Pharmacology Biochemistry & Behavior, Vol. 30, pp. 679–682. © Pergamon Press plc, 1988. Printed in the U.S.A. 0091-3057/88 $3.00 + .00

High Density of Benzodiazepine Binding Sites in the Substantia Innominata of the Rat

M. SARTER[1] AND H. H. SCHNEIDER

Research Laboratories of Schering AG, Berlin and Bergkamen, F.R.G.

Received 4 August 1987

SARTER, M. AND H. H. SCHNEIDER. *High density of benzodiazepine binding sites in the substantia innominata of the rat.* PHARMACOL BIOCHEM BEHAV **30**(3) 679–682, 1988.—In order to study the neuronal basis of the pharmacological interactions between benzodiazepine receptor ligands and cortical cholinergic turnover, we examined the regional distribution of specific benzodiazepine binding sites using in vitro autoradiography. In the basal forebrain, the substantia innominata contained a high density of [^{3}H]lormetazepam (LMZ) binding sites (B_{max}=277 fmol/mg tissue; K_d=0.55 nM). The label could be displaced by diazepam (IC_{50}=100 nM), the benzodiazepine receptor antagonist β-carboline ZK 93426 (45 nM) and the partial inverse agonist β-carboline FG 7142 (540 nM). It is hypothesized that the amnesic effects of benzodiazepine receptor agonists are exerted through benzodiazepine receptors which are situated on cholinergic neurons in the substantia innominata and are involved in a tonic inhibition of cortical acetylcholine release. The benzodiazepine receptor antagonist ZK 93426 may exert its nootropic effects via benzodiazepine receptors in the substantia innominata and, consequently, by disinhibiting cortical acetylcholine release.

Substantia innominata Benzodiazepine receptors ZK 93426 In vitro Autoradiography Acetylcholine FG 7142

CORTICAL cholinergic afferents have been proposed to be involved in learning and memory functions. Compared to other neurotransmitter systems, the degree of dementia in Alzheimer's disease seems to correlate best with changes in cortical cholinergic transmission [2,4]. It seems evident that cortical acetylcholine release (and high affinity choline uptake, the rate limiting step in acetylcholine synthesis), are inhibited by the γ-aminobutyric acid (GABA) agonist muscimol and by benzodiazepine receptor agonists, either given systemically or injected into the basal forebrain [3, 11, 16, 17]. Accordingly, it has been hypothesized that the amnesic properties of benzodiazepines are associated with an impaired cortical acetylcholine turnover [14].

In contrast, the β-carboline ZK 93426 which is an antagonist at the benzodiazepine receptor [7] exerts antiamnesic and promnesic effects in various animal models [12, 14, 15]. Moreover, the compound enhanced performance of healthy volunteers in tasks measuring concentration, attention and memory [5,6]. These properties of ZK 93426 could be based on an increase in cortical acetylcholine turnover [13,14].

In order to investigate a neuronal basis of such a bidirectional control of cortical acetylcholine turnover by benzodiazepine receptor agonists and antagonists, we examined the binding of a [^{3}H]-labeled benzodiazepine to the region of the basal forebrain acetylcholinesterase projection system (BFAPS) [1] by means of in vitro autoradiography.

METHOD

Wistar strain male rats (250±10 g; Department of Tierzucht- und haltung, Schering AG) were used. After decapitation, brains were quickly removed and frozen in liquid nitrogen cooled isopentane. Cryostat sections (20 μm) at the level of the BFAPS were prepared and mounted on chromate/gelatine coated glass slides.

[^{3}H]Lormetazepam (LMZ) Binding

Slide mounted sections were preincubated for 5 min in 5 mM sodium phosphate buffer, pH 7.5, and for 20 min in 25 mM sodium phosphate buffer, pH 7.5, with 200 mM NaCl added (buffer 2) for removal of endogenous GABA. Labeling with [^{3}H]lormetazepam (LMZ) (0.7 nM; 2.94 TBq/mmol, prepared by Dr. P. E. Schulze, Schering AG) with or without addition of test compounds were conducted for 30 min in triplicate in plastic beakers, to avoid the adsorption of β-carbolines to glass surfaces. Nonspecific binding was de-

[1]Requests for reprints should be addressed to Martin Sarter, Department of Neuropsychopharmacology, Schering AG, P.O. Box 650311, D-1000 Berlin 65.

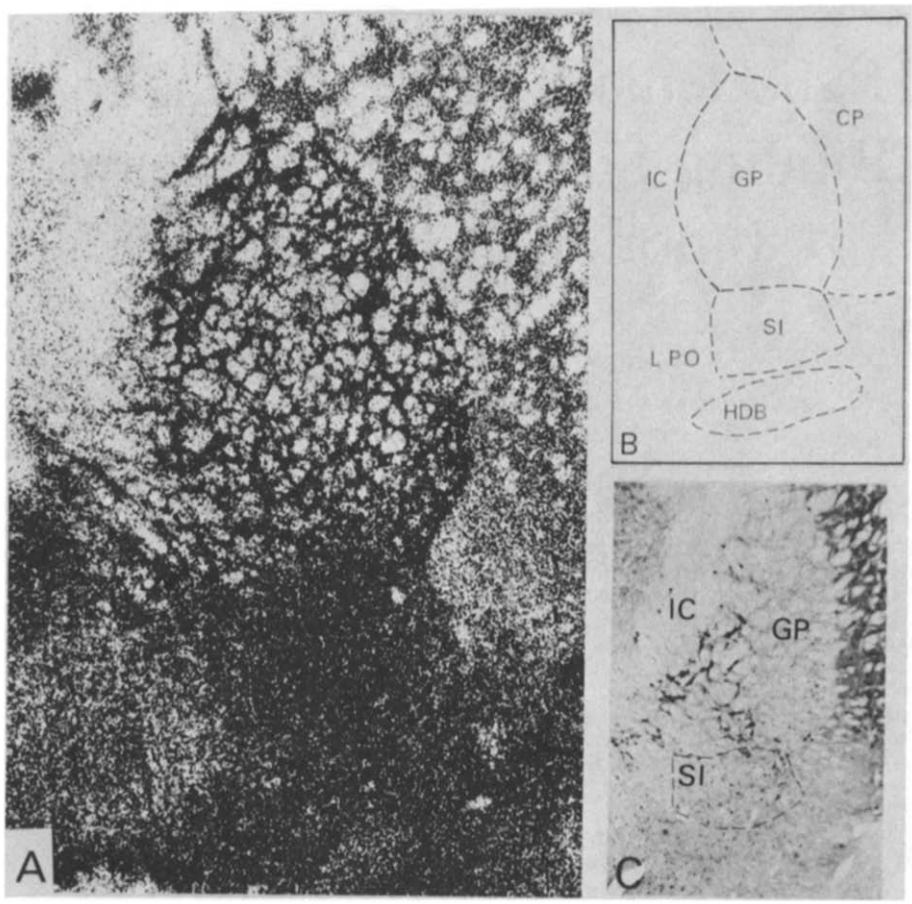

FIG. 1. (A) Photomicrograph from an autoradiogram of a section incubated with [^{3}H]lormetazepam (3.6 nM). (B) Anatomical structures; CP, caudate-putamen; GP, globus pallidus; SI, substantia innominata; IC, capsula interna; HDB, nucleus of the horizontal limb of the diagonal band; LPO, lateral preoptic area; (C) Corresponding section stained for acetylcholinesterase according the Butcher's method [1]. Note that distribution of stained, magnocellular neurons in the ventromedial globus pallidus (i.e., the region of the basal nucleus of Meynert) does not correspond to the distribution of the [^{3}H]lormetazepam label in this region.

termined in the presence of 1 μM Clonazepam. All incubations were conducted at room temperature.

Scatchard analyses were performed using 5 concentrations of [^{3}H]LMZ (0.2–3.6 nM). Following incubations, the sections were washed three times for 15 sec with cold buffer 2, dipped in distilled water, and dried in a stream of air.

Data Analysis

Autoradiographs were prepared by exposing Ultrofilm (LKB) to the sections, together with a tritium standard (microscales; Amersham). The optical density of different regions was determined by using an image analyzer (ASBA, Leitz) based on a microscope (Orthoplan, Leitz). Optical densities were transformed to radioactivity measures assuming a linear relationship between the optical density of the tritium microscales and their tissue equivalent value as given by the supplier (which was corrected according to the tritium decay factor). Identification of anatomical structures was performed with parallel Nissl-stained sections and with acetylcholinesterase stained sections from animals processed according to Butcher's pharmaco-histochemical regimen [1].

RESULTS

The substantia innominata (nomenclature in accordance to [1]) was found to contain a high density of [^{3}H]LMZ binding sites (see Fig. 1). In Table 1A, the result of Scatchard analyses is given for the substantia innominata in comparison to frontoparietal cortex, globus pallidus and nucleus caudatus. Whereas the dissociation constants K_d do not differ significantly between brain regions, the binding site concentration varies considerably within different structures. In the substantia innominata B_{max} is nearly as high as in the frontoparietal cortex.

Diazepam, ZK 93426, and FG 7142 were able to displace specifically bound [^{3}H]LMZ. Whereas the IC_{50} differed between the compounds, the displacement potency of these ligands did not differ between the brain structures investigated (see Table 1B).

DISCUSSION

The substantia innominata of the rat exhibits a high density of benzodiazepine receptors. In contrast to this area, the region of the neurons of basal nucleus of Meynert could not

TABLE 1

(A) [^{3}H]Lormetazepam Binding to Different Areas of the Rat Brain

	B_{max} (fmol/mg tissue)	K_d (nM)
Substantia innominata	277	0.55
Globus pallidus	132	0.38
Nucleus caudatus	115	0.46
Frontoparietal cortex, layers II–IV	324	0.36
Frontoparietal cortex, layers V–VI	257	0.64

(B) Displacement of [^{3}H]Lormetazepam Binding to Different Areas of the Rat Brain (IC_{50}; nM)

	Diazepam	ZK 93426	FG 7142
Substantia innominata	100	45	540
Nucleus caudatus	100	51	690
Globus pallidus	100	30	780
Frontoparietal cortex, layers II–IV	120	41	720
Frontoparietal cortex, layers V–VI	160	46	800

be differentiated from the globus pallidus on the autoradiograms (see Fig. 1), suggesting that a high density of benzodiazepine binding sites is a characteristic property of the substantia innominata, but not of the BFAPS in general.

The question whether the benzodiazepine receptors are situated on cholinergic cell bodies of the substantia innominata or on other types or parts of neurons, e.g., presynaptically, cannot be settled on the basis of autoradiographical data from intact animals. However, Zaborszky *et al.* [17], combining choline acetyltransferase immunohistochemistry with retrograde tracing of horseradish peroxidase and immunostaining against glutamic acid decarboxylase (GAD), demonstrated GAD-positive structures situated on cell bodies of cholinergic neurons in the basal forebrain. One of the sources of GABAergic afferents to the substantia innominata might be the nucleus accumbens [9,10].

Specific [^{3}H]LMZ binding in the substantia innominata, as well as in other brain areas, was displaced by the benzodiazepine diazepam and the β-carbolines ZK 93426 and FG 7142. The ligands bound with different affinities to this site with ZK 93426 being the most potent of the three ligands investigated. There were no major area-related differences in the affinity of these compounds to the LMZ binding site. It remains unsettled whether the benzodiazepine binding site in the substantia innominata may be classified as a type BZ1 or BZ2, as no information on the distribution of the BZ1 related low affinity $GABA_A$ sites is available for this area (see [8]).

If the pharmacological evidence of a bidirectional control of cortical acetylcholine turnover by different substances acting at the GABA/benzodiazepine receptor complex [3, 11, 16–18] is supported in future studies, and if the benzodiazepine receptors in the substantia innominata represent the neuronal basis of these interactions, then benzodiazepine receptor ligands with antagonist and partial inverse agonist quality would offer a new approach to the treatment of disorders related to a decreased cortical acetylcholine turnover. At least, results with the antagonist β-carboline ZK 93426 in animal experiments [12–15] and studies involving human volunteers [5,6] confirm that this compound exerts antiamnesic and promnesic properties.

ACKNOWLEDGEMENTS

We are grateful to J. Seidler and G. Bodewitz for technical assistance and to Drs. D. N. Stephens and J. Turner for fruitful discussions and critical reading of the manuscript.

REFERENCES

1. Bigl, V.; Woolf, N. J.; Butcher, L. L. Cholinergic projections from the basal forebrain to frontal, parietal, temporal, occipital, and cingulate cortices: A combined fluorescent tracer and acetylcholinesterase analysis. Brain Res. Bull. 8:722–749, 1982.
2. Bird, T. D.; Stranakan, S.; Sumi, S. M.; Raskind, M. Alzheimer's disease: Choline acetyltransferase activity in brain tissue from clinical and pathological subgroups. Ann. Neurol. 14:284–293; 1983.
3. Casamenti, F.; Deffenu, G.; Abbamondi, A. L.; Pepeu, G. Changes in cortical acetylcholine output induced by modulation of the nucleus basalis. Brain Res. Bull. 16:689–695; 1986.
4. Collerton, C. Cholinergic function and intellectual decline in Alzheimer's disease. Neuroscience 19:1–28; 1986.
5. Duka, T.; Krause, W.; Stephens, D. N.; Dorow, R. Human studies on the benzodiazepine receptor antagonist β-carboline ZK 93426: preliminary observations on psychotropic activity. Psychopharmacology (Berlin) 93:421–427; 1987.
6. Duka, T.; Schütt, B.; Edelmann, V; Dorow, R. The effects of a benzodiazepine receptor antagonist with weak inverse agonist properties on information processing in humans. In: Hindmarch, I.; Ott, H.; Roth, T., eds. Benzodiazepine receptor ligands, memory and information processing. Berlin: Springer Verlag, 1987 (in press).
7. Jensen, L.; Petersen, E. N.; Braestrup, C.; Honore, T.; Kehr, W.; Stephens, D. N.; Schneider, H. H.; Seidelmann, D.; Schmiechen, R. Evaluation of the β-carboline ZK 93426 as a benzodiazepine receptor antagonist. Psychopharmacology (Berlin) 83:249–256; 1984.
8. McCabe, R. T.; Wamsely, J. K. Autoradiographic localization of subcomponents of the macromolecular GABA receptor complex. Life Sci. 39:1937–1945; 1986.
9. Mogenson, G. J.; Swanson, L. W.; Wu, M. Neural projections from nucleus accumbens to globus pallidus, substantia innominata, and lateral preoptic-lateral hypothalamic area: An anatomical and electrophysiological investigation in the rat. J. Neurosci. 3:189–202; 1983.
10. Nagy, J. I.; Carter, D. A.; Fibiger, H. C. Anterior striatal projections to the globus pallidus, entopeduncular nucleus and substantia nigra in the rat: The GABA connection. Brain Res. 158:15–29; 1978.
11. Petkov, V.; Georgiev, V.; Getova, D.; Petkov, V. On the effects of diazepam, hyoscine and oxotremorine on acetylcholine release from cerebral cortex. Acta Physiol. Pharmacol. Bulg. 9:3–13; 1983.

12. Sarter, M.; Bodewitz, G.; Stephens, D. N. Attenuation of scopolamine-induced impairment of spontaneous alternation behavior by antagonist but not inverse agonist and agonist β-carbolines. Psychopharmacology (Berlin) 94:491–495; 1988.
13. Sarter, M.; Schneider, H. H.; Stephens, D. N. Treatment strategies for senile dementia: Antagonist β-carbolines. Trends Neurosci. 11:13–17; 1988.
14. Sarter, M.; Stephens, D. N. β-carbolines as tools in memory research: Animal data and speculations. In: Hindmarch, I.; Ott, H.; Roth, T., eds. Benzodiazepine receptor ligands, memory and information processing. Berlin: Springer Verlag, 1987:230–245.
15. Stephens, D. N.; Sarter, M. Bidirectional nature of benzodiazepine receptor ligands extends to effects on vigilance. In: Hindmarch, I.; Ott, H.; Roth, T., eds. Benzodiazepine receptor ligands, memory and information processing. Berlin: Springer Verlag, 1987:205–217.
16. Wenk, G. L. Pharmacological manipulations of the substantia innominata-cortical cholinergic pathway. Neurosci. Lett. 51:99–103; 1984.
17. Zaborszky, L.; Heimer, L.; Eckenstein, F.; Leranth, C. GABA-ergic input to cholinergic forebrain neurons: An ultrastructural study using retrograde tracing of HRP and double immunolabeling. J. Comp. Neurol. 250:282–295; 1986.
18. Zsilla, G.; Cheney, D. L.; Costa, E. Regional changes in the rat of turnover of acetylcholine in rat brain following diazepam or muscimol. Arch. Pharmacol. 294:251–255; 1976.