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Effects of benzodiazepines receptor agonists on the hypothalamic–pituitary–adrenocortical axis

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Abstract

Previous studies have demonstrated that classical benzodiazepines decrease hypothalamic–pituitary–adrenocortical cortex (HPA) axis activity. Paradoxically, high doses of benzodiazepines also stimulate basal circulating corticosterone levels in some conditions. Because benzodiazepine agonists display little selectivity to any of the α subtypes of the γ-amino butyric acid $(GABA)_A$ receptor to which they bind, we propose that the unequivocal results are due to an α subtype-dependent modulation of the hypothalamic–pituitary–adrenocortical cortex axis output. To test this, basal hormonal output and induction of Fos in the hypothalamic paraventricular nucleus were measured after administration of various benzodiazepine ligands in mice. Zolpidem, a selective α_1 subtype agonist, produced a very strong increase in plasma adrenocorticotropic hormone and corticosterone whereas the inverse agonist FG7142 induced a small rise in plasma corticosterone. More surprisingly, the non-selective full agonists diazepam and zopiclone induced a lower increase in circulating corticosterone than after zolpidem. In contrast, the $\alpha_{2,3,5}$-selective benzodiazepine agonist and α_1 antagonist L-838,417 had no effect on corticosterone levels. Strong induction of Fos in the paraventricular nucleus was found in response to zolpidem, diazepam, and zopiclone, but not after L-838,417. Finally, pre-administration of L-838,417 prior to zolpidem strongly inhibited the effect of zolpidem on corticosterone. Likewise, the non-selective agonists diazepam and zopiclone at a dose that alone had no effect on corticosterone also inhibited the effect of zolpidem. Taken together, these results suggest that benzodiazepine ligands modulate the hypothalamic–pituitary–adrenocortical cortex axis through partly opposite mechanisms; and that the net effect is dependent on the composition of the $GABA_A$ receptor subunits to which they bind.

Keywords: ACTH (adrenocorticotropic hormone); Corticosterone; GABA; Paraventricular nucleus; Stress; L-838,417; Diazepam; Zolpidem; Zopiclone; (Mouse)

1. Introduction

GABA is the major inhibitory neurotransmitter in the mammalian brain and the $GABA_A$ receptor is the site of action of benzodiazepines. Multiple isoforms of $GABA_A$ receptor exist; each receptor comprises of a pentameric complex formed by the co-assembly of subunits selected from 16 genes (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, and θ) surrounding a central chloride ion-channel (Sieghart, 1995). Agonist activation of the $GABA_A$ receptor is augmented by the anxiolytic benzodiazepine ligands, causing a parallel shift of the GABA concentration–response curve (Barnard et al., 1998; Sieghart, 1995). The α subunit isoform present within an individual $GABA_A$ receptor subtype is the primary determinant of benzodiazepine ligand recognition (Fleck, 2002; Mehta and Ticku, 1999; Pritchett and Seeburg, 1990). $GABA_A$ containing α_1, α_2, α_3, or α_5 together with β, and a γ_2 subunit are all recognised by the classical benzodiazepines. Other drugs distinguish the $GABA_A$ receptors on the basis of their α subunit composition. For example, the sedative–hypnotic imidazolpyridine zolpidem has higher affinity for α_1 than α_2, α_3, or α_5 containing $GABA_A$ receptors (Squires et

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al., 1979). More recently, compounds with subtype selection for α_2, α_3, and α_5 over α_1 containing $GABA_A$ receptors such as L-838,417 are considered to be involved in anxiolytic properties (McKernan et al., 2000). It is therefore plausible that distinct α subtype-specific $GABA_A$ receptors mediate various effects of classical benzodiazepines.

Disturbances in the hypothalamic–pituitary–adrenocortical (HPA) axis are well known in human depression and anxiety, as well as in experimental animal models of anxiety and depression (Arborelius et al., 1999; Holsboer, 2000; Jensen et al., 2001; Van de Kar and Blair, 1999). The abnormal hypersecretion of cortisol that occurs in a variety of psychiatric disorders including major depression, sleep disturbances, and anxiety is suppressed by administration of benzodiazepines (De Boer et al., 1990). Neurons in the medial parvocellular part of the hypothalamic paraventricular nucleus contain corticotrophin-releasing hormone (CRH), and these neurons integrate excitatory and inhibitory signals and behavioural responses to stress into appropriate secretion of CRH that leads to adrenocorticotropic hormone (ACTH) release to the general circulation (Carrasco and Van de Kar, 2003). GABA is essential as a negative regulator of neuronal excitability in the paraventricular nucleus, thus mediating the amplitude and the duration of the stress response (Kovacs et al., 2004). GABA is also contained in neurons comprised in the negative feedback loop to the paraventricular nucleus (Bowers et al., 1998), and it is plausible that $GABA_A$ receptors may act on converging projections to the paraventricular nucleus. GABAergic afferents in the paraventricular nucleus originating from a number of forebrain areas have been reported and ultrastructural studies have shown that GABAergic axons, to a large extent, terminate on CRH neurons (Miklos and Kovacs, 2002; Roland and Sawchenko, 1993), and in situ hybridisation and in vitro autoradiography have detected α_1, α_2, and α_3 subtypes in the paraventricular nucleus (Cullinan, 2000; Duncan et al., 1995; Fenelon et al., 1995).

The role of benzodiazepines on basal corticosteroid release is complex, and even though it has been extensively studied over the last three decades, there is still a lot of controversy in the literature. Benzodiazepines have, under basal conditions, been shown to stimulate (Calogero et al., 1990; Le Fur et al., 1979; Vargas et al., 2001), to inhibit (Owens et al., 1989; Pivac and Pericic, 1993), and to be ineffective (Matheson et al., 1988). The state of the animal under which these drugs are tested should be considered with special care, because benzodiazepines interact with stress-related behaviours and cause a dose-dependent inhibition of stress-induced rise in corticosteroid levels (De Souza, 1990, for review). In addition, the effect of acute benzodiazepines on basal corticosterone levels might be related to several factors such as dose, compound and gender of the animal (Wilson et al., 1996), and these features might underlie the unequivocal results.

In agreement with the literature, we show here that doses of diazepam higher than those producing anxiolytic effects increase corticosterone levels in the mouse blood (Kalman et al., 1997; Lakic et al., 1986). We propose that the conflicting results reported earlier may also be a consequence of the net effect of non-selective benzodiazepines on the various $GABA_A$ receptor α subtypes. In order to address this issue, the responsiveness of the murine HPA axis output was examined after acute and combined treatments with a number of benzodiazepine receptor modulators specific for the α_1 and/or $\alpha_{2,3,5}$ containing $GABA_A$ receptors. In addition, we used induction of Fos as a marker to identify the level of activation in the paraventricular nucleus after acute administration of benzodiazepine ligands.

2. Materials and methods

2.1. Compounds

Zolpidem and zopiclone were purchased from Tocris Ltd. (Bristol, UK). FG7142 (methyl-3-carboline-3-carboxamide), and diazepam was purchased from Sigma-Aldrich (St. Louis, USA). L-838,417 (7-*tert*-butyl-3-(2,5-difluoro-phenyl)-6-(2-methyl-2*H*-[1,2,4]triazol-3-ylmethoxy)-[1,2,4]triazolo[4,3-b]pyridazine) was synthesised by the Department of Medicinal Chemistry, NeuroSearch A/S. All compounds were dissolved in 5% chremophor and administered intraperitoneally at 10 ml/kg.

2.2. Animals and single administration of benzodiazepine ligands

Adult male NMRI mice weighing 30–35 g were purchased from Harlan Inc (Hamburg, Germany). The animals were received at the animal facility, and housed 5 per cage under 12:12-h light/dark cycle, humidity in a temperature-controlled room for at least 7 days before the experiment. Food and water were available ad libitum. All procedures were conducted in accordance with the Danish National Guide for Care and Use of Laboratory animals. In order to analyse the dose-dependent effect of all benzodiazepine ligands on circulating corticosterone, mice ($n=5$) were administered at doses 0, 025, 0.1, 0.5, 2.5, 12.5, and 25 mg/kg i.p. The mice were returned to their home cages and sacrificed by decapitation 60 min after drug administration. Trunk blood was collected in centrifuge tubes containing 2 mg EDTA.

In order to examine the dynamics of the HPA axis, another experiment was carried out to determine the temporal profile of HPA activation by zolpidem. These mice were treated with 12.5 mg/kg zolpidem and the animals were sacrificed and trunk blood was taken at various time points after drug administration ($t=5$, 10, 15, 30, 60, and 120 min). The blood was collected in EDTA containing tubes, kept at 4 °C, and plasma was isolated by centrifugation and stored at −20 °C until processed.

2.3. Combination of drugs

In order to investigate the relationship between activation of α_1 and non-α_1 containing $GABA_A$ receptors, combinations of L-838,417 (12.5 mg/kg i.p.) or vehicle were given 15 min before a single dose of zolpidem i.p. at increasing concentrations (0.5, 2.5, and 12.5 mg/kg). L-838,417 acts at the benzodiazepine binding site and is a functionally selective agonist at α_2, α_3, and α_5 subtypes. In addition, L-838,417 is an antagonist on the α_1 site; it might be that

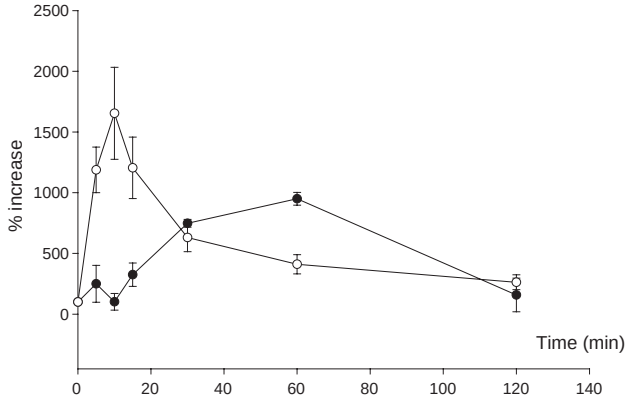

Fig. 1. Time course profile of ACTH and corticosterone responses to a single dose of zolpidem (12.5 mg/kg) in mice ($n=5$). The figure illustrates the relative rise compared to naïve animals of plasma ACTH (open circles) and corticosterone (closed circles) levels at various time points after zolpidem. The time course illustrates that release of ACTH occurs immediately after administration of zolpidem and that precedes release of corticosterone.

any inhibition results from its antagonistic properties. Therefore, the non-selective GABA potentiating benzodiazepine ligands, diazepam and zopiclone, were tested together with zolpidem. In this case mice were treated with vehicle, diazepam (0.5 mg/kg i.p.), or zopiclone (0.5 mg/kg i.p.) 15 min before administering the mice with 0.5 mg/kg zolpidem. The animals were sacrificed 60 min after zolpidem and trunk blood was taken for corticosterone measurements. All single and combined dosing experiments were carried out twice. ED_{50} values were calculated by non-linear regression using GraphPad Prism software (version 2.01, Gradpad Software Inc., San Diego, CA).

2.4. Radioimmunoassay

Plasma aliquots were stored at −20 °C until hormone levels were determined. Plasma corticosterone and ACTH were measured directly without prior extraction. In the case of corticosterone, it was assayed using a commercial [^{125}I] radioimmunoassay kit from DPC Diagnostics. Plasma ACTH concentrations were determined by a commercial radioimmunoassay kit from Amersham.

2.5. Immunohistochemistry for Fos

Sixty male mice were used for Fos studies. The animals were administered i.p. with 12.5 mg/kg diazepam ($n=9$), zopiclone ($n=9$), zolpidem ($n=12$), L-838,417 ($n=9$), or vehicle (5% chremophor) ($n=11$) at 4 h after the onset of the light phase and returned to their home cage. Sixty minutes after the injection the animals were deeply anaesthetised with mebumal and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 5 min. The brains were post-fixed overnight and subsequently submerged in 20% sucrose in 0.01 M phosphate-buffered saline (PBS) at 4 °C overnight. Forty-micrometer serial coronal sections were cut through the hypothalamus on a freezing sledge microtome in series of four and stored in a solution containing ethylene glycol glycerol in 30% sucrose at −20 °C until further processing. Prior to the immunocytochemical steps, the sections were rinsed for 3×10 min in 0.01 M PBS, in 1% H_2O_2–PBS for 10 min, and in PBS with 0.3% Triton X-100, 5% swine-serum, and 1% bovine serum albumin (BSA) for 30 min. Then the sections were incubated at 4 °C for 24 h in an antiserum against Fos (code #94012-5) diluted 1:4000 in PBS with 0.3% Triton X-100 and 1% bovine serum albumin. The primary antiserum was raised in rabbit against the N-terminal peptide similar to 2–17 of the rat Fos protein and characterised previously (Mikkelsen et al., 1998; Woldbye et al., 1996). The immunoreactive cells were identified by means of the avidin–biotin protocol and diaminobenzidine was applied as chromogene as earlier described (Mikkelsen et al., 1998).

2.6. Quantifications and statistics

To quantify the effects of the benzodiazepine ligands tested, an observer blind to the treatment of the animals counted the number of positive cells in each of the two paraventricular nuclei at its middle portion in the same tissue section. The number of positive cells is given as the average of these two counts. The middle portion of the paraventricular nucleus was delineated according to Paxinos and Franklin (2004) to about 2.98 mm rostral to the interaural line. The number of immunoreactive nuclei was counted within the total paraventricular nucleus at this level including both the magnocellular and the parvocellular subportions of the nucleus.

The data were analysed by one- or two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls method. All data are represented as group means ± standard error of mean (S.E.M.), and levels at $p<0.05$ were considered significant.

3. Results

3.1. ACTH and corticosterone responses after acute administration of benzodiazepine ligands

Zolpidem significantly and dose-dependently stimulated the mouse peripheral components of the HPA axis (Fig. 1). Plasma ACTH was found to be about 15-fold above baseline levels and

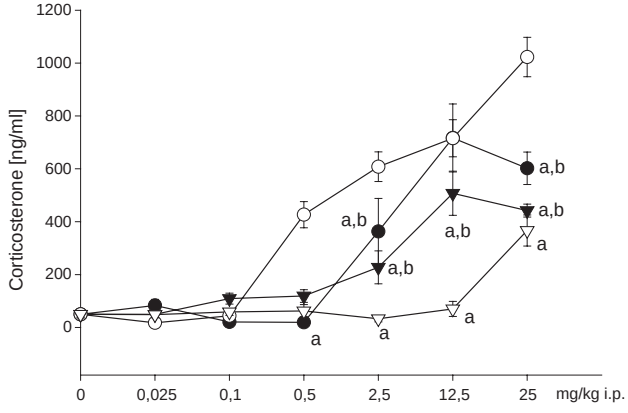

Fig. 2. The effect of increasing doses of the non-selective benzodiazepine ligands diazepam (closed circles) and zopiclone (closed triangles) on plasma corticosterone levels in mice compared to the α_1 selective zolpidem (open circles) and the $\alpha_{2,3}$ selective agonist L-838,417 (open triangles). The four drugs are given as a single dose and the mice are sacrificed 60 min after the treatment. The data represent mean ± S.E.M. of minimum of 5 mice per group. There was a significant ($P<0.05$) difference between the effect of non-selective and selective benzodiazepine receptor ligands. $^{\#}P<0.05$ vs. zolpidem and $^{*}P<0.05$ vs. vehicle. Two-way ANOVA and Student–Newman–Keuls test. The letters a and b denote a significant difference at a given dose to either zolpidem (a) or L-838,417 (b).

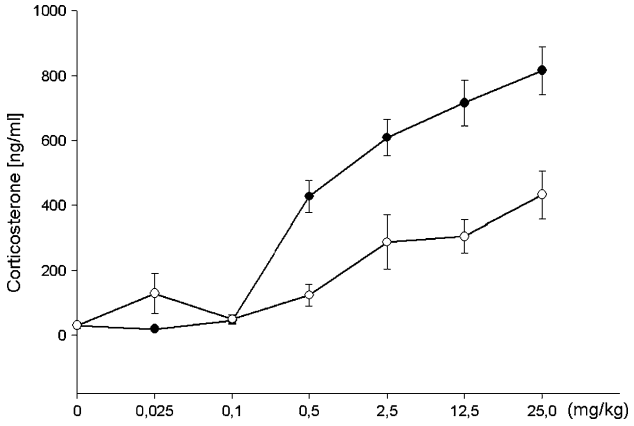

Fig. 3. The effects of FG7142 (open circles) on plasma corticosterone compared to zolpidem (closed circles). The compounds were given at 6 different doses 60 min before sacrifice. FG7142 significantly increased corticosterone at doses of 12.5 mg/kg and higher ($P<0.05$). There was a significant difference of FG7142 compared to zolpidem ($P<0.01$). Two-way ANOVA and Student–Newman–Keuls test.

corticosterone increased about 10-fold compared to naïve animals (Fig. 1) The time course illustrates a significant effect of zolpidem on ACTH release within 5 min after administration and reached its maximal level at 10 min. The level of plasma ACTH 15 min after zolpidem was significantly higher than sham (zolpidem 332±70 pg/ml vs. sham 27.6±6.8 pg/ml). Thereafter the ACTH level declined over time and reached basal levels after 120 min (Fig. 1). The time course for corticosterone was slightly delayed compared to ACTH, and peaked about 60 min after zolpidem, and returned to baseline after 120 min.

Treatment with zolpidem increased plasma corticosterone at a minimum effective dose of 0.5 mg/kg reaching a maximum at 12.5 mg/kg (Fig. 2). The ED_{50} was calculated to be 1.5 mg/kg. Zolpidem was effective and increased the circulating corticosterone up to about 800 ng/ml, a higher level comparable to that seen in restraint-stressed rats (Kalman et al., 1997). Both diazepam and zopiclone also produced a rise in plasma corticosterone. However, the potencies and efficacies of diazepam and zopiclone were significantly lower than for zolpidem (Fig. 2). Thus, a rise in plasma corticosterone was seen after administration of these drugs at 2.5 mg/kg and the level of corticosterone at this dose was in the range of 400 ng/ml at maximal concentrations (Fig. 2). Notably, while zolpidem induced a slightly higher level of corticosterone at 25 mg/kg compared to 12.5 mg/kg, the two non-selective benzodiazepine ligands produced a slightly lower level of corticosterone (Fig. 2). The two non-selective benzodiazepine ligands produced a different dose–response curve, with zopiclone being slightly stronger than diazepam. The two drugs were found to be equally effective on the release of corticosterone. Based on the dose–response curves the ED_{50} were calculated to be 2.4 mg/kg for zopiclone and 7.7 mg/kg for diazepam. In contrast, the $\alpha_{2,3,5}$ subtype-specific agonist L-838,417 was ineffective at all doses tested and produced only an insignificant increase in corticosterone levels at the highest dose of 25 mg/kg (Fig. 2). Statistical analysis revealed that both diazepam and zopiclone produced significantly stronger effects on circulating stress hormones than L-838,417, but significantly lower effect than zolpidem.

Treatment with a single dose of the α_1 inverse agonist FG7142 compared to zolpidem had relatively little effect on circulating corticosterone (Fig. 3). A significant release of corticosterone was only observed at doses of 12.5 and 25 mg/kg, but even at these doses the effects on corticosterone were much lower than after zolpidem (Fig. 3).

3.2. Effects of benzodiazepine ligands on Fos expression in the paraventricular nucleus

In order to determine whether the effects of benzodiazepine ligands occur at the level of the paraventricular nucleus and how

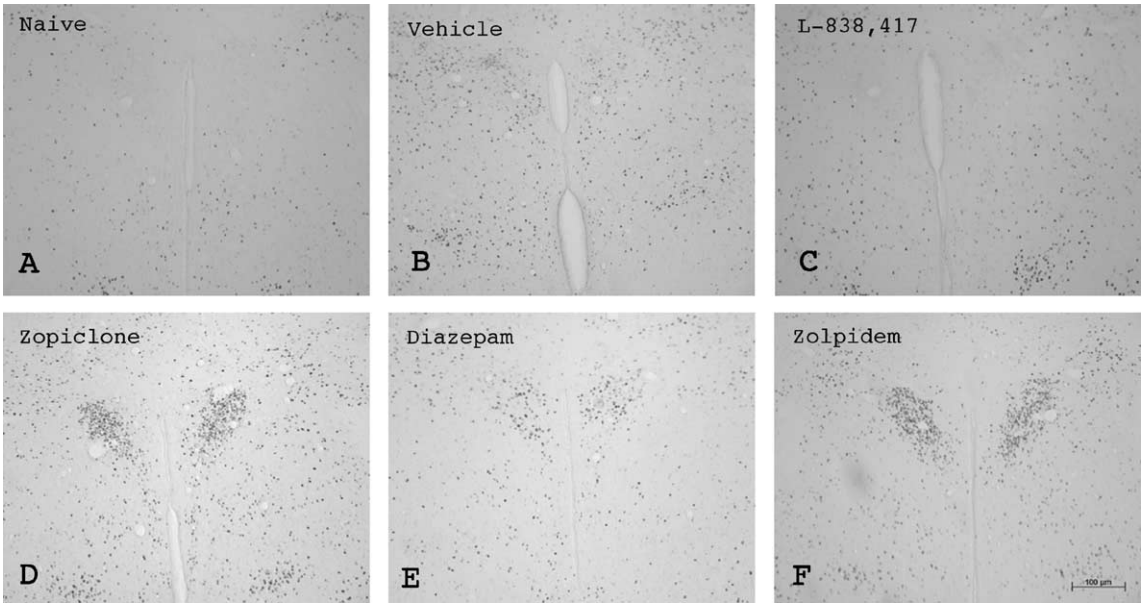

Fig. 4. α_1 receptor agonists induce Fos in the mouse paraventricular nucleus. Distribution of Fos in the paraventricular region in naïve mice (A), and after administration with vehicle (B), L-838,417 (C), zopiclone (D), diazepam (E), and zolpidem (F). All drugs are given in a dose of 12.5 mg/kg 60 min before sacrifice. Scale bar=100 μm.

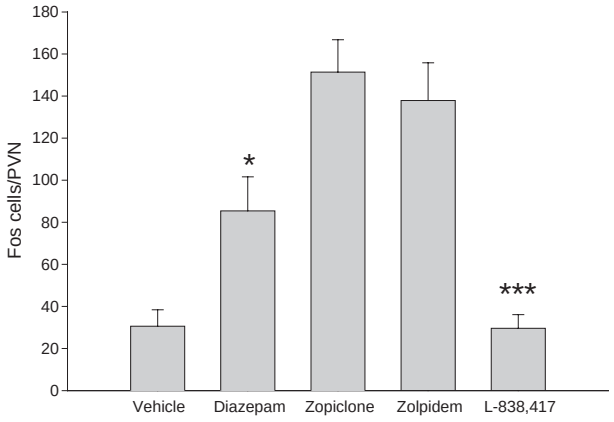

Fig. 5. Quantificative analysis of Fos expression in the paraventricular nucleus after acute treatment with benzodiazepine ligands. The effect of Fos induction was analysed 60 min after single administration of drugs at 12.5 mg/kg, the same dose that produces a maximal increase of corticosterone in plasma. The values are given as the mean number of cells expressing Fos in one section of the middle part of the paraventricular nucleus (mean ± S.E.M.). ***$P<0.001$; *$P<0.05$ compared to zolpidem. ANOVA non-parametric Tukey's multiple comparison test.

strong this input might be, Fos induction was studied in mice treated with zolpidem, diazepam, zopiclone, and L-838,417 (all drugs 12.5 mg/kg i.p.). A small number of Fos-immunoreactive nuclei were observed in the medial paraventricular nucleus in naïve and vehicle treated animals (Fig. 4A and B). Similarly, in animals treated with L-838,417, a low number of Fos positive cells were observed in the paraventricular nucleus (Fig. 4C). Zolpidem, diazepam, and zopiclone produced a strong increase of Fos containing nuclei in the paraventricular nucleus (Figs. 4D–F and 5). The activated cells were present in an area overlapping with the CRH containing medial parvocellular subportion of the paraventricular nucleus, but also in the magnocellular portion of nucleus (Fig. 4). Labelling was also found in the magnocellular supraoptic nucleus (not shown). Quantitative measurements of the number of Fos cells in the entire paraventricular nucleus revealed a strong increase after all drugs tested except L-838,417, which

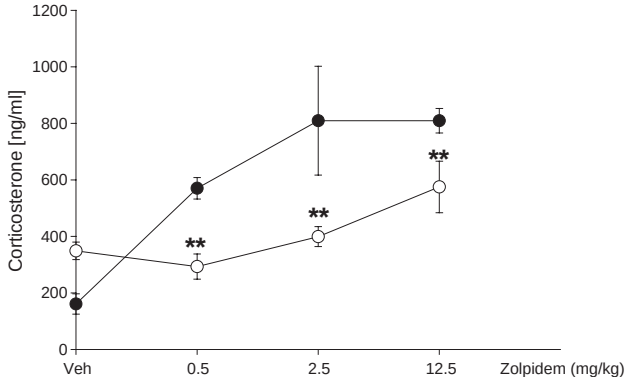

Fig. 6. Release of corticosterone by zolpidem (closed circles) is inhibited by pre-treatment with L-838,417 (open circles). Pre-administration of L-838,417 (12.5 mg/kg) 15 min before one injection of increasing concentrations of zolpidem shows that L-838,417 inhibits the activation by zolpidem at all doses tested. The values are given as mean ($n=5$) ± S.E.M. Significance between groups was determined by two-way ANOVA and **$P<0.01$.

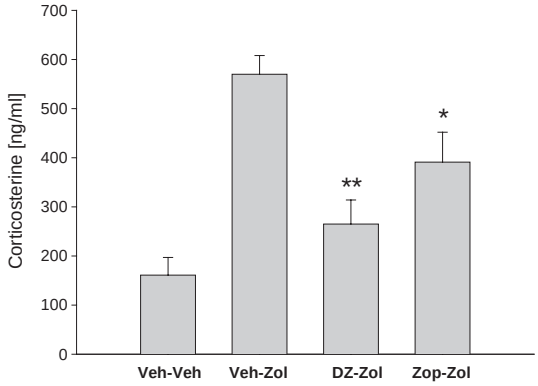

Fig. 7. Pre-treatment with diazepam or zopiclone (both 0.5 mg/kg) 15 min before administration of 0.5 mg/kg zolpidem inhibited release of corticosterone. As negative control mice were treated with vehicle twice, and as positive control zolpidem was administered 15 min after vehicle. **$P<0.01$; *$P<0.05$ vs. zolpidem.

was found to be ineffective (Fig. 5). Furthermore, Fos induction after diazepam was significantly lower at a concentration of 12.5 mg/kg, corresponding to the relative effect on corticosterone levels (Fig. 2).

3.3. Interaction/inhibition studies

Administration of 12.5 mg/kg L-838,417 immediately before zolpidem (0.5, 2.5, or 12.5 mg/kg) produced a strong inhibition of the release of corticosterone at all doses of zolpidem (Fig. 6). The maximal effects of the combined treatment of L-838,417 and zolpidem were comparable to the maximal effect of the non-selective benzodiazepines when given alone.

The level of corticosterone in mice pre-treated with 0.5 mg/kg diazepam before zolpidem was significantly lower than after vehicle at a dose of 0.5 mg/kg zolpidem (Fig. 7). Also, pre-treatment with 0.5 mg/kg zopiclone significantly inhibited the effect of zolpidem at the same dose (Fig. 7).

4. Discussion

Activation of the HPA axis to threatening stressful environmental stimuli is essential, but accurate inhibitory mechanisms are equally required to limit the magnitude in activation of autonomic, neuroendocrine, and behavioural responses. The major mechanisms responsible for inhibition of stress are the steroid-dependent negative feedback via steroid receptors in the hippocampus and the direct neural inhibition of paraventricular nucleus neurons driven by the neurotransmitters GABA and substance P (Jessop et al., 2000; Kovacs et al., 2004). Notably, part of the neuronal inhibitory GABAergic input to the paraventricular nucleus is a likely target of the corticoid-sensitive limbic forebrain structures (Herman et al., 2003).

It has not been understood how anxiolytics like benzodiazepines can activate the HPA axis in high concentrations, in particular whether it can be an effect independent of the $GABA_A$ receptor. We show here that acute administration of the $GABA_A$ receptor α_1 subtype selective

benzodiazepine site agonist zolpidem strongly activates the HPA axis in mice. Furthermore, it is shown that zolpidem induces expression of the transcription factor Fos in the medial part of the paraventricular nucleus, suggesting that zolpidem is acting in the brain. Zolpidem stimulates rapidly and robustly the secretion of both plasma ACTH and corticosterone levels. The ED_{50} was found to be 1.5 mg/kg, which is similar or lower than doses producing behavioural effects. The maximal response reached a level that has earlier been reported to occur in C57/B mice exposed to restraint stress or forced swim (Droste et al., 2003). The non-selective benzodiazepine site ligands, diazepam and zopiclone, did not have the same maximal efficacy as zolpidem on corticosterone levels. Finally, the $\alpha_{2,3,5}$ selective agonist L-838,417 was ineffective. Since zolpidem is selective for $GABA_A$ receptor complexes that contain α_1 subunits over those containing α_2, α_3, or α_5 subunits, and zopiclone and diazepam are non-selective (Fleck, 2002; Pritchett and Seeburg, 1990), it is suggested that activation of α_1 and non-α_1 containing $GABA_A$ receptors may oppose each other in stimulating CRH neurons in the paraventricular nucleus. We consider the α_2 subunit the main α subunit opposing this action because it is highly expressed in all CRH synthesising neurons in the paraventricular nucleus (Cullinan, 2000). In contrast, α_3 subunit mRNA is not expressed in the paraventricular nucleus and α_5 subunit mRNA expression is, to a great extent, limited to the hippocampus (Tietz et al., 1999).

The proposed model illustrated in Fig. 8 illustrates that at least two $GABA_A$ receptors bearing at least two distinct α subunits are involved in the regulation of the HPA axis output in vivo. Zolpidem activates the HPA axis by inhibiting a GABAergic input onto CRH neurons. Several lines of evidence support the presence of a disinhibiting GABAergic afferent input to the paraventricular nucleus. CRH neurons are under tonic inhibitory GABAergic control, because systemic administration of the $GABA_A$ receptor antagonist bicuculline both stimulates corticosterone secretion and mimics stress-induced ACTH release (Ixart et al., 1983). Moreover, bicuculline administered directly into the paraventricular nucleus induces a rapid increase in plasma ACTH (Cole and Sawchenko, 2002), and suppression of GABAergic transmission appears to play a permissive role for inducing an increase in secretory activity of CRH in organotypic cultures (Bartanusz et al., 2004). Therefore, by disinhibiting the GABAergic input onto CRH neurons, zolpidem effectively stimulates the HPA axis (Fig. 8). By contrast, direct inhibition of the paraventricular nucleus likely via an α_2 subunit containing $GABA_A$ receptor expressed in CRH neurons inhibits the HPA axis. Therefore, the balance between pharmacological activation of α_1 and α_2 containing $GABA_A$ receptors determines the response of benzodiazepine receptor ligands on the HPA axis. This hypothetical relationship is demonstrated by the antagonism of zolpidem-induced corticosterone release by L-813,417 (Fig. 6). Since L-838,417 in addition to its

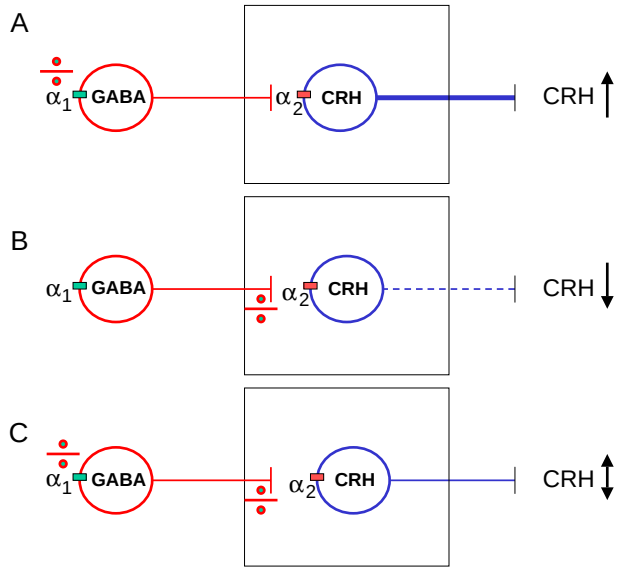

Fig. 8. Schematic illustration of the model proposed for the action of selective and non-selective benzodiazepine ligands on the CRH neurons in the paraventricular nucleus. Non-selective benzodiazepine receptor ligands act on at least two different sites. One is outside the paraventricular nucleus and is disinhibiting the CRH neurons. The other is inside the nucleus leading to a direct inhibition of the HPA output. The balance between activation of these two targets determines the release or inhibition of stress hormone secretion to the blood.

selective action on α_2, α_3, and α_5 subunits is also an antagonist at α_1 receptors (McKernan et al., 2000), we cannot exclude the possibility that a competition between L838,417 and zolpidem at this receptor subtype explains our findings. However, diazepam and zopiclone, both of which are full agonists at α_1 receptors (Sieghart, 1995) at doses that do not themselves stimulate corticosterone release, also inhibit zolpidem's effect, emphasising the role of $GABA_A$ receptors containing an α subunit distinct from α_1.

There appeared to be a good correlation between the relative rise in plasma corticosterone concentrations and the number of Fos containing cells in the paraventricular nucleus after administration of all drugs. However, this does not mean that Fos induction is required to elicit a hormone output. The Fos signal in the paraventricular nucleus appears to be a good marker for neuronal excitability, emphasising, as illustrated in Fig. 8, that the net result of increased neuronal inhibition via activation of α_1 $GABA_A$ receptors outside the paraventricular nucleus is an increased excitability inside the paraventricular nucleus. It is unlikely that zolpidem acts directly on the CRH neurons, but rather on an inhibitory interneuron. Light and electron microscopic studies indicate that nearly half of the synapses within the paraventricular nucleus are GABAergic (Decavel and Van den Pol, 1990) and CRH neurons express $GABA_A$ receptor α and β subunits (Cullinan, 2000). This raises the question: What are the inhibitory neurons innervating the paraventricular nucleus that is modified by zolpidem? Anatomical and functional studies suggest that sources of GABAergic inputs to the CRH neurons emanate

from the bed nucleus of the stria terminals, and these neurons might be the same as those converging the negative steroid-dependent feedback to the paraventricular nucleus (Herman and Cullinan, 1997). In addition, parvocellular neurons in the medial paraventricular subdivision receive synaptic inputs from neurons in the lateral and dorsal hypothalamus and the suprachiasmatic nucleus, all of which are considered to be GABAergic (Larsen et al., 1994; Roland and Sawchenko, 1993; ter Horst and Luiten, 1986; Vrang et al., 1995). Glutamate microstimulation of neurons in the paraventricular nucleus shows that GABA producing neurons are abundant in this region, particularly those in a region ventrolateral to the nucleus (Boudaba et al., 1996). The same population of GABAergic neurons, in addition to those in the bed nucleus of the stria terminals, is considered to be a part of the feedback inhibition to the paraventricular nucleus, because the projections from the subiculum and amygdala terminate rather around than inside the nucleus (Canteras and Swanson, 1992; Herman and Cullinan, 1997; Roland and Sawchenko, 1993). These GABAergic neurons are the targets for zolpidem and remain to be established by combining neural tracers and visualisation of α_1 subunits. The available evidence suggests that the GABAergic neurons stimulated by stress are likely located in the paraventricular nucleus or at least in its close vicinity (Herman et al., 2003; Kovacs et al., 2004). It seems therefore plausible that the GABAergic neurons activated by stress are the same as those stimulated by benzodiazepine ligands.

Significant effort has been given on developing drugs which interact at the benzodiazepine recognition site but which are devoid of some of the side effects, particularly in the tolerance and withdrawal (Atack, 2003). Separation of the α_1-dependent effect from the other α subunits is an important tool to select such new selective $GABA_A$ receptor modulators. Thus, it is believed that development of α subtype selective agents will provide better anxiolytics. However, experimental models that can distinguish between activities attributed to different α subtypes are required. Therefore, the delineation of the inhibitory inputs to the paraventricular nucleus that are modulated by the α_1 subunit activation may provide insight into the mechanisms of selective $GABA_A$ receptor ligands. Previous studies have shown that benzodiazepine receptor ligands including L-838,417 inhibit the effects of various stressors (Le Fur et al., 1979; McKernan et al., 2000; Yagi and Onaka, 1996), and our data suggest that this action could be mediated at the level of the paraventricular nucleus. The doses of L-838,417 and diazepam selected in the present study, exerting an inhibitory effect on the HPA axis, correlate well with the effective doses in models of anxiety (McKernan et al., 2000). In line with the recent research emphasizing a role for α_2 $GABA_A$ receptors in mediating the anxiolytic effect of benzodiazepines (Löw et al., 2000; McKernan et al., 2000; Rudolph et al., 1999), it is tempting to speculate that α_2 containing $GABA_A$ receptors in the paraventricular nucleus are important in this effect, and that this effect represents a good and easy detectable biomarker for α_2 subtype efficacy in vivo.

In summary, despite the fact that diazepam, zolpidem, and zopiclone all stimulate α_1 containing $GABA_A$ receptors, their immediate effects on the HPA axis differ. We conclude that the previous results reporting that various benzodiazepines produce different effects on the basal level of HPA activity in rodents are due to the balance between α_1 and non-α_1-dependent (presumably α_2) modulation of the CRH neurons in the paraventricular nucleus.

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