Mini-Reviews

Benzodiazepine receptors resolved

by J. G. Richards, P. Schoch, H. Möhler and W. Haefely

F. Hoffmann-La Roche & Co. Ltd, Pharma Research Dept, CH-4002 Basel (Switzerland)

Summary. To date, attempts to map the distribution and density of benzodiazepine receptors in the CNS have been dominated by radiohistochemical techniques with conventional receptor binding. Their limited resolution, however, prompted us to try an immunohistochemical approach. Purified GABA/benzodiazepine receptors, prepared from bovine cerebral cortex, have been used to raise monoclonal antibodies for this purpose. Immunoreactive sites in rat brain, spinal cord and retina as well as in bovine and post-mortem human brain were found to be concentrated on neuronal cell bodies and processes in those regions known to be innervated by GABAergic neurons. Electron microscopic analysis revealed a selective staining of axosomatic and axodendritic pre- and postsynaptic contacts.
Key words. Receptor mapping; $GABA_A$/benzodiazepine receptor; in vivo binding; in vitro binding; radiohistochemistry; immunohistochemistry; monoclonal antibodies; resolution.

According to current opinion[6], the therapeutic effects of benzodiazepine minor tranquillizers are due to an increase in the efficiency of submaximal GABAergic transmission mediated by a variety of projecting (long-fiber) neurons and interneurons[1] in the CNS. The pharmacological receptors triggering these effects are localized in neuronal membranes as part of an *oligomeric complex with $GABA_A$ receptors and their associated chloride channel*[5,17]. The benzodiazepine receptors are functionally involved in the coupling mechanism between GABA receptors and chloride channels. By this means, benzodiazepines allosterically modulate the GABA receptor-effector system. However, they do not influence neuronal activity when the GABA receptor-effector system is either in its resting state or maximally activated, since benzodiazepine receptor ligands do not directly affect chloride conductance, but rather modulate the efficiency of the stimulus provided by GABA.

Benzodiazepine receptors contain the recognition sites for three basic types of high-affinity exogenous ligands (simplified scheme fig. 1)[26] each with different functional consequences: *agonists* are anxiolytic, hypnotic, anticonvulsant and muscle relaxant and enhance the chloride conductance-increasing effect of GABA (postive intrinsic activity: + + +); *inverse agonists* have opposite effects, they reduce the effect of GABA and are e.g. anxiogenic and convulsant (negative intrinsic activity: − − −); *competitive antagonists* are practically inactive per se (intrinsic activity: 0) but prevent or abolish the receptor-mediated pharmacological and therapeutical effects of agonists and inverse agonists.

Benzodiazepine receptor agonists and inverse agonists are, thus, positive and negative *allosteric modulators of $GABA_A$ receptors*. This allosteric coupling explains the unique property of benzodiazepine receptors to mediate a spectrum of effects with opposite efficacy. It remains to

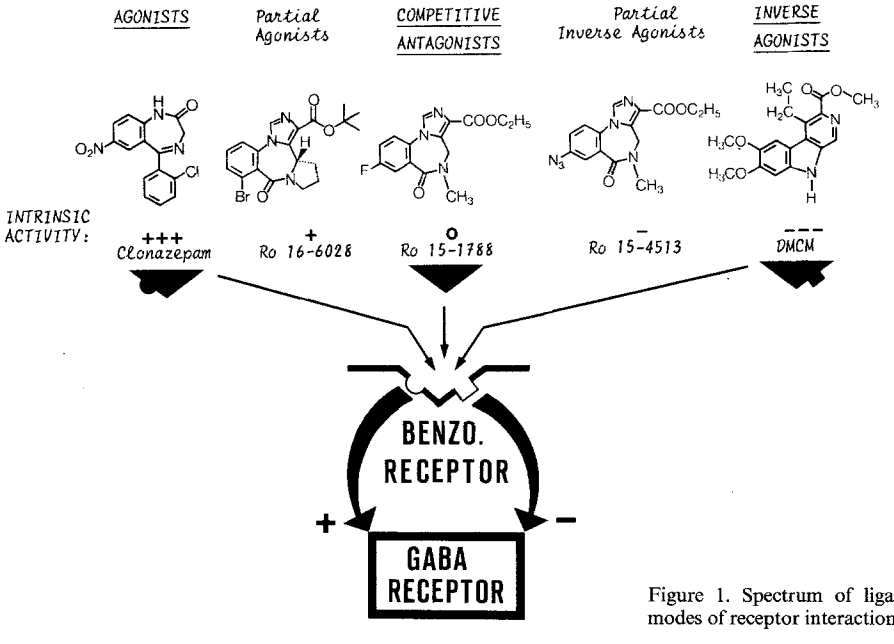


Figure 1. Spectrum of ligands with different modes of receptor interaction.

be seen whether the pharmacological profile of partial agonists (e.g. non-sedative anxiolytic), now under clinical investigation[4], can be explained by qualitative differences in the type of conformational changes induced in the receptor, their differential affinity for benzodiazepine receptor subtypes or by their fractional receptor occupancy in different brain regions with varying receptor reserves[6]. Here, we review recent research trends in mapping the $GABA_A$/benzodiazepine receptor/chloride ionophore complex in the CNS by radiohistochemistry and immunohistochemistry.

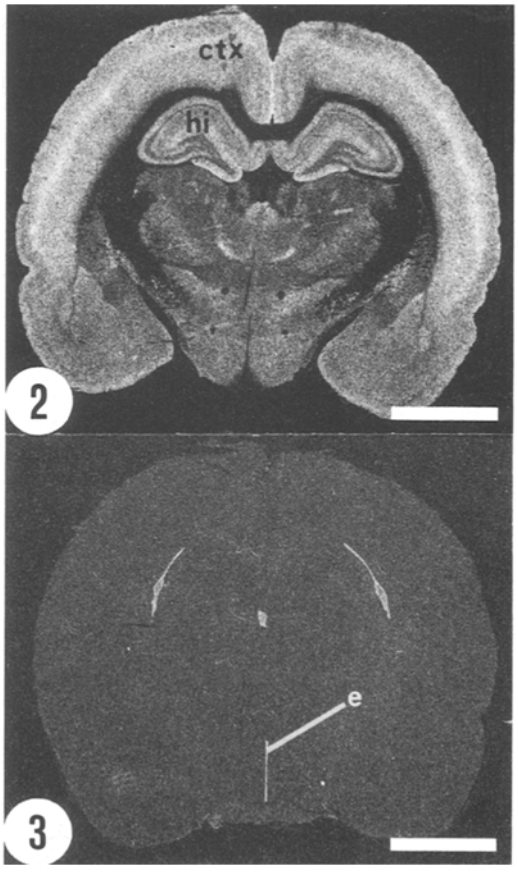
Resolving benzodiazepine receptors

Radiohistochemical approach. In recent years it has become possible not only to identify receptor proteins directly by biochemical binding methods using radioligands[42] but also to map their distribution by autoradiography[10,49]. Together with knowledge of the chemical anatomy of the CNS[9], receptor autoradiography reveals the affinity of neurotransmitters, hormones and drugs for specific regions with an *anatomical resolution* not previously possible. Autoradiographic methods for reversibly-bound ligands (such as the benzodiazepines) were pioneered by Roth and collaborators[32,43] and adapted for in vitro receptor binding by Young and Kuhar[54] and later by Herkenham and Pert[7]. The method soon became quantitative with the use of ^{3}H-sensitive sheet film (LKB Ultrofilm^R)[21] and calibrating brain paste standards[3,11,27,28,47].

In vitro. Young and Kuhar[53,55] were the first to apply their method to the study of benzodiazepine receptors in vitro[30] and this was soon followed by several other studies concerning their modulation by GABA[46], their apparent heterogeneity[56] and their ontogeny[38]. In a previous article[29], we showed that the distribution of neuronal binding sites of benzodiazepines (*receptors*) in the CNS in vitro and their pharmacological specificity were completely different from those of presumed glial binding sites (*acceptors*) with which no pharmacological effects could be associated to date. Although subsequent studies[25,52] have revealed GABA depressant effects of Ro 5-4864, a potent ligand for the high-affinity glial binding sites[34], these effects are probably due to its interaction with low affinity sites on the chloride ionophore[2,23,24,50,51]. The in vitro binding assay for receptor autoradiography has also been used to characterize the high- and low-affinity GABA sites with ^{3}H-muscimol[22] and ^{3}H-bicuculline[20] respectively, the latter correlating better with the distribution of benzodiazepine receptors. A further binding site of the oligomeric receptor complex, the chloride ionophore, was mapped with ^{35}S-t-butyl-bicyclophosphorothionate (TBPS)[48], a cage convulsant; the ionophore has been proposed to contain the binding sites for anesthetic barbiturates and some convulsants[19,44,45].

In vivo. Although radioligand binding studies in vitro offer several advantages (controlled binding conditions, metabolic stability of ligands, circumvention of the blood-brain barrier, possibility to study post-mortem human brain and the affinity of different ligands [or even antibodies] in adjacent sections), *in vivo studies* provide the only means of investigating receptor occupancy by drugs and their metabolites, after systemic administration, under physiological conditions. Demonstrating the feasibility of visualizing ligand-receptor interactions in vivo by autoradiography would be a first step. Indeed, recent investigations in our laboratory indicate that the autoradiographic distribution and density of neuronal and glial binding sites for benzodiazepines in the CNS in vivo is very similar to that observed with in vitro binding (figs 2, 3 and table). These observations demonstrate not only the validity of the in vitro binding assay but also the potential of in vivo binding for autoradiographic studies of receptor occupancy, for example, during acute and chronic drug tolerance.

In order to study the *in vivo distribution of benzodiazepines in the human brain*[13,33], one has to use ligands labeled with positron emitting isotopes e.g. ^{11}C-flunitrazepam or ^{11}C-Ro 15-1788. Such tomographic studies (albeit with a poor resolution), using a quantitative model for the in vivo assessment of drug binding studies[14], provide a non-invasive method for future clinical investigations of disorders of benzodiazepine receptor function possibly associated with diseases of the human CNS.

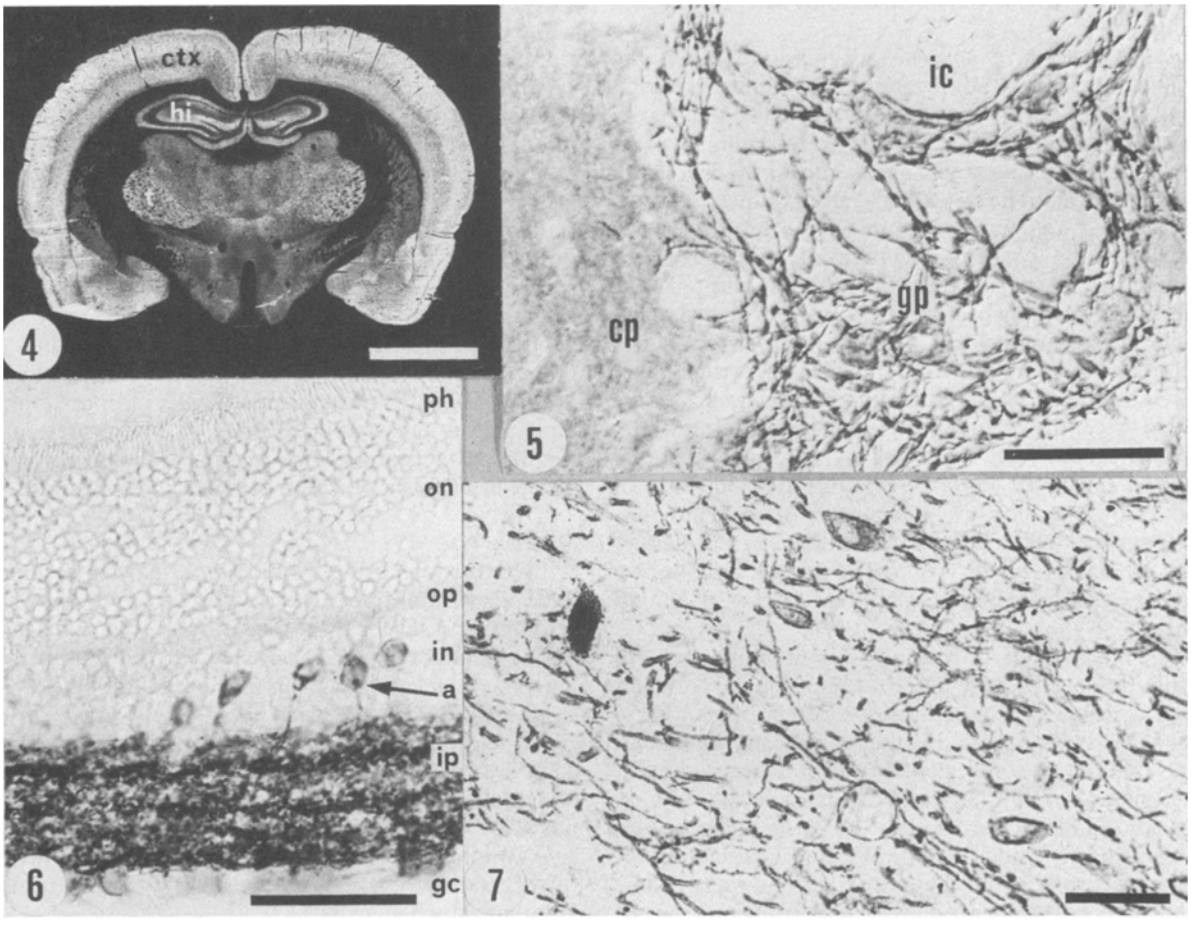


Figures 2 and 3. Radiohistochemical localization of ^{3}H-Ro 15-1788 (fig. 2) and ^{3}H-Ro 5-4864 (fig. 3) in frontal brain sections 5 min after their i.v. injection to rats (500 µCi[= 8 µg]/kg); Ro 15-1788 is a benzodiazepine receptor antagonist and Ro 5-4864 a high affinity ligand for the so-called peripheral benzodiazepine binding sites. Discretely labeled (white) regions include cerebral cortex (ctx), hippocampal formation (hi) and ependyma (e). Bars = 3 mm.

Microdensitometric measurements of regional brain concentrations, i.e. total binding (fmol/mg protein), of ^{3}H-Ro 15-1788 and ^{3}H-Ro 5-4864 administered i.v.

	^{3}H–Ro 15–1788	^{3}H–Ro 5–4864
Olf bulb (plex. layer)	262	30
Inferior colliculus	186	51
Frontal cortex (IV)	176	12
Superior colliculus	155	49
Substant. nigra ret.	126	54
Islands of Calleja	121	14
Cerebellum (mol. layer)	118	62
Ventral pallidum	115	18
Dentate gyrus	109	60
Hippocampus CA1	88	54
Globus pallidus	76	68
Olf bulb (glom. layer)	61	70
Cerebellum (gran. layer)	35	66
Olf bulb (nerve layer)	5	85
Ependyma (lat. ventricle)	2	295
Subcommissural organ	2	46
Choroid plexus	2	183
Pineal organ	1	190
Median eminence	1	55

Radiohistochemistry at high resolution. Reversibly-bound receptor ligands, such as those discussed so far, can only be localized with low resolution techniques. The first attempt to improve the resolution of the radiohistochemical approach was to use radiolabels (such as the agonists ^{3}H-flunitrazepam and ^{3}H-clonazepam) which are photoaffinity markers for the benzodiazepine receptor[15,16,39]. By this means, tissues containing the irreversibly-bound ligand could be suitably fixed for electron microscopy and subcellular studies. Benzodiazepine receptors were localized in regions of synaptic contacts some of which were GABAergic. Recently, a partial inverse agonist, Ro 15-4513, which is an azide derivative of the benzodiazepine receptor antagonist Ro 15-1788, was developed as a photoaffinity label[18]. It had the advantage that, whereas only 25% of the total number of benzodiazepine binding sites are photolabeled by tritiated 7-nitro benzodiazepines, all the sites could now be photolabeled. The regional distribution of this photolabel in the rat CNS in vitro and the photolabeled proteins were virtually identical to that of ^{3}H-flunitrazepam. Altogether, this is seen as strong evidence that the binding sites for the benzodiazepine agonist and partial inverse agonist are on the same proteins. This new, more efficient, photoaffinity label could be the ligand of choice for future radio-



Figures 4–7. Immunohistochemical localization (PAP method) of a mAb to a $GABA_A$/benzodiazepine receptor complex. The overview (fig. 4) illustrates the selective distribution of antigenic sites (white areas) in a frontal section of rat brain (compare with fig. 2). Note the discrete staining, around cells and processes, in rat brain (fig. 5), retina (fig. 6) and in the the post-mortem human substantia nigra (fig. 7). a, amacrine cell; cp, caudate-putamen; ctx, cerebral cortex; gc, ganglion cell layer; gp, globus pallidus; hi, hippocampus; ic, internal capsule; in, inner nuclear layer; ip, inner plexiform layer; on, outer nuclear layer; op, outer plexiform layer; ph, photosensitive cells; t, thalamus, Bars: fig. 4 = 3 mm; figs 5, 6, 7, = 50 μm.

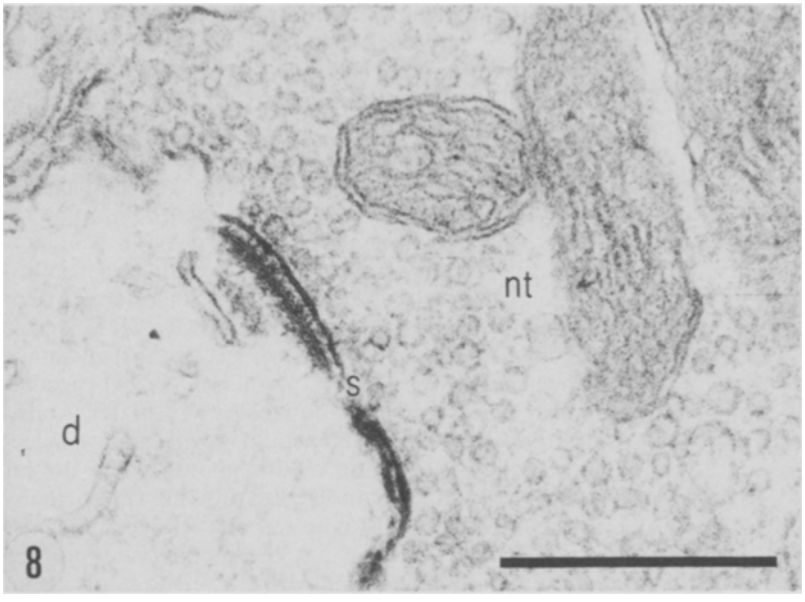


Figure 8. Immunocytochemical localization (PAP method) of a mAb to a $GABA_A$/benzodiazepine receptor complex in rat substantia nigra. The region of an axodendritic synaptic contact (s) is highly electron dense indicating the presence of receptor antigenic sites pre- and postsynaptically. d, dendrite; nt, nerve terminal. Bar = 0.5 µm.

histochemical studies of benzodiazepine receptors at high resolution.

Immunohistochemical approach. Radiation scatter and possible diffusion of radioligands clearly limit the resolution of the radiohistochemical approach such that receptors cannot be attributed to defined subcellular structures. The immunohistochemical approach overcomes these limitations. The GABA receptor complex has now been isolated and purified from bovine brain[36,40,41]. To immunize mice, a receptor preparation was used which contained high- and low-affinity binding sites for GABA in addition to the neuronal binding sites for benzodiazepines[36]. The monoclonal antibodies (mAb) immunoprecipitated a complex from membrane extracts that showed all the radioligand binding characteristics of the isolated receptor[8,35,37] i.e. the low and high affinity $GABA_A$ receptor, the neuronal high affinity benzodiazepine receptor and ^{35}S-TBPS binding sites indicative of the chloride ionophore (Schoch, unpublished). The epitopes recognized by the mAb were localized on a protein of 50 kd or 55 kd which correspond to the two known subunits of the receptor complex. The mAb were subunit specific.

Histochemically, the distribution of immunoreactivity to a β-subunit-specific mAb in the rat brain, spinal cord and retina, as well as bovine and post-mortem human brain (figs 4–7), was very similar to that of neuronal benzodiazepine binding sites revealed by receptor autoradiography (Richards et al.[31]; Schoch et al.[37]; Richards et al., in preparation). Moreover, the appearance of antigenic sites during ontogeny coincided with the appearance of radiolabeled receptors. From these observations we conclude that most, if not all, benzodiazepine receptors are part of $GABA_A$ receptor complexes. Immunoreactive, presumably GABAceptive, neuronal cell bodies and processes were observed in numerous rat, bovine and human brain regions with a detail far superior to that obtained with radiohistochemistry (figs 5–7).

Tissues lacking benzodiazepine receptors, e.g. brain white matter, pineal, pituitary gland, adrenals and superior cervical ganglia were devoid of an immune reaction.

Immunohistochemistry at high resolution. Clearly, one of the aims of these studies was to visualize not only the cellular but also the subcellular sites of benzodiazepine interaction with a resolution superior to that of radiohistochemistry. Immunocytochemical analysis of rat substantia nigra (fig. 8) revealed selective staining of pre- and postsynaptic membranes of axosomatic and axodendritic contacts. This observation would suggest that, at least in rat substantia nigra, a $GABA_A$/benzodiazepine receptor complex is present not only postsynaptically but also presynaptically[12]. Thus, benzodiazepine receptor ligands affect not only the postsynaptic GABA receptor complex but also a presynaptic GABA receptor, presumably GABA autoreceptors. This finding opens up new views on the mechanism of action of the receptor ligands.

Outlook

1. Investigations using colloidal gold-labeled secondary antibodies should provide a further improvement in the resolution for the localization of $GABA_A$/benzodiazepine receptors and a possibility of carrying out double-labeling experiments e.g. with monoclonal antibodies to the GABA-synthesizing enzyme GAD.
2. The feasibility of mapping the benzodiazepine receptor complex with monoclonal antibodies in post-mortem human brain (fig. 7) provides a new diagnostic tool to study possible receptor-associated diseases of the human CNS.
3. Anti-idiotypic monoclonal antibodies could provide further tools to characterize these drug binding proteins immunohistochemically.
4. Finally, cDNA probes for receptor-coding mRNA might become available in the near future to visualize, by in situ hybridization, those neurons expressing the $GABA_A$/benzodiazepine receptor/chloride ionophore complex.

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Full Papers

Psychophysiological reactivity in Type A and B women during a rapid information processing task

D. Pfiffner, P. Elsinger, R. Nil, R. Buzzi and K. Bättig

Behavioral Science Institute, Behavioral Biology Laboratory, Swiss Federal Institute of Technology, ETH-Zentrum CH-8092 Zürich, (Switzerland), 21 May 1985

Summary. Type A and Type B women assessed by a newly developed German questionnaire 'need for control' (NC) were compared with respect to time-pressured information processing performance and to simultaneously recorded psychophysiological reactivity. The task was computer controlled, monetarily reinforced and subject paced. The physiological measurements included the cardiovascular parameters, ECG and finger plethysmographic amplitudes and the noncardiovascular parameters, EMG (frontal muscle), skin conductance reactivity, and respiration. NC-Type A and Type B women did not differ in performance, but the Type As showed stronger vasoconstrictive responses to the task than did the Type Bs. Other physiological intergroup differences were not seen. In addition, the Type As scored significantly higher in nervousness and irritability and marginally higher in depression, reactive aggressivity and neuroticism than did the Type Bs. This particular pattern of NC-Type A/B differences is discussed with regard to relevant differences observed by other studies between SI and JAS Type As and Bs.
Key words. Type A; Type A questionnaire; 'need for control' (NC); rapid information processing; performance; psychophysiological reactivity; personality.

A variety of measurements and procedures have been used to assess patterns of the coronary prone Type A behavior (TABP), which is mostly characterized by aggressivity, achievement striving, time urgency and impatience.

Although different types of TABP measurements appear to be intrinsically reliable and reproducible, they assess different behavioral aspects of the TABP syndrome[24]. Particularly the profile of TABP as defined by the Rosenman Structured Interview differs from that defined by the JAS questionnaire. The JAS has been directed mainly at assessing the competitive striving of Type A persons. So it is not surprising that JAS-A individuals usually report higher educational levels and also that they mostly attain a higher occupational status than JAS-B individuals.

Furthermore, several studies examining laboratory performance on different tests reported higher performance levels in JAS-As than in JAS-Bs, and this was particularly when the tests were difficult or called for persistence and endurance[6]. In contrast to performance, psychophysiological reactivity does not seem to differ between JAS-Type As and JAS-Type Bs.