Autoradiographic Studies on Benzodiazepine Receptor Subtypes in the Rat Brain

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Abstract—The technique of in vitro autoradiography which was developed by Kuhar and others was applied to the rat brain with use of 3H-flunitrazepam (flu) as a radioactive ligand. The cerebral cortex, hippocampus, substantia nigra and cerebellar cortex were rich in 3H-flu binding sites. To differentiate the benzodiazepine receptor (BZR) subtype, the authors used a type 1 specific ligand, either triazolopiridazine (C1 218872) or methyl-β-carboline-caboxylate (β-CCM), as an unlabeled displacer. The preparations were exposed on a 3H-sensitive film and then the film was developed. Computer-analysis of thus obtained autoradiographic pictures revealed that type 2 binding sites were distributed evenly within the rat brain, but with slight predominance in the hippocampus. After adding β-CCM, no silver grains were noticed in the cerebellum and substantia nigra. These data meant that these two structures contained essentially type 1 BZR, while the hippocampus contained both type 1 and type 2 receptors. Autoradiographically, characteristic distribution of BZR represented by 3H-flu binding was considerably lost by adding a type 1 specific ligand, and this treatment caused the silver grains to be evenly distributed. These data suggest that the BZR which is directly associated with characteristic pharmacological actions such as anxiolytic and hypnotic effects is type 1, and type 2 binding sites have a less characteristic distribution pattern and might be pharmacologically less specific.

These days, multiplicity of the benzodiazepine receptor (BZR) has been advocated (1-3). Lippa and others pointed out that Cl 218872, a triazolopiridazine derivative, bound with higher affinity to BZR in the cerebellum than to those in the other structures of the brain and termed the receptors with high affinity for Cl 218872 type 1 BZR (4, 5). Subsequently, it was found that ethyl-β-carboline-3-carboxylate(β-CCE) was a most probable candidate for the endogenous benzodiazepine (BZ)-like substance (6), and the derivatives of β-CCE had a more specific affinity to type 1 receptors (7-9). The receptors which showed low affinity to these type 1 specific ligands have been called type 2. Nevertheless, the functional significance of these subtypes of BZR is still obscure. In this paper, the authors tried to map out type 2 binding sites and compared the results with the distribution of 3H-flunitrazepam (flu) binding sites which equally bound to both subtypes.

Materials and Methods
Wistar strain male rats of 200 g body weight were used for the experiments. After decapitation, brains were rapidly removed, frozen and embedded in OCT compound. Cryostat sections of 10 μm thickness were prepared. As preliminary work, receptor binding experiments were done on cryostat sections, and Scatchard analysis was done. For 3H-flu binding site autoradiography, 2 nM of the radioactive ligand was incubated at 0°C for 90 min after preincubation. Ultraviolet light was irradiated on the sections for 12 min to obtain irreversible binding (10). Clonazepam of 1 μM was used as displacer. Either Cl 218872 or methyl-β-carboline-
carboxylate (β-CCM) as a non-radioactive type 1 receptor specific ligand was needed to map out type 2 binding sites. After incubation, the sections were washed in buffer and rapidly air-dried. The thus obtained samples were apposed to 3H-sensitive films. The films were exposed for 2 weeks and developed. These autoradiograms were computer-analysed by IBAS II of the Zeiss company.

Results

Previously published literature (3-5) demonstrated that both CI 218872, a BZ agonist, and β-CCM, an antagonist, bound to type 1 receptors with high affinity, the latter being more specific to type 1. It is also known that the affinity difference between type 1 and 2 receptors is less for β-CCM than for CI 218872(3). These facts mean that we are able to map out type 2 receptor distribution by adding either of these ligands in the 3H-flu binding experimental system. In these experiments, cryostat serial sections were made, and a three-serial-section set was treated with 3H-flu alone, 3H-flu with 200 nM CI 218872 and 3H-flu with 20 nM β-CCM one after another.

In Fig. 1, Scatchard plots of saturation experiments of 3H-flu binding on cryostat sections are shown. The $K_d$ and $B_{max}$ values were 2.3 nM and 30.4 fmol/section, respectively.

![Fig. 1](image.png)

**Fig. 1.** Scatchard plots of saturation experiments of 3H-flu binding to cryostat sections of the rat cerebellum. Each plot represents the mean value in three repeated experiments.

Figure 2-a shows an autoradiogram of 3H-flu binding sites at the section through the

![Fig. 2](image.png)

**Fig. 2.** Autoradiograms of 3H-flu binding sites of sections through the striatum. a: Total 3H-flu binding sites without non-radioactive ligand added. In this picture, 3H-flu binds both type 1 and type 2 receptors equally. The cerebral cortex shows the highest density of 3H-flu specific binding sites. b: An autoradiogram of the neighboring section with CI 218872. Difference of grain densities between the cerebral cortex and striatum was much less when compared to Fig. 2-a. c: An autoradiogram of the next section incubated with β-CCM. Grain densities were almost evenly distributed throughout the cerebral cortex to striatum.
striatum. There were a large number of $^3$H-flu binding sites in the cerebral cortex, especially in the middle layers, and preoptic area, while the striatum contained a few binding sites. In Fig. 2-b, an autoradiogram of the neighboring section with CI 218872 added is presented. In this figure, the difference of grain densities between the cerebral cortex and striatum was much less when compared to Fig. 2-a. Autoradiography of the next section in serial cutting was performed using incubation with $\beta$-CCM, which was more specific to type 1 receptors. By adding $\beta$-CCM, the characteristic distribution of grain densities as observed in Fig. 2-a disappeared, and grain densities were almost evenly distributed throughout the cerebral cortex to striatum (Fig. 2-c).

Figures 3-a, 3-b and 3-c were autoradiograms of serial cryostat sections through the substantia nigra in the order of $^3$H-flu without a type 1 specific ligand, with CI 218872 and with $\beta$-CCM. It was found that $^3$H-flu binding sites were highly concentrated in the cerebral cortex, especially the middle layers, hippocampus, substantia nigra and pretectal area, and the receptor population was less in the thalamic nuclei (Fig. 3-a). By adding CI 218872, the cerebral cortex, hippocampus and substantia nigra showed remarkable reduction in receptor population, but still remained at higher levels than the thalamic nuclei (Fig. 3-b). In the case of $\beta$-CCM, silver grains were still retained in the hippocampus, where type 2 receptors seemed to be more concentrated than in other areas (Fig. 3-c).

The same serial sections were prepared for the cerebellum. $^3$H-flu binding sites were abundant throughout the gray matter of the cerebellum (Fig. 4-a). Such a relationship between structure and receptor population was kept after adding CI 218872, although the receptor concentration was remarkably decreased in total (Fig. 4-b). In contrast to CI 218872, $\beta$-CCM caused remarkable changes in receptor population, that is, there was no structure-dependent density distribution, and actually, grain densities were diffusely observed at the background level (Fig. 4-c). This means that $\beta$-CCM is more specific to the type 1 receptor, and cerebellar BZR are considered to be essentially type 1.

Discussion

Young and Kuhar introduced a microscopic
in vitro autoradiographic technique of neurotransmitter receptor assay which made it possible to study the pharmacology and kinetics of the binding on slide-mounted sections (11), and they identified BZR through this method in 1979 (12). Recently, this method has been widely utilized as a tool of neurobiological research and has been developed into a method for localization of bound radioactivity by apposition of a tritium sensitive film. The authors applied this method to autoradiographic observation of BZR distribution in the rat brain using \(^3\)H-flu and tried to study the functional significance of BZR subtypes. Our \(^3\)H-flu binding experiments on cryostat sections of the rat brain showed a single binding site with a K_d value of 2.3 nM, which was concentrated in the cerebral cortex, hippocampus, substantia nigra and cerebellar cortex. Both CI 218872 and \(\beta\)-CCM were useful ligands to differentiate BZR subtypes. By adding a type 1 specific ligand, either CI 218872 or \(\beta\)-CCM, in the experimental system of \(^3\)H-flu autoradiography, reduction of \(^3\)H-flu binding sites was observed much more remarkably in such areas as the cerebral cortex, hippocampus, substantia nigra and cerebellar cortex where the binding site was originally more concentrated than in other areas. As the result, it was shown that the autoradiographic \(^3\)H-flu binding site was evenly distributed throughout the brain after addition of any of these non-radioactive ligands. Nevertheless, only the hippocampus retained the receptor density to a considerable degree after this procedure.

It is concluded that BZR richness in the brain actually means richness of type 1 BZR in the areas that are pharmacologically active sites of BZ, while type 2 receptors are distributed diffusely in the brain less specifically. Only the hippocampus contained both type 1 and 2 receptors equally.

Sieghart extracted 4 kinds of protein, P_51, P_53, P_55 and P_59, which irreversibly bound to \(^3\)H-flu and presumed that P_51 with an apparent molecular weight of 51,000 was especially associated with type 1 BZR of the brain. He estimated the contribution of the individual proteins to total irreversible binding and confirmed that P_51 was highest in percentage of the total radioactivity of proteins irreversibly labeled by \(^3\)H-flu. Relative contribution of P_51 to total binding was highest in the inferior colliculus, cerebellum and substantia nigra, and it was

![Fig. 4. Distribution of \(^3\)H-flu binding sites in the cerebellum. a: Total \(^3\)H-flu binding sites were abundant throughout the gray matter of the cerebellum. b: \(^3\)H-flu binding sites with CI 218872. Receptor binding was reduced to some extent. c: \(^3\)H-flu binding sites with \(\beta\)-CCM, \(\beta\)-CCM caused remarkable changes in receptor population. There is no structure-dependent density distribution.](image)
lowest in the striatum and hippocampus (13).

These results are consistent with our autoradiographical findings. How these receptor subtypes are associated with various pharmacological actions of BZ is left for further investigations. From our data, it is assumed that the distribution of type 1 receptors corresponds to probable sites of the main pharmacological actions of BZ, and such effects as antianxiety or hypnotics are considered to be associated with type 1 receptors.

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