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ALPHA$_2$-ADRENOCEPTORS IN HUMAN LYMPHOCYTES: DIRECT CHARACTERISATION BY [^{3}H]YOHIMBINE BINDING

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SUMMARY: [^{3}H]yohimbine, a potent and selective alpha$_2$-adrenergic antagonist was used to label alpha-adrenoceptors in intact human lymphocytes. Binding of [^{3}H]yohimbine was rapid ($t_{1/2}$ 1.5 -2.0 min) and readily reversed by 10uM phentolamine ($t_{1/2}$ = 5 - 6 min) and of high affinity (K_d = 3.7 + 0.86nM). At saturation, the total number of binding sites was 19.9 + 5.3 fmol/10^7 lymphocytes. Adrenergic agonists competed for [^{3}H]yohimbine binding sites with an order of potency: clonidine > (-) epinephrine > (-) norepinephrine > (+) epinephrine >> (-) isoproterenol; adrenergic antagonists with a potency order of yohimbine > phentolamine > prazosin. These results indicate the presence of alpha$_2$-adrenoceptors in human lymphocytes.

INTRODUCTION: Radioligands have been successfully used to label alpha-adrenoceptors in different tissues (1-5). In the last few years it became clear that there are two subtypes of alpha receptors, termed alpha$_1$ and alpha$_2$ receptors (6). Alpha$_1$ receptors include typical postsynaptic alpha receptors mediating smooth muscle contraction. alpha$_2$ receptors include not only all known presynaptic autoregulatory alpha receptors but also some less typical postsynaptic receptors existing on, for example, human platelets. Using radioligand binding techniques, two different approaches have been applied to determine the presence of alpha$_1$ and alpha$_2$ receptors. The first involves the use of a non-selective radioligand such as [^{3}H] dihydroergocryptine ([^{3}H] DHE) to label the entire alpha receptor population. The alpha receptor subtypes are distinguished by constructing competition curves using antagonist compounds which have selectively greater potency for one or the other alpha receptor subtype (7-11), for example, the antagonists prazosin (alpha$_1$ selective) and yohimbine (alpha$_2$ selective). The second, the most useful and widely applied criterion for distinguishing between alpha receptor subtypes, is by using radioligands which themselves selectively label either alpha$_1$ or alpha$_2$ receptors (12-21). [^{3}H]prazosin is selective for alpha$_1$ receptors whereas [^{3}H]yohimbine is selective for alpha$_2$ receptors. Previous studies using [^{3}H] DHE were able to identify alpha-adrenoceptors in human lymphocytes (22), but the subtype was not defined.

The purpose of this study was to characterize the alpha adrenoceptor subtypes in intact human lymphocytes.

MATERIALS AND METHODS: [^{3}H]yohimbine (84.5 Ci/mmol) was obtained from New England Nuclear, West Germany. The following drugs were kindly donated by the sources indicated: prazosin hydrochloride (Pfizer, UK); (-) epinephrine

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BP (Smith and Nephew, UK); and (+) epinephrine bitartrate (Kodak, UK). Other drugs and chemicals were obtained from commercial sources. Lymphocytes were isolated as described elsewhere (23).

Alpha$_2$-Adrenoceptor Assay - The saturation assays were done in polypropylene tubes (Sarsted, 12 x 75mm) in a final volume of 500 ul, comprising for the total binding: lymphocyte suspension (250-350 ug protein) and [^{3}H]yohimbine at appropriate concentrations (1-20 nM) in Buffer A (Earle's Balanced Salt Solution minus sodium bicarbonate and supplemented with 0.1% calf serum and 20 mM Hepes at pH 7.6). In tubes used to determine non-specific binding, 10 uM phentolamine was also added. Total and non-specific binding were estimated in duplicate at each [^{3}H]yohimbine concentration. The tubes were incubated at 25C for 25 minutes. The reaction was stopped by the addition of 2.5 ml Buffer B (50 mM Tris and 0.9% sodium chloride, pH 7.6) at 25C. The samples were then filtered through Whatman GFC fibre-glass filters (pre-wetted in Buffer B) by applying a vacuum of 3-4 psi, and the filters were rinsed with 2 x 10 ml of Buffer B at 25C. Filtering and rinsing were complete within 12 s. The filters were air dried, shaken with 10 ml scintillator 299 (Packard) and the radioactivity determined (50% efficiency).

Data Analysis - All experiments were performed in duplicate and replicated at least twice. The number (B_{max}) and sensitivity (K_d) of the lymphocyte alpha$_2$-adrenoceptors were determined from Scatchard analysis (24). The dissociation constant (K_d) of various competing drugs for binding sites was calculated from the equation of Cheng and Prusoff (25).
All data shown are the mean $\pm$ standard deviation where indicated.

RESULTS:

Kinetic Analysis of Binding - Binding of [^{3}H]yohimbine to human lymphocytes at 25C was rapid, with half - maximal specific binding ($t_{1/2}$) of 1.5-2.0 min (Fig.1), reaching equilibrium within 16-20 min. Binding remained stable for

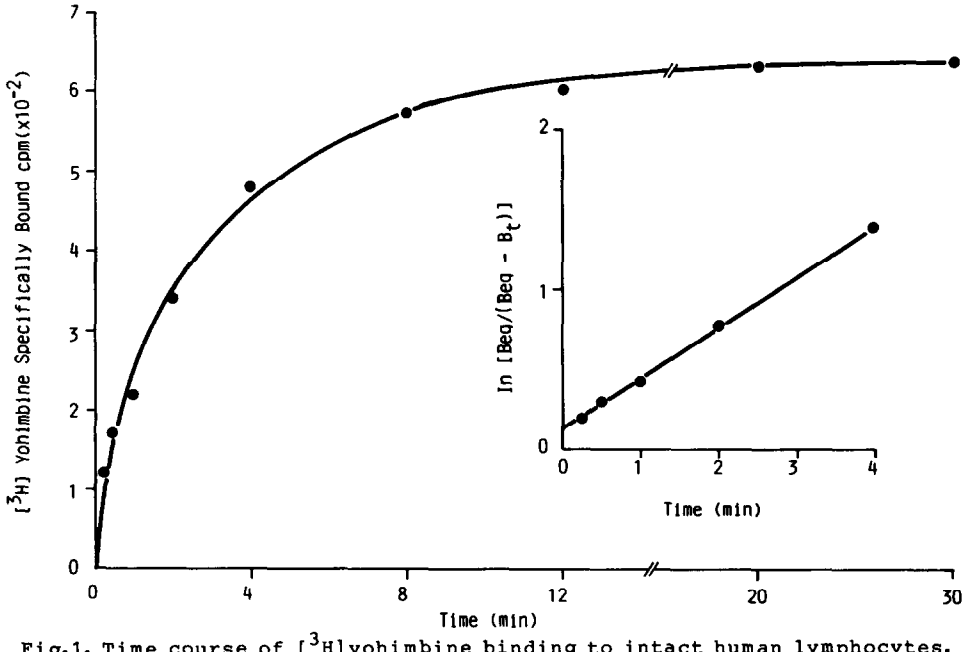

Fig.1. Time course of [^{3}H]yohimbine binding to intact human lymphocytes. Lymphocytes in buffer A were incubated with [^{3}H]yohimbine (5nM) in the absence and presence of 10uM phentolamine. At the indicated times, the samples were diluted with buffer B and filtered. Specific binding was determined in duplicate at each time interval. (Inset) the regression line (r=0.99) was determined by the plot of ln [$B_{eq}/(B_{eq}-B_t)$] vs. time, where B_{eq} = binding at equilibrium and B_t = binding at time t. K_{ob} is equal to the slope of the line.

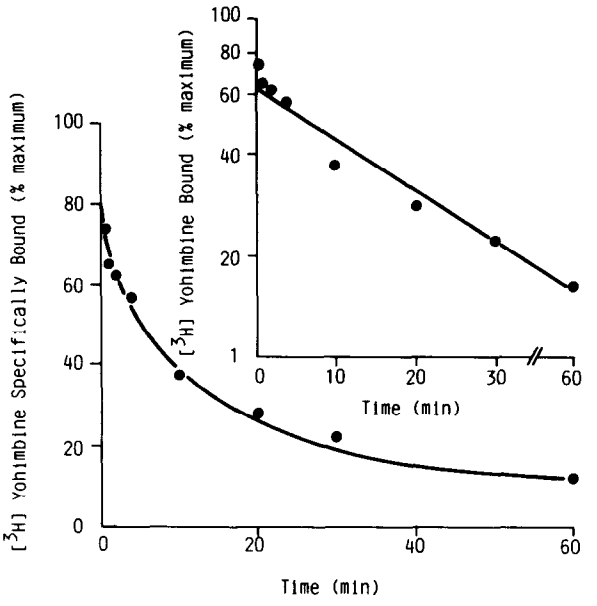

Fig.2. Time course for dissociation of [^{3}H]yohimbine binding to intact human lymphocytes. Lymphocytes in buffer A were incubated with [^{3}H]yohimbine (5nM) for 25 min to allow equilibrium. At zero time, 10uM phentolamine was added to the samples and specific binding was determined at various time intervals. 100% binding refers to the specific binding just prior to the addition of phentolamine at zero time. (Inset) first-order rate plot of dissociation of [^{3}H]yohimbine binding. K_2 is equal to the slope of the line.

30 min at 25C. An incubation time of 25 min was chosen to represent equilibrium binding in subsequent experiments.

The initial rate constant (K_{ob}) for the association reaction obtained from the slope (Fig.1,inset) was 0.32 min^{-1} for 5nM [^{3}H]yohimbine. The dissociation of bound [^{3}H]yohimbine at 25C was determined by incubating lymphocytes to equilibrium and then adding 10uM phentolamine at time zero and measuring residual specific binding at subsequent time intervals (Fig.2). Dissociation was rapid with a $t_{1/2}$ of 5.0-6.0 min (Fig.2) and the first - order dissociation rate constant (K_2) was 0.138 min^{-1} (Fig.2, inset).

The second - order association rate constant (K_1) was then calculated from the equation (26): $K_1 = (K_{ob} - K_2)/[yohimbine]$, where [yohimbine] is equal to the concentration of [^{3}H]yohimbine in the assay (5nM), K_1 was 0.364 x 10^8 M^{-1} min^{-1}. The equilibrium dissociation constant (K_d), determined from the ratio of K_2/K_1, was 3.79nM.

Saturability of Binding - The binding characteristics of [^{3}H]yohimbine to intact human lymphocytes is described in Fig.3. Specific binding of [^{3}H]yoimbine (the binding that could be displaced by 10uM phentolamine) was clearly saturable and of high affinity. Apparent saturation of binding sites seemed to occur at [^{3}H] yohimbine concentration of 14-16nM, with half maximal binding occuring at about 2-3nM. Scatchard analysis (Fig.3, inset) of the saturation data yielded a straight line, indicating that

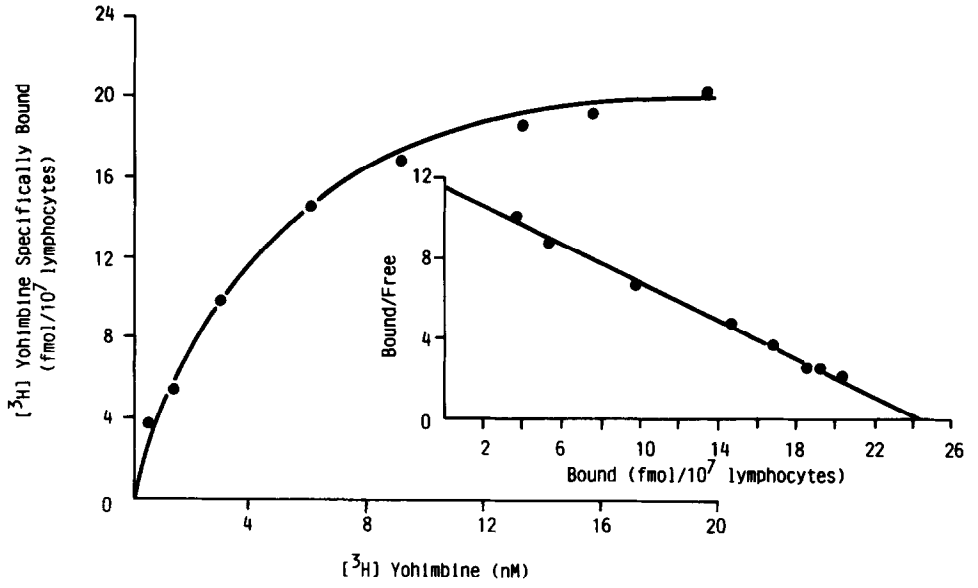

Fig 3. Specific binding of [^{3}H]yohimbine to intact human lymphocytes as a function of concentration of [^{3}H]yohimbine. Lymphocytes in buffer A were incubated with various concentrations of [^{3}H]yohimbine in the presence and absence of 10uM phentolamine. Specific binding was determined at each concentration of [^{3}H]yohimbine. (Inset) Scatchard analysis of the binding data yielding a K_d of 4.24nM and the total number of receptors was 24.8 fmol/10^7 lymphocytes.

[^{3}H]yohimbine bound to a single class of receptors. The dissociation constant (K_d) of binding was 3.7 ± 0.86nM (8 experiments) and the total number of binding sites was 19.9 ± 5.3 fmol/10^7 lymphocytes.

Inhibition of [^{3}H]Yohimbine Binding by Competing Ligands - Binding of [^{3}H]yohimbine was stereospecifically inhibited by epinephrine (Table 1); the (-) isomer was more potent than the (+) stereoisomer. Adrenergic agonists competed for [^{3}H]yohimbine binding sites with a potency order of clonidine >

Table 1

The dissociation constants (K_d) of drugs determined by competition for [^{3}H]yohimbine binding

Drug	Dissociation Constant (K_d,uM)
Clonidine	0.28 ± 0.027
(-)Epinephrine	1.51 ± 0.15
(+)Epinephrine	16.20 ± 1.3
(-)Norepinephrine	6.74 ± 0.65
(-)Isoproterenol	126.00 ± 12.0
Yohimbine	0.01 ± 0.0011
Phentolamine	0.03 ± 0.0028
Prazosin	2.10 ± 0.2

The dissociation constant (K_d) was calculated according to the equation of Cheng and Prusoff (25). Results are expressed as the Mean ± SD (4 expts).

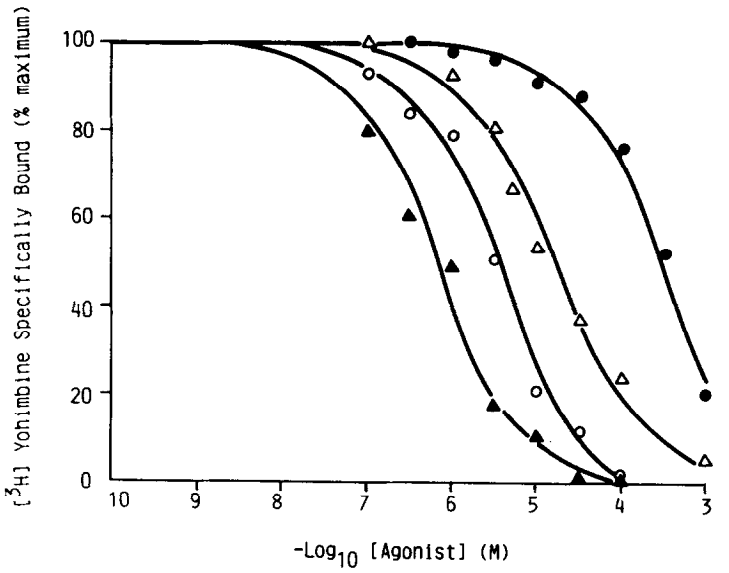

Fig.4. Competition for [^{3}H]yohimbine binding sites by adrenergic agonists in intact human lymphocytes. Various concentrations of clonidine (▲), (-) epinephrine (○), (-)norepinephrine (△) and (-)isoproterenol (●) were incubated with lymphocytes and [^{3}H]yohimbine (5nM) for 25 min. Then, the samples were diluted and filtered. Specific binding was determined in duplicate at each drug concentration.

(-) epinephrine > (-) norepinephrine > (+) epinephrine >> (-) isoproterenol, with K_d values of 0.28, 1.50, 6.74, and 16.00 uM for clonidine, (-) epinephrine, (-) norepinephrine and (+) epinephrine respectively (Fig.4 and Table 1). The beta-adrenergic agonist, (-) isoproterenol, was a very weak inhibitor of [^{3}H]yohimbine binding (K_d = 126.00 uM, Fig.4 and Table 1).

The alpha-adrenergic antagonists phentolamine, yohimbine and prazosin competed for [^{3}H]yohimbine binding sites with a potency order of yohimbine > phentolamine > prazosin (Fig.5 and Table 1). Yohimbine, an $alpha_2$ adrenergic antagonist competed for the binding sites with a K_d of 0.01 uM. Phentolamine, a non-selective alpha-adrenergic antagonist competed with a K_d of 0.03 uM. Prazosin, a specific $alpha_1$-adrenergic antagonist, competed with a K_d of 2.00 uM.

DISCUSSION: Earlier studies by us (23) have indicated the presence of a circadian variation in $beta_2$-adrenoceptor number and affinity in lymphocytes of both normals and asthmatic patients. To continue these studies it was essential to investigate if there was a similar variation in $alpha_2/beta_2$ receptor ratios in one or other (or both) of the groups as other workers have described differences between the alpha/ beta ratios in normals and asthmatic patients (22) but have not defined the receptor subtypes. These workers used [^{3}H] DHE to look at the general alpha receptor population. Perhaps as a result of the low specific activity of commercially available preparations of [^{3}H]DHE we were unable to reproduce the method satisfactorily, and so developed the method described here using the much higher activity ligand [^{3}H]yohimbine.

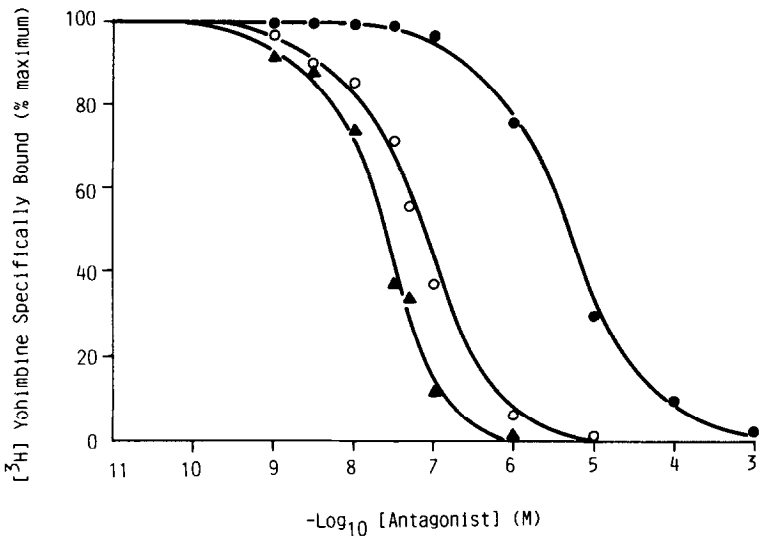

Fig.5. Competition for [^{3}H]yohimbine binding sites by adrenergic antagonists in intact human lymphocytes. Various concentrations of yohimbine (▲), phentolamine (○) and prazosin (●) were incubated with lymphocytes and [^{3}H]yohimbine (5nM) for 25 min. Then, the samples were diluted and filtered. Specific binding was determined in duplicate at each drug concentration.

[^{3}H]yohimbine has been used to label alpha$_2$-adrenoceptors in a variety of tissues (17-21). Other workers (15,16) using [^{3}H]yohimbine to label alpha$_2$ adrenoceptors in human platelets have shown that this radioligand appears to have a number of advantages as compared to other radioligands, including lower non-specific binding, higher specific activity and fewer cells required. Accordingly, we have used [^{3}H]yohimbine in our present study to demonstrate the existence of binding sites for this ligand on intact human lymphocytes. Our results indicate that [^{3}H]yohimbine binding was rapid and reversible. Specific binding was high (60-80%) and saturable with high affinity.

The dissociation constant of [^{3}H]yohimbine of 3.7 $\pm$ 0.86 nM established at equilibrium was very similar to that of 3.31 $\pm$ 0.49 nM determined by kinetic data (i.e., ratio of association and dissociation rate constants). Similar dissociation constants have been observed for human platelets (15) and for human adipocyte membranes (18).

The stereospecificity of the receptor was shown by (-) epinephrine displacing [^{3}H]yohimbine more potently than (+) epinephrine. Binding of [^{3}H]yohimbine was inhibited by adrenergic agonists with the potency order of clonidine > (-) epinephrine > (-) norepinephrine >> (-) isoproterenol and by adrenergic antagonists with a potency series of yohimbine > phentolamine > prazosin. The dissociation constants for these adrenergic drugs (Table 1) are in accord with data reported by Motulsky et al (15) for human platelets.

Therefore these observations indicate the presence of putative alpha$_2$-adrenoceptors on human lymphocytes.

Finally to our knowledge, this is the first report to characterize alpha$_2$-adrenoceptors in intact human lymphocytes using the radioligand [^{3}H]yohimbine.

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REFERENCES:

1. Newman, K.D., Williams, L.T., Bishopric, N.H. and Lefkowitz, R.J. (1978) J. Clin. Invest. 61, 395-402.
2. Williams, R.S. and Lekowitz, R.J. (1978) Circ. Res. 43,721-727.
3. Begin-Heick, N., Houlihan, P.A. and Sigouin, J. (1979) Can. J. Biochem. 57, 1047-1049.
4. Holck, M.I., Marks, B.H. and Wilberding, C.A. (1979) Mol. Pharmacol. 16, 77-90.
5. Carman-Krzan, M. (1980) Arch. int. Pharmacodyn. 246, 237-250.
6. Berthelsen, S. and Pettinger, W.A. (1977) Life Sci. 21, 595-606.
7. Miach, P.J., Dausse, J-P. and Meyer, P. (1978) Nature 274, 492-494.
8. Hoffman, B.B., DeLean, A., Wood, C.L., Schocken, D.D. and Lefkowitz, R.J. (1979) Life Sci. 24, 1739-1746.
9. Hoffman, B.B. and Lefkowitz, R.J. (1980) Biochem. Pharmacol. 29, 452-454.
10. Hoffman, B.B., Mullikin-Kilpatrick, D. and Lefkowitz, R.J. (1980). J. Biol. Chem. 255, 4645-4652.
11. Hoffman, B.B., Michel, T., Mullikin-Kilpatrick, D., Lefkowitz, R.J., Tolbert, M.E.M., Gilman, H. and Fain, J.N. (1980) Proc. Natl. Acad. Sci. USA 77, 4569-4573.
12. Barnes, P., Karliner, J., Hamilton, C.A. and Dollery, C. (1979) Life Sci. 25, 1207-1214.
13. Greengrass, P. and Bremner, R. (1979) Eur. J. Pharmacol. 55, 323-326.
14. Karliner, J.S., Barnes, P., Hamilton, C.A. and Dollery, C.T. (1979) Biochem. Biophys. Res. Commun. 90, 142-149.
15. Motulsky, H.J., Shattil, S.J. and Insel, P.A. (1980) Biochem. Biophys. Res. Commun. 97, 1562-1570.
16. Daiguji, M., Meltzer, H.Y. and U'Prichard, D.C. (1981) Life Sci. 28, 2705-2717.
17. Hoffman, B.B., Dukes, D.F. and Lefkowitz, R.J. (1981) Life Sci. 28, 265-272.
18. Tharp, M.D., Hoffman, B.B. and Lefkowitz, R.J. (1981) J. Clin. Endocrinol. Metab. 52, 709-714.
19. Mukherjee, A. (1981) Biochim. Biophys. Acta 676, 148-154.
20. Motulsky, H.J. and Insel, P.A. (1982) Biochem. Pharmacol. 31, 2591-2597.
21. Engfeldt, P., Arner, P., Kimura, H., Wahrenberg, H. and Ostman, J. (1983) Scand. J. Clin. Lab. Invest. 43, 207-213.
22. Sano, Y., Krokos, K. and Townley, R. (1981) J. Allergy Clin. Immunol. 67, 59 (Abstract).
23. Titinchi, S., Al Shamma, M., Patel, K.R., Kerr, J.W. and Clark, B. (1984) Clin. Sci. 66, 323-328.
24. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660-672.
25. Cheng, Y. and Prusoff, W.H. (1973) Biochem. Pharmacol. 22, 3099-3108.
26. Bennett, J.P., Jr. (1978) in Neurotransmitter Receptor Binding (Yamamura, H.I., Enna, S.J. and Kuhar, M.J., eds.), pp. 57-90, Raven Press, New York.