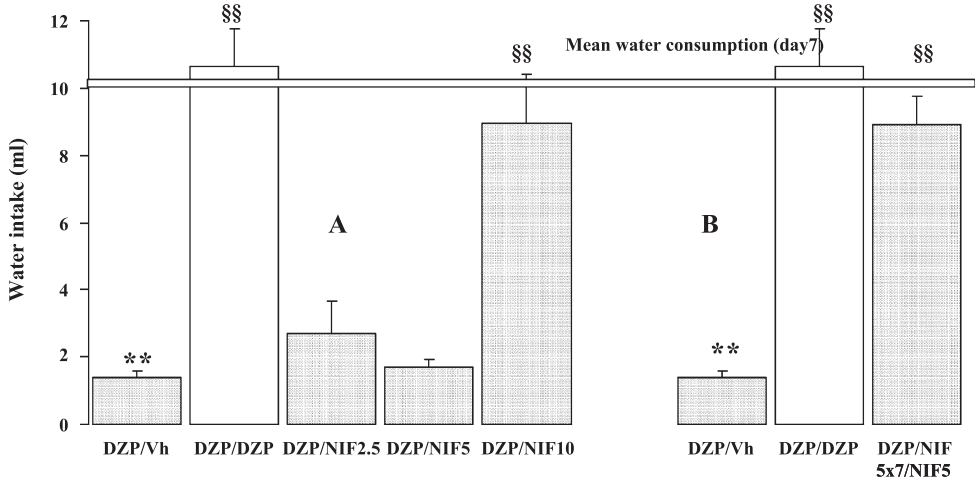

Fig. 3. Nifedipine-induced protection from diazepam-withdrawal syndrome, as assessed from measurement of water intake (in ml) in the unfamiliar white arm of the Y maze. (A) Animals were injected for 15 consecutive days with either saline (Vh) or diazepam at 2 mg/kg/day (DZP), and were trained for the last seven days to drink water in the Y maze. On day 8 (test session), animals received, 30 min before the session, either 1% Tween 80 (DZP/Vh), diazepam (DZP/DZP), nifedipine at 2.5 (DZP/NIF 2.5), 5 (DZP/NIF 5) or 10 mg/kg (DZP/NIF 10). Each bar represents the mean (+S.E.M.) amount of water drunk in groups of 10 rats. $^{\S\S}p<0.001$ as compared with the DZP/Vh, DZP/NIF 2.5, and DZP/NIF 5 groups; $^{**}p<0.001$ as compared with water intake on the last training day (day 7). (B) Nifedipine (5 mg/kg i.p.) was coadministered daily with diazepam (2 mg/kg i.p.) during the last 7 days of a 2-week treatment with benzodiazepine. On day 8, an additional injection of either 1% Tween 80 (DZP/Vh), diazepam (DZP/DZP), or nifedipine (DZP+NIF5û7/NIF5) was made 30 min before the test session. Each bar represents the mean (+S.E.M.) amount of water (in ml) drunk by groups of 10 rats. The horizontal straight line represents the mean water intake by all rats during the last training session (day 7). $^{\S\S}p<0.001$ as compared with the DZP/Vh group; $^{**}p<0.001$ as compared with water intake on the last training day (day 7).

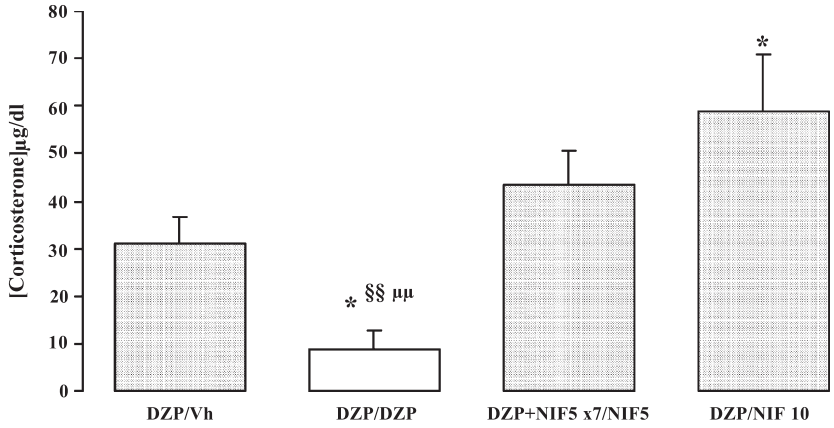

Fig. 4. The effects of acute or repeated nifedipine treatment compared with diazepam on serum corticosterone levels. Abbreviations for the identification of group treatments are as indicated in Figs. 2 and 3. Each bar is the mean (+S.E.M.) of 10 independent determinations (one determination per rat). *$p<0.05$ as compared with the DZP/Vh group; $^{§§}p<0.01$ as compared with the DZP+NIF 5×7/NIF 5 group; $^{µµ}p<0.001$ as compared with the DZP/NIF 10 group.

nifedipine ($p<0.001$ and $p<0.01$, respectively). When nifedipine (10 mg/kg i.p.) was given to the diazepam-withdrawn animals, serum corticosterone levels increased significantly ($p<0.05$) as compared with those measured in diazepam-withdrawn rats given vehicle.

Quantitative determinations of GR mRNA in the hippocampus and the cerebral cortex of diazepam-withdrawn rats showed that neither a further injection of diazepam, an acute i.p. administration of 10 mg/kg of nifedipine, nor a subchronic treatment with the latter drug at 5 mg/kg/day for 7 days significantly altered the levels of this transcript compared with those found in diazepam-withdrawn rats treated with the vehicle (not shown).

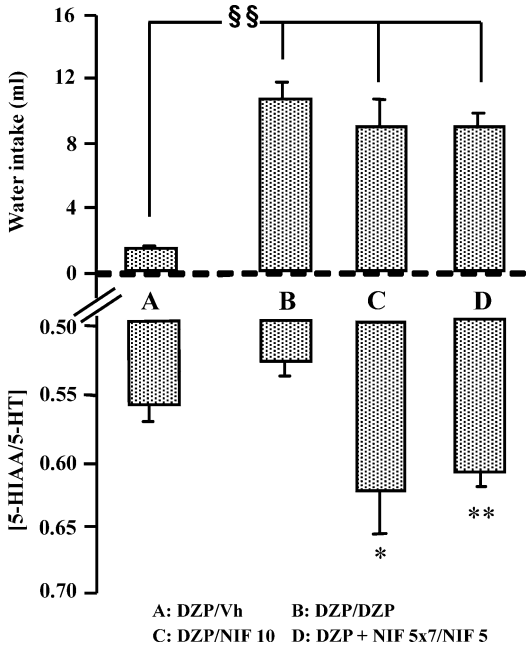

Fig. 5. Respective effects of acute injection of diazepam or nifedipine or repeated treatment with nifedipine on water intake in arm 3 of the Y maze (top part) and hippocampal 5-HIAA/5-HT ratio (bottom part) in diazepam-withdrawn rats. Abbreviations for the identification of group treatments are as indicated in Figs. 2 and 3. Each bar is the mean (+S.E.M.) of 10 independent determinations (one determination per rat). *$p<0.05$, **$p<0.01$ as compared with the DZP/DZP group; $^{§§}p<0.01$ as compared with the DZP/Vh group.

Fig. 5 shows the values of the 5-HIAA/5-HT ratio determined in the hippocampus of diazepam-withdrawn rats. Means comparison by Student's t-test indicated a significant increase (22–25%) in 5-HIAA levels after acute ($p<0.05$) or repeated nifedipine administration to diazepam-withdrawn rats, as compared with those receiving acute diazepam or vehicle on the testing day. As 5-HT levels were not significantly changed by these treatments, the 5-HIAA/5-HT ratio was significantly increased in the hippocampus (by approx. 16%) after acute, as well as chronic, nifedipine treatment. Post hoc analysis confirmed that nifedipine administration to diazepam-withdrawn rats significantly increased 5-HIAA levels, as well as the 5-HIAA/5-HT ratio, in the hippocampus ($p<0.05$ after acute nifedipine; $p<0.01$ after chronic nifedipine).

In contrast, measurements in the cerebral cortex of diazepam-withdrawn rats showed that neither a further injection of diazepam, an acute administration of 10 mg/kg i.p. of nifedipine, nor a coadministration of the latter drug at 5 mg/kg/day for the second half of a 2-week treatment with diazepam significantly modified 5-HT and 5-HIAA levels compared with those found in diazepam-withdrawn rats injected with the vehicle (not shown).

4. Discussion

Previous data from our laboratory have shown that acute administration of CCAs increased water intake by rats placed in a novel environment, indicating an anxiolytic-like effect of these drugs (El Ganouni et al., 1998). We confirmed herein that one of these drugs, nifedipine, dose dependently increased water intake by rats in a novel environment, and we also demonstrated that this effect was more striking after subchronic (for 8 days) than after acute administration of this drug. In contrast, other data indicated

that some CCAs are devoid of anxiolytic-like activity in certain tests. Thus, nitrendipine was reported to exert no effects in rats subjected to the social interaction test (File et al., 1989, 1992), the elevated plus-maze, or the open-field test (Pucilowski, 1992). These results may suggest that the anxiolytic-like effects of certain CCAs can be only observed under conditions of high degree of behavioural inhibition, such as those achieved in the test used herein.

Interestingly, a reduction in anxiogenic-like behaviours normally associated with withdrawal syndrome after chronic exposure to benzodiazepines or drugs of abuse has also been reported in animals injected with CCAs (Pucilowski et al., 1989; Czyrak et al., 1990). Indeed, CCAs may offer the possibility to substitute for benzodiazepine anxiolytics because they do not induce physical dependence (Jaffe, 1987). In line with this perspective, Hitchcott et al. (1992) noted that administration of the phenylalkylamine calcium channel antagonist, verapamil, effectively protected rats from anxiogenic-like reactions caused by diazepam withdrawal. In our study, we investigated the effects of nifedipine on diazepam-withdrawal syndrome to evaluate its potential antiwithdrawal action and to elucidate the mechanisms by which it exerts such behavioural effects.

Our results showed that withdrawal from chronic diazepam was followed by a decrease in water intake in a novel environment, suggesting increased anxiety. This rebound of anxiety could be blocked by nifedipine at 10 mg/kg, like that achieved by a further administration of diazepam. Moreover, nifedipine, at the daily dose of 5 mg/kg, administered concominantly with diazepam, also provided good protection against the withdrawal syndrome. Indeed, repeated administration of nifedipine at 5 mg/kg/day for 8 days was more effective than acute treatment with this drug because, at this dose, the latter treatment did not reduce benzodiazepine withdrawal syndrome. These observations are in line with previous data showing an antiwithdrawal-like effect of CCAs after chronic ethanol or diazepam administration (Pucilowski, 1992).

Attempts to elucidate the mechanisms underlying the anxiolytic-like effects of nifedipine first focused on the HPA axis, with measurements of serum corticosterone levels and GR mRNA concentrations in selected brain areas. We found that acute administration of nifedipine to diazepam-withdrawn rats significantly enhanced serum corticosterone levels, while, in contrast, the hormone levels were significantly decreased by an additional injection of diazepam to prevent withdrawal behaviour in rats rendered dependent on the benzodiazepine. Moreover, a slight increase in serum corticosterone levels was observed in diazepam-withdrawn rats (as compared with untreated naive rats; data not shown), which indicated that diazepam withdrawal might cause an increased activity of the HPA axis associated with anxiogenic-like behaviour in these animals. Interestingly, GR mRNA levels in the hippocampus and the cerebral cortex were affected neither by acute or repeated administration of nifedipine (see also Przegalinski et al., 1993) nor by diazepam treatment, thereby suggesting that the observed changes in serum corticosterone levels were not caused by alterations in GR-mediated feedback control of the HPA axis activity (see Froger et al., 2004). In any case, the striking differences between the effects of nifedipine and those of diazepam on serum corticosterone levels in diazepam-withdrawn rats (Fig. 4) may indicate that the mechanisms by which nifedipine mediates its antiwithdrawal effects involve other neurobiological substrates than the diazepam targets, i.e., the GABA A–benzodiazepine receptor complexes (Malcolm, 2003).

We previously demonstrated that the anxiolytic-like effects of nifedipine could be modulated by the 5-HT_{1A} receptor agonist, ipsapirone (El Ganouni et al., 1998). Thus, some influence of CCAs on 5-HT neurotransmission might be implicated in the anxiolytic properties of these drugs. In line with this hypothesis, the results obtained herein indicate that acute, as well as repeated, administration of nifedipine to diazepam-withdrawn rats significantly increased 5-HIAA levels and the 5-HIAA/5-HT ratio in the hippocampus (but not in the cerebral cortex). Interestingly, previous studies also provided evidence that nifedipine significantly increased 5-HT turnover in morphine-dependent guinea pigs (Bongianni et al., 1986; Baeyens et al., 1987).

In contrast, no significant changes in 5-HT and 5-HIAA levels in the hippocampus (and the cerebral cortex) were found after an additional injection of diazepam to prevent withdrawal-induced reduction in water intake in rats rendered dependent on benzodiazepine. Such differences, compared with nifedipine, provide further support to the above conclusion that the prevention of diazepam withdrawal syndrome by the CCA involves neurobiological mechanisms different from those underlying the effects of diazepam (i.e., facilitation of GABA A receptor functioning).

Given all these considerations, it is possible to speculate that the enhanced serum corticosterone levels in response to nifedipine administration reflected some stimulatory effect of the CCA on 5-HT neurotransmission during the diazepam-withdrawal syndrome. Indeed, it is well established that, through the stimulation of 5-HT_{1A} and 5-HT_{2A} receptors, an increased output of 5-HT can enhance corticosterone secretion (Fuller, 1996). Then corticosterone enters the brain, where it binds to intracellular receptors that are abundant in limbic areas, in particular, the hippocampus, the frontal cortex, and amygdala nuclei. In these areas, corticosterone can increase the amplitude of sustained high-voltage activated calcium currents (L-type, at which dihydropyridine CCAs act) and enhance calcium influx in neurones within the limbic areas (Karst et al., 1994, 2002). Such actions could modify the activity of limbic pathways that are implicated in anxiety behaviour and regulated by 5-HT projections from the midbrain. Furthermore, it is well known that glucocorticoids exert an influence on the activity of serotoninergic projections from the midbrain to the hippocampus (Meijer and de Kloet, 1998), and the resulting changes in serotoninergic neurotransmission might also

contribute to modulate limbic networks involved in anxiety-related behaviours. On this basis, one can therefore speculate that nifedipine exerts its antiwithdrawal effect by breaking off the modulatory effect of corticosterone on 5-HT neurotransmission. This would explain the increase in 5-HT turnover after nifedipine treatment in diazepam-withdrawn rats.

Another possibility is that nifedipine exerts its protective action against diazepam-withdrawal-induced anxiogenous behaviour by antagonizing the up-regulation of calcium channels in the brain. Indeed, several data point to modifications in the number and/or sensitivity of dihydropyridine binding sites in the brain as a possible mechanism underlying drug dependence. Accordingly, the blockade of dihydropyridine binding sites by CCAs would account for the preventive effects of these drugs against withdrawal syndrome. In vitro data suggested that a slowly developing up-regulation of calcium channels in response to drugs of abuse, including ethanol, benzodiazepine, and morphine, appears to be an adaptive response to long-term cell inhibition (Littleton and Brennam, 1993). However, one can speculate that the dihydropyridine binding sites in the brain may be involved in the CNS hyperexcitability observed during benzodiazepine-withdrawal states. Therefore, the effects of dihydropyridine calcium channel inhibitors on withdrawal syndrome would be the consequence of their interaction with hypersensitive and/or up-regulated calcium channels in brain.

Given all these considerations, it can be proposed that CCAs may be useful to reduce and/or prevent withdrawal syndrome caused by abrupt cessation of exposure to diazepam (and possibly also to other drugs generating dependence). Moreover, some somatic consequences of drug withdrawal, such as tachycardia and other cardiac disorders, would be successfully treated at the same time, given the known cardiac effects of dihydropyridines. Relevant clinical trials should be performed to assess the possible human application of such data.

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References

Andrews N, File SE. Increased 5-HT release mediates the anxiogenic response during benzodiazepine withdrawal: a review of supporting neurochemical and behavioural evidence. Psychopharmacology 1993; 112:21–5.

Andrews N, File SE, Fernandes C, Gonzalez LE, Barnes NM. Evidence that the median raphé nucleus–dorsal hippocampal pathway mediates diazepam withdrawal-induced anxiety. Psychopharmacology 1997; 130:228–34.

Baeyens JM, Esposito E, Ossowska G, Samanin R. Effects of peripheral and central administration of calcium channel blockers in the naloxone-precipitated abstinence syndrome in morphine-dependent rats. Eur J Pharmacol 1987;137:9–13.

Baldwin HA, Hitchcott PK, File SE. Evidence that the increased anxiety detected in the elevated plus-maze during chlordiazepoxide withdrawal is not due to enhanced noradrenergic activity. Pharmacol Biochem Behav 1989;34:931–3.

Bongianni F, Carla V, Moroni F, Pellegrini-Giampietro DE. Calcium channel inhibitors suppress the morphine-withdrawal syndrome in rats. Br J Pharmacol 1986;88:561–7.

Chopin P, Briley M. Animal models of anxiety: the effects of compounds that modify 5-HT neurotransmission. Trends Pharmacol Sci 1987;8: 383–8.

Czyrak A, Mogilnicka E, Siwanowicz J, Maj J. Some behavioral effects of repeated administration of calcium channel antagonists. Pharmacol Biochem Behav 1990;35:557–60.

Danielsen M, Northrop JP, Ringold GM. The mouse glucocorticoid receptor: mapping of functional domains by cloning, sequencing and expression of wild-type and mutant receptor proteins. EMBO J 1986; 10:2513–22.

Dolin SJ, Hunter AB, Hasley MJ, Little HJ. Anticonvulsant profile of the dihydropyridine calcium channel antagonists, nitrendipine and nimodipine. Eur J Pharmacol 1988;152:19–27.

El Ganouni S, Tazi A, Hakkou F. Potential serotonergic interactions with the anxiolytic-like effects of calcium channel antagonists. Pharmacol Biochem Behav 1998;60:365–9.

File E. The history of benzodiazepine dependence: a review of animal studies. Neurosci Biobehav Rev 1990;14:135–46.

File SE, Baldwin HA, Hitchcott PK. Flumazenil but not nitrendipine reverses the increased anxiety during ethanol withdrawal in the rat. Psychopharmacology 1989;98:262–4.

File SE, Zharkowsky A, Hitchcott PK. Effects of nitrendipine, chlordiazepoxide, flumazenil and baclofen on the increased anxiety resulting from alcohol withdrawal. Prog Neuro-psychopharmacol Biol Psychiatry 1992;16:87–93.

Froger N, Palazzo E, Boni C, Hanoun N, Saurini F, Joubert C, et al. Neurochemical and behavioral alterations in glucocorticoid receptor-impaired transgenic mice after chronic mild stress. J Neurosci 2004;24: 2787–96.

Fuller RW. Serotonin receptors involved in regulation of pituitary–adrenocortical function in rats. Behav Brain Res 1996;73:215–9.

Gupta MB, Nath C, Patnaik GK, Saxena RC. Effect of calcium channel blockers on withdrawal syndrome of lorazepam in rats. Ind J Med Res 1996;103:310–4.

Hamon M, Fattaccini CM, Adrien J, Gallissot MC, Martin P, Gozlan H. Alterations of central serotonin and dopamine turnover in rats treated with ipsapirone and other 5-hydroxytryptamine$_{1A}$ agonists with potential anxiolytic properties. J Pharmacol Exp Ther 1988;246:745–52.

Hitchcott PK, Zharkovsky A, File SE. Concurrent treatment with verapamil prevents diazepam withdrawal-induced anxiety, in the absence of altered calcium flux in cortical synaptosomes. Neuropharmacology 1992;31:55–60.

Jaffe JH. Pharmacological agents in treatment of drug dependence. In: Meltzer HY, editor. Psychopharmacology: The third generation of progress. New York: Raven Press; 1987. p. 1605–16.

Karst H, Wadman WJ, Joels M. Corticosterone receptor-dependent modulation of calcium currents in rat hippocampal CA1 neurons. Brain Res 1994;649:234–42.

Karst H, Nair S, Velzing E, Rumpff-van Essen L, Slagter E, Shinnick-Gallagher P, et al. Glucocorticoids alter calcium conductances and calcium channel subunit expression in basolateral amygdala neurons. Eur J Neurosci 2002;16:1083–9.

Kunchandy J, Kulkarni SK. Reversal by alpha-2 agonists of diazepam withdrawal hyperactivity in rats. Psychopharmacology 1986;90: 198–202.

Ladewig D. Dependence liability of the benzodiazepines. Drug Alcohol Dependence 1984;13:139–49.

Le Poul E, Boni C, Hanoun N, Laporte AM, Laaris N, Chauveau J, et al. Differential adaptation of brain 5-HT_{1A} and 5-HT_{1B} receptors and 5-HT transporter in rats treated chronically with fluoxetine. Neuropharmacology 2000;39:110–22.

Littleton J, Brennam C. Adaptive changes in number of calcium channels in drug dependence. Biochem Soc Symp 1993;59:193–203.

Malcolm RJ. GABA systems, benzodiazepines, and substance dependence. J Clin Psychiatry 2003;64:36–40.

Meijer OC, de Kloet ER. Corticosterone and serotonergic neurotransmission in the hippocampus: functional implications of central corticosteroid receptor diversity. Crit Rev Neurobiol 1998;12:1–20.

Post RM, Frye MA, Denicoff KD, Leverich GS, Kimbrell TA, Dunn RT. Beyond lithium in the treatment of bipolar illness. Neuropsychopharmacology 1998;19:206–19.

Przegalinski E, Budziszewska B, Siwanowicz J, Jaworska L. The effect of repeated combined treatment with nifedipine and antidepressant drugs or electroconvulsive shock on the hippocampal corticosteroid receptors in rats. Neuropharmacology 1993;32:1397–400.

Pucilowski O. Psychopharmacological properties of calcium channel inhibitors. Psychopharmacology 1992;109:12–29.

Pucilowski O, Kostowski W. Increased anxiety during ethanol and diazepam withdrawal in rats: effects of diltiazem and nicardipine. Alcohol Clin Exp Res 1991;15:331.

Pucilowski O, Krzascik P, Trazskowska E, Kostowski W. Different effect of diltiazem and nifedipine on some central actions of ethanol in the rat. Alcohol 1989;6:165–8.

Siebert PD, Larrick JW. Competitive PCR. Nature 1992;359:557–8.

Tazi A, Farh M, Moumni M, Hakkou F. Potentiation of behavioural effects of a calcium channel antagonist, nifedipine, by ipsapirone. Behav Pharmacol 1992;3:269–73.