Laminar Distribution of Benzodiazepine Receptors in Visual Cortex of Adult Rat

T. Rothe and R. Schliebs

Karl Marx University, Paul Flechsig Institute for Brain Research, Department of Neurochemistry, Karl-Marx-Städter-Str. 50, 7039, Leipzig, German Democratic Republic

Abstract. The characteristics and distribution of benzodiazepine receptors in individual layers of the visual cortex of adult rats were examined with the ^{3}H-flunitrazepam binding technique employed on intact tissue slices. The different visual cortical layers were separated by cutting serial cryocut sections horizontally to the cortical surface and collecting the slices from each individual cortical layer under anatomical control. Highest benzodiazepine receptor densities were found in layers IV and VI. A moderate receptor density was detected in layer V (80 % of highest density). The lowest receptor binding was observed in cortical layers I and II/III, still representing 66 % of the highest receptor density. Binding affinities varied slightly between layers with dissociation constants somewhat higher for layers IV to VI in comparison to layers I and II/III. The distinct laminar pattern of benzodiazepine receptors in rat visual cortex suggests a differential neuromodulatory significance of these receptors in each individual cortical layer.

Key words: Benzodiazepine receptor — ^{3}H-flunitrazepam binding — Laminar distribution — Visual cortex — Rat brain

Introduction

Over a considerable period of time, the visual cortex served as a useful model to study not only the processes of visual information transfer, but also the mechanism underlying postnatal development and neuronal plasticity (see e.g., Singer 1985). The development of the laminar pattern of neurochemical parameters together with morphological data might be helpful in understanding the development of cortical function. Recently, we examined the laminar pattern of some cholinergic and noradrenergic markers in the visual cortex of adult and developing rats (Schliebs and Gödicke 1988; Walch and Schliebs 1989). The inhibitory neurotransmitter γ-aminobutyric acid (GABA) is also involved in a variety of response properties in visual cortical neurons (Sillito 1975) and has

been implicated in the mechanisms of cortical plasticity (Duffy et al. 1976; Kratz et al. 1976).

GABA receptors represent the target structures which receive GABAergic input and are partly associated with benzodiazepine receptors (Stephenson 1988). The benzodiazepine receptors are thought to play a neuromodulatory role in the brain by modifying the activities of a certain population of GABA receptors (Costa 1988).

In the present study we examined the characteristics and distribution of benzodiazepine receptors in individual layers of the visual cortex of adult rats by applying the ^{3}H-flunitrazepam binding technique to intact tissue slices. To perform biochemical analyses in individual cortical layers, the layers were separated by cutting serial cryostat sections horizontally to the cortical surface and collecting the slices from each individual cortical layer.

Materials and Methods

Materials

^{3}H-Flunitrazepam (specific activity 3.06 TBq/mmol) was obtained from the Radiochemical Centre Amersham-Buchler, FRG. Chlordiazepoxide was a gift from VEB Arzneimittelwerk Dresden, GDR. All other chemicals used were commercial products of the highest purity available.

Tissue preparation

The visual cortex, comprised of cortical areas 17, 18, and 18a, was isolated from rats (strain BD III, kept under standard animal housing conditions) at the age of 90 days, as described previously (Schliebs and Gödicke 1988). Briefly, the dissected visual cortex was transferred "upside down" to a plastic plate and frozen on dry ice resulting in a flattening of the cortical surface. The frozen piece of cortical tissue was trimmed precisely into 2 × 2 mm blocks by means of a tissue chopper, in order to obtain sections of similar size. The different cortical layers were then separated by cutting serial cryostat sections of 10 µm thickness. Slices were thaw-mounted onto glass slides and stored at −20 °C until used (normally not longer than two weeks). No loss of specific binding due to the storage for this interval was noted.

The thickness of each layer was estimated from cresyl violet stained sagittal sections of the visual cortex: layer I, 60 µm; layer II/III, 300 µm; layer IV, 240 µm; layer V, 300 µm; and layer VI, 450 µm. In each experiment slices from the border between adjacent layers were taken and stained as a histological control of the precise separation of the cortical layers.

Binding assay

For the receptor assay, two slide-mounted tissue sections collected from one individual cortical layer were incubated in humid chambers by flooding the sections with 50 mmol/l Tris-HCl, pH 7.4, containing ^{3}H-flunitrazepam in a final concentration ranging between 1 and 20 nmol/l. After one hour of incubation at 4 °C, the slides were given two rinses of 1 min each in ice-cold buffer before drying in a light air stream. The amount of radioligand bound to the tissue section was assessed by

scraping the tissue slices from the slides and measuring the radioactivity via liquid scintillation counting.

Nonspecific binding was assayed in adjacent sections using an incubation buffer which contained additionally 100 µmol/l chlordiazepoxide. Specific binding was determined by subtracting the nonspecific binding from the total binding. Nonspecific binding ranged between 5 and 10 % of total binding depending on the ligand concentration used. All determinations were performed in duplicate.

Specific binding was calculated as fmoles of radioligand specifically bound per mg of protein content. The protein content of the tissue slices was measured in parallel experiments using the method of Lowry et al. (1951).

Preliminary experiments were performed to characterize the binding sites and to determine optimal binding and washing conditions using coronal sections of the rat visual cortex.

Calculation of binding parameters

The binding parameters, such as equilibrium dissociation constant (K_d) and maximum receptor number (B_{max}), were fitted by nonlinear least squares regression analysis to the mass action expression for the reaction of one radioligand with one population of independent binding sites, as follows:

$$b_{sp} = \frac{B_{max} * f}{K_d + f}$$

b_{sp} denotes specific binding and f is the free concentration of radioligand not bound to the receptor site.

Nonlinear analyses were performed using least squares procedures, as follows: the squares of deviation (Q_d) of the experimental data from the fitted curve

$$Q_d = \sum_{k=0}^{n} \left[\frac{B_{max} * f_k}{K_d + f_k} - b_{sp,k} \right]^2$$

[n represents the number of the experimental pairs of values ($b_{sp,k}$; f_k)] were minimized by setting both the partial deviations of Q_d after B_{max} and K_d zero:

$$\partial Q_d / \partial K_d = 0 \quad \text{and} \quad \partial Q_d / \partial B_{max} = 0 \tag{1}$$

B_{max} and K_d then can be calculated by solving the two equation system (1). This algorithm was developed in our laboratory and applied in the computer program DATAFIT2. The program accepts as input the binding data (total and nonspecific binding; the dependence of nonspecific binding on free ligand concentration is fitted by a straight line using linear least squares regression analysis), from which the binding parameters are computed, and the best fit of specific binding against the free ligand concentration together with the experimental data points is plotted.

Statistical analysis

A one-way analysis of variance (ANOVA) was used to examine differences in receptor density of binding affinity between the distinct visual cortical layers. Two-tailed Student's t-test was applied to test for significant differences between two layers.

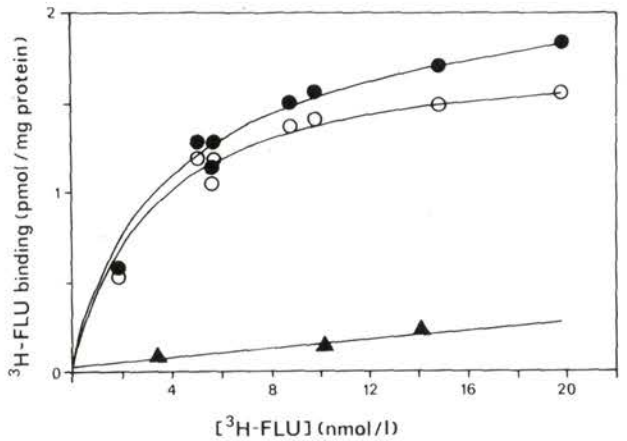

Fig. 1. Effect of thickness of coronal cryocut sections of rat visual cortex on total (•), specific (○) and nonspecific (▲) ^{3}H-flunitrazepam binding. ^{3}H-Flunitrazepam concentration in the assay was 2 nmol/l. For details of the assay conditions, see Materials and Methods.

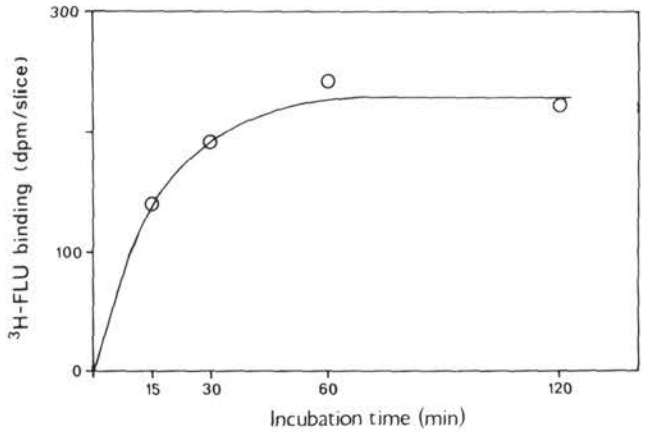

Fig. 2. Effect of incubation time on specific ^{3}H-flunitrazepam binding in coronal 10 μm cryocut sections of rat visual cortex. ^{3}H-Flunitrazepam concentration in the assay was 1 nmol/l. For details of the assay conditions, see Materials and Methods.

Results

Extensive preliminary experiments were performed in order to establish incubation conditions that give an optimal and reproducible labelling of the benzodiazepine receptors in intact tissue slices. When performing binding experiments in intact tissue slices and assessing the amount of bound radioligand to

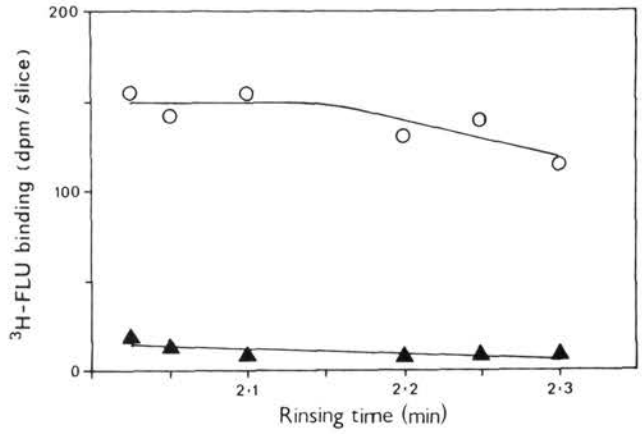

Fig. 3. Effect of rinsing time after incubation of coronal 10 μm cryocut sections of rat visual cortex on specific (○) and nonspecific (▲) ^{3}H-flunitrazepam binding. ^{3}H-Flunitrazepam concentration in the assay was 1 nmol/l. For details of the assay conditions, see Materials and Methods.

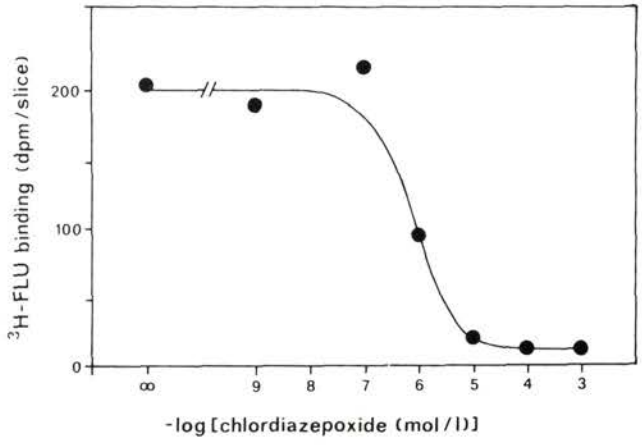

Fig. 4. Effect of increasing concentrations of chlordiazepoxide in the incubation medium on ^{3}H-flunitrazepam binding in coronal 10 μm cryocut sections of rat visual cortex. ^{3}H-Flunitrazepam concentration in the assay was 1 nmol/l. For details of the assay conditions, see Materials and Methods.

the tissue by direct liquid scintillation counting, it is necessary to evaluate the optimal thickness of the slice, the incubation time to reach equilibrium, the optimal time of rinsing the sections after incubation, and the optimal displacer concentration to estimate nonspecific binding.

As shown in Fig. 1, specific ^{3}H-flunitrazepam binding was linearly depen-

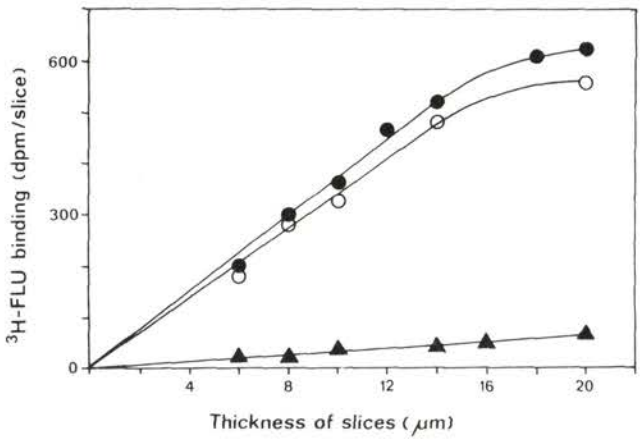

Fig. 5. Representative binding isotherms of benzodiazepine receptors in horizontal tissue slices of rat visual cortex (10 μm thick) showing ^{3}H-flunitrazepam binding in the presence (▲, nonspecific binding) and absence (●, total binding) of 100 μmol/l chlordiazepoxide. Specific binding (○) was calculated as the difference of total and nonspecific binding. The points represent experimental data, and the lines are the best fits obtained by nonlinear least squares regression analysis to the mass action expression for the reaction of one ligand with one population of independent binding sites. For details of calculation and binding assay, see Materials and Methods.

dent on the thickness of slices up to 14 μm indicating a complete penetration of the radioligand through the slices up to 14 μm. Therefore, for routine experiments cryocut sections of 10 μm thickness were used.

Specific ^{3}H-flunitrazepam binding to intact tissue slices reached equilibrium after one hour of incubation at 4 °C at a ligand concentration of 1 nmol/l (Fig. 2).

Rinsing the slides with ice-cold buffer after incubation should remove radioligand not specifically bound to the tissue receptor. In Fig. 3 specific and nonspecific ^{3}H-flunitrazepam binding to tissue slices is plotted against the time of slice rinsing. Giving slides two rinses of one min each resulted in a considerable reduction of nonspecific binding, while the specific binding remained nearly unchanged.

As competition experiments revealed, the presence of 100 μm chlordiazepoxide in the incubation medium was sufficient to displace the agonist from nearly all specific binding sites (Fig. 4).

The plot of specific ^{3}H-flunitrazepam binding against free ligand concentration shows that at a ligand concentration of about 10 nmol/l ^{3}H-flunitrazepam nearly all benzodiazepine binding sites present in the tissue slices are occupied

Table 1. Laminar distribution of benzodiazepine receptors in visual cortex of adult rats

Layer	B_{max} (fmol/mg protein)	K_d (nmol/l)
I	1764 ± 221 (3)	2.2 ± 0.3 (3)
II/III	1570 ± 102 (6)	1.8 ± 0.2 (6)
IV	2435 ± 132 (6)	4.7 ± 0.5 (6)
V	2090 ± 142 (6)	4.3 ± 0.4 (6)
VI	2628 ± 85 (6)	3.8 ± 0.3 (6)
ANOVA		
α(F)	0.005	0.005

The different cortical layers were separated by cutting serial cryostat sections of 10 µm thickness. Slices from one individual layer were thaw-mounted onto glass slides and incubated at 4 °C with varying concentrations of ^{3}H-flunitrazepam ranging between 1 and 20 nmol/l. The amount of radioligand bound to the tissue section was assessed by scraping the tissue slices from the slides and measuring the radioactivity via liquid scintillation counting. Maximum receptor densities (B_{max}) and binding constants (K_d) were obtained from saturation experiments by fitting experimental data to the saturation curve using nonlinear least squares regression analysis. For details of the assay conditions, see Materials and Methods.

The data represent the means ± SEM for the number of separate saturation experiments given in parentheses. $\alpha(F)$ indicates the level of significance of the F-distribution using one-way analysis of variance (ANOVA] between all cortical layers.

by the radioligand. Fig. 5 shows a representative example of such a saturation experiment.

Table 1 gives an image of the laminar distribution pattern of benzodiazepine receptors in visual cortex of adult rats obtained from saturation experiments performed with each individual cortical layer. Analysis of variance revealed that the density of benzodiazepine receptors differs significantly between visual cortical layers ($\alpha < 0.005$). Highest receptor densities were found in layers IV and VI. A moderate receptor density was detected in layer V (about 80 % of highest binding, $p < 0.01$). Lowest binding was observed in cortical layers I and II/III, still representing about 66 % ($p < 0.01$) of the highest observed receptor density.

Binding affinities displayed slight but significant differences between the distinct visual cortical layers as revealed by analysis of variance. Higher binding constants were found for layers IV to VI in comparison to layers I and II/III (Table 1).

Discussion

Quantitative receptor autoradiography is a powerful tool to study the distribution of binding sites which, in comparison with binding studies in membrane fractions, combines anatomical resolution with increased sensitivity. However, the interpretation of results obtained by quantitative autoradiography can bear potential errors, and special care has to be taken in identifying discretely labeled layers of the cerebral cortex (Geary et al. 1985; Hall et al. 1986). In this study, therefore, we separated the different layers of the visual cortex by cutting serial cryostat sections and performing binding studies in slide-mounted tissue sections of each individual cortical layer, thus combining the increased sensitivity of the receptor binding technique in slices with a precise determination, by liquid scintillation counting, of the amount of radioligand bound to the tissue. Furthermore, this procedure allows also to take into account the recently reported laminar variation of protein content in rat visual cortex (Schliebs and Gödicke 1988). In the present study, the characteristics and laminar distribution of benzodiazepine receptors were examined in the visual cortex of adult rats. Benzodiazepine receptor binding carried out in tissue slices of rat visual cortex displays binding characteristics similar to those described for the receptor binding in cortical membrane fractions (Morin 1986; Watanabe et al. 1986). Highest densities of benzodiazepine receptors were found in layers IV and VI of visual cortex of adult rats what is in agreement with the laminar pattern in rat cerebral cortex observed in autoradiographic experiments (McCabe and Wamsley 1986; Tietz et al. 1986). In contrast, ^{3}H-muscimol binding to high-affinity $GABA_A$ receptors in the rat cerebral cortex was found to be highest in the superficial layers I to IV and lowest in layer V—VI (Palacios et al. 1981).

Several types of GABAergic neurons differing in their synaptic connections are present in all layers of rat visual cortex (Somogyi et al. 1984). GABAergic nerve terminals are also present in all layers, but their density and distribution vary throughout the layers. The greatest density is centered in layer IV of rat visual cortex, but a high density was also found in the superficial half of layer I and at the border between layer VI and white matter (Lin et al. 1986). This pattern of GABAergic fibres correlates well with the laminar distribution of benzodiazepine receptors reported here.

Immunocytochemical studies revealed, that the low- and high-affinity forms of the $GABA_A$ receptor are possibly the same protein in different conformational states, and that only the low-affinity site of the $GABA_A$ receptor is functionally coupled to benzodiazepine receptors (De Blas et al. 1988). At least in the frontal and the parietal cortex the low-affinity $GABA_A$ receptors are found to be concentrated in layer IV (McCabe and Wamsley 1986). The low density of benzodiazepine receptors detected in layers I-III corresponds to the low

density of low-affinity $GABA_A$ receptors in these layers emphasizing the allosterical coupling of both receptor types. In contrast, high-affinity $GABA_A$ receptors were found to be highest in layers I-III of the frontal cortex. The functional significance of this different laminar pattern of GABA and benzodiazepine receptors in the rat visual cortex remains to be clarified. The exact knowledge of the laminar distribution of GABAergic markers might be helpful in understanding the functional anatomy of GABAergic transmission in the visual cortex.

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