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Increased number of brain benzodiazepine receptors after in-vivo administration of estazolam to rats

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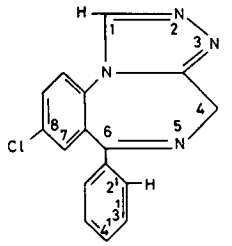
Estazolam significantly increased the K_d of [^{3}H]flunitrazepam in-vitro, like other benzodiazepines (BDZs) acting competitively at the receptor site. At variance with other BDZs, estazolam significantly raised the B_{max} for [^{3}H]flunitrazepam, at concentrations lower than its K_i for BDZ receptors. This effect may be responsible for the observed increase in [^{3}H]diazepam binding after in-vivo administration of estazolam to rats.

Benzodiazepines (BDZ) exert their different pharmacological effects by occupying specific receptor sites in the cns to various extents (Mennini & Garattini 1982). Under standard conditions, 50% protection against pentetrazol (leptazol, metrazol)-induced seizures in rats (ED50 AP) is achieved when 20–25% of total brain receptors are occupied by various BDZs corresponding to ≈20% displacement of [^{3}H]flunitrazepam (Braestrup & Nielsen 1983) or ≈50% displacement of [^{3}H]diazepam (Garattini et al 1981) bound in-vivo to cerebral membranes.

There are some exceptions to this general rule. For example, estazolam, an *s*-triazolo benzodiazepine with a chloro-substituent in position 8 (see Fig. 1) with high affinity for BDZ receptors in-vitro (Cotecchia et al 1981) when injected to rats at a dose corresponding to its ED50 AP, raises the amount of [^{3}H]diazepam bound (Mennini & Garattini 1982).

The present findings further describe the in-vivo and in-vitro interactions of estazolam with BDZ receptors in rat brain.

FIG. 1.



* Correspondence.

Materials and methods

Male rats (CD-COBS, Charles River, Italy), 200 ± 20 g, were used. In-vivo binding of [^{3}H]diazepam was determined as previously described (Mennini et al 1982a), by injecting iv 50 μCi/rat of [^{3}H]diazepam (spec. act. 78 Ci mmol^{-1}, NEN) 1 min before the animals were killed. Non-specific binding was determined by incubating the homogenates at 0 °C for 30 min in the presence of 3 μM unlabelled diazepam. In-vitro [^{3}H]flunitrazepam binding (spec. act. 84 Ci mmol^{-1}, NEN) concentration range 0·5–10 nM, was determined on thoroughly washed, thawed crude membrane preparations in 0·15 M Tris HCl, pH 7·4 as detailed elsewhere (Mennini et al 1982b). Non-specific binding was determined in the presence of 3 μM unlabelled diazepam, and represented about 10% of total. GABA levels were measured by radioreceptor assay as described by Herschel & Baldessarini (1979) on brain tissues from animals killed by microwave irradiation.

Results and discussion

Table 1 reports the in-vivo displacement of [^{3}H]diazepam bound to hippocampus or cerebellum of rats after pretreatment with equiactive doses (ED50 AP) of

diazepam, estazolam and its oxidized metabolite, 1-oxo-estazolam. All compounds showed antipentetrazol activity in rats (Caccia & Garattini 1983) but the metabolite contributed little or nothing to the effect of estazolam since only trace amounts were detectable in the brains of rats treated with estazolam (Caccia, unpublished results).

Even though estazolam was about six times more active than its metabolite in terms of dosage, the brain levels of the two compounds after a dose corresponding to their ED50 AP were similar: 170 ± 30 pmol g^{-1} for estazolam and 220 ±40 pmol g^{-1} for 1-oxo-estazolam (Caccia & Garattini 1983), reflecting their in-vitro affinities for BDZ receptors, with K_i 20 and 30 nM, respectively (Cotecchia et al 1981). Despite these similarities, while 1-oxo-estazolam at ED50 AP occupied BDZ receptors (Table 1), resulting in about 30% displacement of [^{3}H]diazepam in the rat hippocampus, estazolam significantly increased [^{3}H]diazepam binding in both brain regions considered.

Table 1. In-vivo displacement of [^{3}H]diazepam bound to hippocampus or cerebellum after pretreatment with equiactive doses of diazepam, estazolam and its oxidized metabolite.

Drug	Treatment μmol kg^{-1}	[^{3}H]Diazepam specifically bound % of total radioactivity Hippocampus	Cerebellum
Vehicle	—	18·9 ± 2·5	17·0 ± 0·6
Estazolam	2·13 (oral, 30′)	30·8 ± 4·5**	32·2 ± 3·6**
1-Oxo-estazolam	13·25 (oral, 30′)	13·6 ± 3·4*	19·5 ± 4·8
Diazepam	4·3 (ip, 15′)	12·1 ± 1·1*	19·9 ± 0·9

Data are mean ± s.d. of 4 animals per group.
*$P < 0.05$, **$P < 0.01$. Student's t-test on arc sin transformation of data.

GABA levels (nmol g^{-1} tissue ± s.d.) were: hippocampus: controls 1791 ± 116, estazolam 1744 ± 139; cerebellum: controls 1405 ± 178, estazolam 1275 ± 173. This excludes the possibility that the increase of [^{3}H]diazepam binding in-vivo was due to changes in BDZ receptor affinity modulated by endogeneous GABA.

Therefore we decided to study in-vitro the effects of different doses of estazolam on kinetic parameters of [^{3}H]flunitrazepam binding. As shown in Table 2, estazolam, at a concentration equal to its in-vitro IC50 ($2 \cdot 10^{-8}$ M), significantly raised the K_D of [^{3}H]flunitrazepam in hippocampus and cerebellum, like other BDZs acting competitively at the receptor site. At variance with other BDZs estazolam significantly raised the B_{max} for [^{3}H]flunitrazepam in hippocampus, but not in cerebellum. Using a lower concentration of estazolam (1/10 its IC50) we found the effect on K_D was non-significant (Table 2), but the increase in B_{max} was even more pronounced in hippocampus, but not in cerebellum.

The lack of effect of estazolam on in-vitro [^{3}H]flunitrazepam binding in cerebellum contrasts with the similarity of results regarding in-vivo benzodiazepine binding. One possible explanation is that the effect of

Table 2. The effect of estazolam on the kinetic parameters of [^{3}H]flunitrazepam binding.

	[^{3}H]Flunitrazepam binding Hippocampus B_{max}	K_D	Cerebellum B_{max}	K_D
Control	1·7 ± 0·2	5·4 ± 1·1	1·9 ± 0·1	2·6 ± 0·4
Estazolam $2 \cdot 10^{-8}$ M	*2·8 ± 0·4	*14·1 ± 2·9	1·6 ± 0·3	*13·0 ± 3·8
Estazolam $2 \cdot 10^{-9}$ M	*3·1 ± 0·5	6·7 ± 1·9	2·1 ± 0·6	9·2 ± 4·2

*$P < 0.05$ Student's t-test.
Data are mean ± s.d.
B_{max} and K_D were calculated by non-linear fitting of binding data from saturation experiments, using 8 concentrations of [^{3}H]flunitrazepam (0·5–10 nM).

estazolam on the B_{max} for [^{3}H]flunitrazepam is indirectly mediated by another site which is no longer effectively coupled to cerebellar BDZ receptors in-vitro. The heterogeneity of BDZ receptors in those two brain regions is well documented (Supavilai & Karobath 1980; Stapleton et al 1982; Sieghart et al 1983; Mennini et al 1982a; Mennini & Garattini 1983).

Many types of treatment reportedly raise the maximum number of BDZ receptors (Mennini & Garattini 1982), among them tofisopam, a 3,4-benzodiazepine which does not bind directly to BDZ receptors (Mennini et al 1982b). Estazolam is the first drug with high affinity for BDZ binding sites that has been shown to increase the B_{max} of [^{3}H]flunitrazepam at a concentration as low as 2 nM.

The molecular mechanism and the pharmacological relevance of the increase in the number of BDZ receptors induced by estazolam merits further investigation.

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