Biochimica et Biophysica Acta, 770 (1984) 22–28
Elsevier

BBA 71988

IMIPRAMINE BINDING SITE

TEMPERATURE DEPENDENCE OF THE BINDING OF ^{3}H-LABELED IMIPRAMINE AND ^{3}H-LABELED PAROXETINE TO HUMAN PLATELET MEMBRANE

P. PLENGE and E.T. MELLERUP

Psychochemistry Institute, Rigshospitalet, 9, Blegdamsvej, DK-2100 Copenhagen Ø (Denmark)

(Received September 15th, 1983)

Key words: Imipramine; Paroxetine; Drug receptor; Temperature dependence; (Platelet membrane)

The characteristics of ^{3}H-labeled imipramine and ^{3}H-labeled paroxetine binding to human platelet membranes were determined at various temperatures between 0 and 37°C. Both paroxetine and imipramine probably bind to the same molecular complex in the platelet membrane, but the binding characteristics are different for the two molecules. The dissociation constant (K_d) for imipramine increases from 0.3 nM to 7.0 nM with increasing incubation temperature in a continuous way, whereas K_d for paroxetine is almost constant, about 0.05 nM, between 0 and 19°C, and first begins to increase from 0.06 nM to 0.16 nM between 20 and 37°C. This suggests that the binding of paroxetine to the binding site induces a conformational change in the molecular complex of the binding site, whereas the binding of imipramine takes place without conformational changes in the binding site.

Introduction

Imipramine binding sites have been described by several investigators in mammalian brain and in blood platelets [1–3]. The binding site is located at, but probably not identical with the serotonin reuptake site [4]. Recently data have been presented [5], which suggest that the serotonin reuptake inhibitor paroxetine® binds reversibly, but with higher affinity to the binding site for imipramine.

Several receptors show decreasing affinity for their ligand with increasing temperature, most notably the benzodiazepine receptor [6,7]. Consequently, the assays for benzodiazepine receptors are normally performed at 0°C, and this incubation temperature has become practice for many other receptors, and also for the imipramine binding site.

We have investigated the effect of incubation temperature on the binding of ^{3}H-labeled imipramine and ^{3}H-labeled paroxetine to human platelet membranes, and report here that K_d for this binding increases with temperature. The temperature dependence is not the same for imipramine and paroxetine, which may indicate that these two drugs may either bind to different places at the same protein complex, or to the same binding site in two different conformations.

Methods

Platelet membranes. Platelets were obtained from 500 ml blood, sodium citrate was used as anticoagulant. Red blood cells and leucocytes were removed by low-speed centrifugation (200 × *g*, 10 min, room temperature). All the following steps were performed at 0–4°C. Platelets were precipitated from the platelet rich plasma by centrifugation at 30000 × *g*, 10 min. The platelets were washed twice with 8 ml buffer (5 mM Tris-HCl/5 mM EDTA, pH 7.5), and frozen as a dry pellet at

– 80°C. Lysis and homogenization of the platelets took place in 8 ml buffer (5 mM Tris-HCl/5 mM EDTA, pH 7.5) using an Ultra-turrax homogenizer (5 s, high speed). Platelet membranes were washed twice with 8 ml buffer (70 mM Tris-HCl, pH 7.5) (centrifugation 30000 × g, 10 min) and finally resuspended in buffer (50 mM Tris-HCl/120 mM NaCl/5 mM KCl, pH 7.5) to a concentration between 0.5 and 1.0 mg protein/ml. The samples were kept at – 80°C until analysis.

Isotopes. ^{3}H-Labeled imipramine, 23 Ci/mmol (Amersham International). ^{3}H-labeled paroxetine, 15 Ci/mmol were prepared by catalytic halogen-tritium replacement of 6'-bromoparoxetine (Amersham International). The crude ^{3}H-labeled paroxetine solution was purified at Ferrosan by HPLC using a partisil PXS 10/25 ODS, Whatman, column. The eluent used was 40% CH_3CN/60% (0.4% $(C_2H_5)_3N$-H_3PO_4 buffer, 50 mM, pH 3.70). The specific activity was determined by quantification of the HPLC-chromatogram by ultraviolet light absorption at 226 nm and counting of a purified sample from the HPLC using a Packard 3330 liquid scintillation counter at 39% efficiency. ^{3}H-Labeled paroxetine was isographic with authentic paroxetine by TLC on silica plates eluted in $CHCl_3$/$(C_2H_5)_3N$ (20 : 1, v/v), R_F 0.11. ^{3}H-Labeled paroxetine solution was diluted 100–3000-times before incubation.

Binding assay. For saturation studies, 100 µl platelet membrane suspension (50–100 µg protein) was incubated at various temperatures from 0 to 37°C in a total volume of 900 µl (50 mM Tris-HCl/120 mM NaCl/5 mM KCl/0.5% ethanol, pH 7.5) containing ^{3}H-labeled paroxetine (0.05–2 nM) or ^{3}H-labeled imipramine (0.1–10 nM); The concentration range used in the individual assays depended on the incubation temperature. Non-specific binding was determined by addition of paroxetine or desimipramine to a concentration of 10 µM. After incubation for 1–4 h (37–0°C), 5 ml ice-cold buffer was added, and the samples were rapidly filtered through Whatman CF/F glass fiber filter, and washed four times with 5 ml ice-cold buffer. The allowable separation time in this kind of binding studies depends on the affinity (K_d). A low K_d permits a long separation time. When K_d is above 10 nM the total allowable separation time is less than 10 s, which is insufficient [21]. The filters were dried overnight at room temperature, and counted in 3 ml Pico Fluor 15® in a Packard 460 CD liquid-scintillation counter. Protein concentration was determined by a modification of the Lowry method [8]. Specific binding was calculated as the difference between the binding without and with unlabeled ligand. For both ^{3}H-labeled paroxetine and ^{3}H-labeled imipramine specific binding was more than 90% of the total binding.

For the studies of association and dissociation rates of ^{3}H-labeled imipramine or ^{3}H-labeled paroxetine to the thrombocyte membrane binding site, a batch procedure was used. Thrombocyte membrane in one tube, and buffer (50 mM Tris-HCL/120 mM NaCl/5 mM KCl/0.5% ethanol, pH 7.5) with either ^{3}H-labeled paroxetine (0.2 nM) or ^{3}H-labeled imipramine (0.5 nM) in another tube, were temperature equilibrated. At time zero the membranes were mixed with one of the isotopes, and 900 µl samples in duplicate were removed at specified time intervals. After equilibrium was reached paroxetine or desimipramine (10 µM final concentration) was added to the mixture and again 900 µl samples in duplicate were removed at specified time intervals. The 900 µl samples were filtered and counted as described above.

Results

Fig. 1 shows the binding of [^{3}H]imipramine to human platelet membranes at 0, 22 and 37°C, and the subsequent displacement of the binding with a

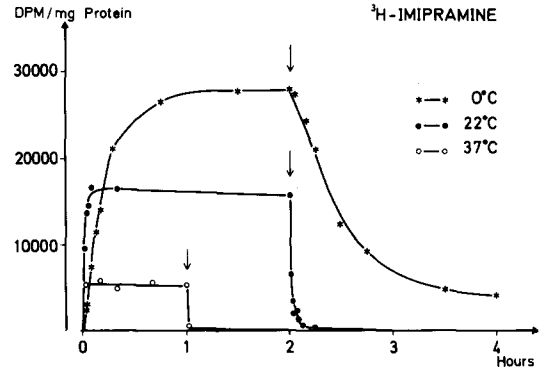

Fig. 1. Binding of 0.5 nM ^{3}H-labeled imipramine to human platelet membranes at 0, 22 and 37°C, and the subsequent displacement with non-radioactive imipramine (arrow, 10 µM).

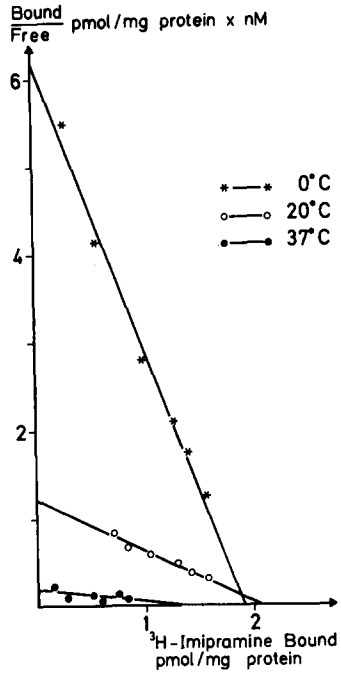

Fig. 2. Scatchard analysis of ^{3}H-labeled imipramine binding to human platelet membrane at 0, 20 and 37°C.

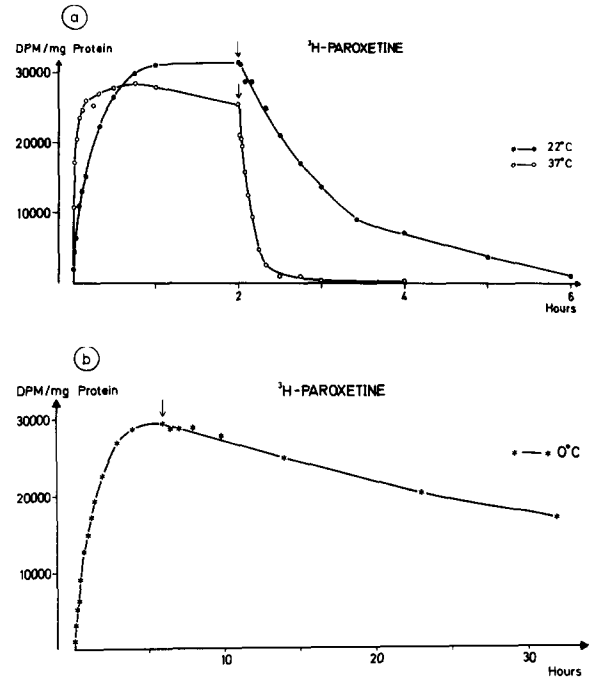

Fig. 3. Binding of 0.2 nM ^{3}H-labeled paroxetine to human platelet membranes at 22 and 37°C (a) and at 0°C (b) (note the different time scales on the x-axis), and the subsequent displacement with non-radioactive paroxetine (arrow, 10 μM).

surplus of desmethylimipramine. Corresponding K_d values were determined by Scatchard analysis of saturation isoterms at 0, 20 and 37°C as seen in Fig. 2. K_d is seen to increase with increasing temperature, whereas B_{max} was unaffected by the incubation temperature, Both Fig. 1 and Fig. 2 indicate that determination of imipramine binding at 37°C is difficult to perform with filtration methods, because the allowable separation time becomes too short [21] due to the relatively high K_d at this temperature.

In Figs. 3 a and b binding curves for [^{4}H]paroxetine to human platelet membranes at 0,

TABLE I

RATES AND RATE CONSTANTS OF ^{3}H-LABELED PAROXETINE BINDING TO HUMAN PLATELET MEMBRANES AT DIFFERENT TEMPERATURE

Assays of ^{3}H-labeled paroxetine binding were performed as described in Methods. Thrombocyte membrane protein concentration was 0.72 mg/ml incubation mixture. The paroxetine (and imipramine) receptor concentration was 0.18 nM. The ^{3}H-labeled paroxetine concentration was 0.21 nM. Calculation of k_{+1} and k_{-1} was performed as described in Ref. 21. The calculated K_d values presented, are the ratios of k_{-1} and k_1 (k_{diss}/k_{ass}).

Incubation temperature (°C)	Association $t_{1/2}$ (min)	Dissociation $t_{1/2}$ (min)	Association k_{+1} ($M^{-1} \cdot s^{-1}$)	Dissociation k_{-1} (s^{-1})	K_d (nm)	
					'Rate constants' (k_{-1}/k_{+1})	'Scatchard analysis'
0	55	1920	$0.40 \cdot 10^6$	$6.0 \cdot 10^{-6}$	0.015	0.045
22	11	55	$2.82 \cdot 10^6$	$0.21 \cdot 10^{-3}$	0.075	0.063
37	1.5	7	$11.0 \cdot 10^6$	$1.63 \cdot 10^{-3}$	0.15	0.16

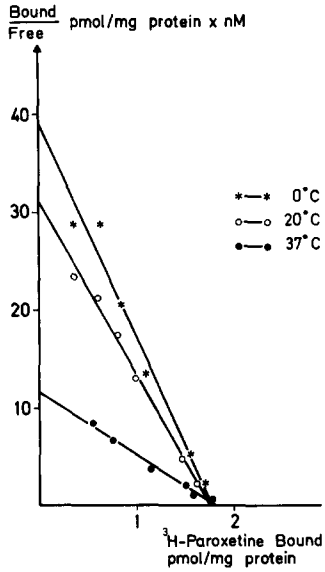

Fig. 4. Scatchard analysis of ^{3}H-labeled paroxetine binding to human platelet membranes at 0, 20 and 37°C.

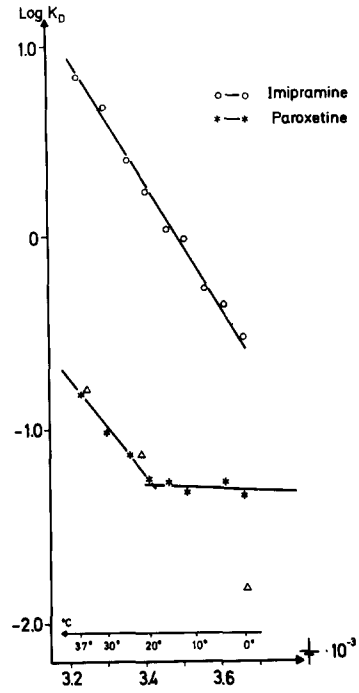

Fig. 5. Van't Hoff plot of K_d of ^{3}H-labeled imipramine (O——O) and ^{3}H-labeled paroxetine (*——*) binding to human platelet membranes, determined by Scatchard analysis, as a function of temperature. Inserted are also three K_d values for ^{3}H-labeled paroxetine binding (Δ), calculated from binding and displacement curves (Figs. 3a, 3b and Table I) for ^{3}H-labeled paroxetine at 0, 22 and 37°C.

22 and 37°C are shown. The figures also show the displacement of the binding with a surplus of non-radioactive paroxetine. From this binding and the displacement curves the rate constants of association (k_{+1}) and dissociation (k_{-1}) were calculated (Table I) and used to determine the dissociation constant (K_d) at the three different temperatures. Both k_{-1} and k_{+1} increased with increasing temperature, however, k_{-1} increased at a more rapid rate than k_{+1} causing K_d to increase.

The K_d values for [^{3}H]paroxetine were also determined by Scatchard analysis of saturation isoterms at 0, 20 and 37°C which is illustrated in Fig. 4, K_d is seen to increase with increasing temperature, whereas B_{max} was unaffected by the incubation temperature.

Saturation analysis were performed at several temperatures between 0 and 37°C for both [^{3}H]imipramine and [^{3}H]paroxetine. Fig. 5 shows log K_d versus the reciprocal of temperature in degrees Kelvin (Van't Hoff plot) for both drugs. For [^{3}H]imipramine the observed values fell on a single straight line, whereas the curve for [^{3}H]paroxetine had a completely different course. From 0 to about 19°C, K_d for paroxetine binding was almost unchanged, leading to a nearly horizontal curve. At 19°C this changed, and K_d now increased with increasing temperature in a linear way. The changes in enthalpy and entropy were calculated for the binding of ^{3}H-labeled imipramine and ^{3}H-labeled paroxetine to the receptor [9]. For imipramine $\Delta H°$ was 15 500 cal/mol and ΔS was -10 cal/mol. For paroxetine $\Delta H°$ was 370 cal/mol and ΔS was $+45$ cal/mol from 0 to 19°C. From 19 to 37°C $\Delta H°$ was 11 300 cal/mol and ΔS was $+6$ cal/mol.

Discussion

^{3}H-Labeled imipramine binding was initially suggested to be identical with, or at least closely related to the serotonin reuptake site, both in brain and in platelet membranes [10,11]. This, however, was questioned by Ahtee et al. [4], who in platelets from patients suffering from alcoholic cirrhosis found reduced serotonin uptake, al-

though the ^{3}H-labeled imipramine binding in the platelets from the same patients did not differ from those of the healthy control group. The close correlation was also questioned by Raisman et al. [12], who in platelets from a group of depressed patients found both decreased ^{3}H-labeled imipramine binding and decreased serotonin uptake, but no correlation between the two parameters in the individual patients.

Recently, Wennogle and Meyerson [13] have shown that serotonin modulates the dissociation of ^{3}H-labeled imipramine from platelet membranes. Addition of serotonin together with fluoxetine increased the half-time of the dissociation of the ^{3}H-labeled imipramine receptor complex from 62 to 170 min at 0°C; other biogenic amines were without effect. This result probably indicates that serotonin increases the receptor affinity for ^{3}H-labeled imipramine and is thus similar to the effect γ-aminobutyric acid has on the benzodiazepine receptor affinity for benzodiazepines [14]. Sette et al. [15] have presented results from rat brain studies indicating that also in brain the imipramine-serotonin receptor binding is more complex than originally thought. These authors find that serotonin and non-tricyclic serotonin reuptake inhibitors like citalopram, fluoxetine and paroxetine ($\beta+$), displaces ^{3}H-labeled imipramine in another way than do tricyclic drugs like imipramine, desimipramine and amitriptyline. The latter give rise to Hill coefficients round 1, 0, whereas the former have Hill coefficients around 0.5.

As mentioned in Results (Fig. 5) we find that K_d for both paroxetine and imipramine increases with increasing incubation temperature. However, the increase for paroxetine was different from that of imipramine. From 0 to 19°C almost no increase in K_d was seen with the ^{3}H-labeled paroxetine receptor complex, whereas the affinity decreased (K_d increased) more rapidly from 19 to 37°C. A similar break in linearity of the change in affinity with changes in temperature has been described for the benzodiazepine receptor [6,7], though the low temperature component was found more steep. The break in linearity for the benzodiazepine receptor also occurred in the same range, i.e. 15–20°C. The explanation of our paroxetine results may thus be the same as the one offered for the benzodiazepine receptor [7], namely that lipids are known to undergo phase transition from gel to liquid-crystalline phase at temperatures of 15–20°C. The break in linearity of the Van't Hoff plot could thus correspond to a membrane lipid phase shift, which affected the ability of the receptor to interact with paroxetine.

Imipramine binding showed a completely different temperature dependence (Fig. 5), namely a constant decrease in affinity (increase in K_d) with increasing temperature, which was linear in the Van't Hoff plot. This result is not in accordance with the published kinetics for the binding of 2-nitroimipramine [16], which also binds to the imipramine binding site [17]. 2-Nitroimipramine is reported to have less affinity at 0°C than at 20°C, i.e. the opposite of imipramine and paroxetine, and also of the kinetics for benzodiazepine.

This behaviour of the 2-nitroimipramine binding is difficult to explain with known physical properties of other receptors and with the present results in mind, and a possible explanation for the peculiar finding may thus be that the assay with 2-nitroimipramine, which binds very slowly, was incubated too shortly. This suggestion is supported by Fig. 2 in Ref. 16.

In the present experiments and in others [5] the same B_{max} for a given platelet membrane preparation was found, whether determined with ^{3}H-labeled paroxetine or ^{3}H-labeled imipramine. This supports the idea that the two drugs bind to the same complex of receptor molecules. However, the different slope of the two curves in the Van't Hoff plot (Fig. 5), when K_d is determined by saturation analysis, indicates that paroxetine and imipramine cannot bind in the same way to the receptor molecules.

Serotonin transport is an active process driven by an electrochemical gradient across the cell membrane [18]. The structure of the transport site is not known, but a model linking Na^+, K^+ and serotonin transport has been proposed [19].

Klingenberg [20] has proposed that membrane proteins with transport functions often could be dimeric molecules, where the molecular complex exists in two states with binding of the substrate taking place in the one state, and release of the substrate in the other state.

If, therefore, also our receptor is a dimer, which can exist in two different conformations (Table II)

TABLE II

HYPOTHETICAL REACTION SCHEMES EXPLAINING THE DIFFERENCE BETWEEN ^{3}H-LABELED IMIPRAMINE AND ^{3}H-LABELED PAROXETINE BINDING TO THE THROMBOCYTE MEMBRANE BINDING SITE FROM 0 TO 37°C

Imipramine (I):

$$I + R_1 \underset{k_{-1}}{\overset{k_{+1}}{\leftrightharpoons}} IR_1$$

$$K_d(0\text{–}37°C) = \frac{k_{-1}}{k_{+1}}$$

Paroxetine (P):

$$P + R_1 \underset{k_{-1}}{\overset{k_{+1}}{\leftrightharpoons}} PR_1 \underset{}{\overset{k_x < 19°C}{\rightleftharpoons}} PR_2 \underset{k_{+2}}{\overset{k_{-2}}{\leftrightharpoons}} P + R_2$$

'Scatchard conditions'

$$\begin{cases} K_d(19\text{–}37°C) = \dfrac{k_x}{k_{+1}} & (k_x > k_{-1} \text{ in this temperature interval}) \\ K_d(0\text{–}19°C) = \dfrac{k_{-1}}{k_{+1}} & (k_{-1} > k_x \text{ in this temperature interval}) \end{cases}$$

'Binding curve' condition $K_d(0°C) = \dfrac{k_x}{k_{+1}}$ ($k_x > k_{-1}$ due to a decrease in k_{-1} caused by an allosteric effect of high concentrations of paroxetine on the binding site)

called R_1 and R_2, and ^{3}H-labeled imipramine (I) binds to the receptor in the R_1 conformation, thereby blocking the transition between R_1 and R_2,

$$I + R_1 \leftrightharpoons IR_1$$

this could correspond to the straight Van't Hoff plot with no break in linearity, i.e., the receptor-imipramine complex is independent of membrane lipid fluidity. If, on the other hand, ^{3}H-labeled paroxetine (P) binds to the receptor without blocking the transition between PR_1 and PR_2, and binding preferably takes place to the receptor in the R_1, conformation and dissociation of the paroxetine receptor complex preferably take place when the receptor is in the R_2 conformation, the paroxetine results might be explained. Above 19°C membrane lipids are fluid and transition of the paroxetine receptor complex between the two stages PR_1 and PR_2 is fast, allowing the paroxetine receptor complex to dissociate. Below 19°C membrane lipids have changed fluidity, inhibiting the transition of the paroxetine receptor complex between R_1 and R_2, thereby inhibiting the normal dissociation of the paroxetine-R_2 complex. Thus, below 19°C the slower dissociation rate of the paroxetine-R_1 complex will dominate, leading to the break in linearity of the Van't Hoff plot for paroxetine.

Indirect support for this model comes when the K_d values for paroxetine binding (Table I) calculated from the binding and dissociation curves in Figs. 3a and 3b, are plotted in the Van't Hoff plot Fig. 5 (the values are marked with a △). These three values are seen to fit a straight line instead of the break in linearity obtained, when K_d values from Scatchard plots are used. However, the experimental set up is fundamentally different in the two experiments. In saturation (Scatchard) experiments equilibrium between free and bound paroxetine is determined with sub-nanomolar concentrations of ^{3}H-labeled paroxetine and micromolar concentration of non-radioactive paroxetine is only used to determine non-specific binding in parallel samples. Below 19°C the equilibrium

$$\text{Paroxetine} + R_1 \leftrightharpoons \text{Paroxetine} \cdot R_1$$

thus determines the K_d values.

In binding experiments, equilibrium between free and bound ^{3}H-labeled paroxetine is first obtained, and then in the same vial, the concentration of paroxetine is increased to 10000 nM, i.e. the free paroxetine concentration is increased about 100000-times. Wennogle et al. [13] found that

serotonin in high concentrations decreased the dissociation rate of the imipramine binding site. Paroxetine, which is rather specific for serotonin receptor, possibly mimics this serotonin effect in high concentrations. This means that the equilibrium

$$\text{Paroxetine} + R_1 \leftrightharpoons \text{Paroxetine} \cdot R_1$$

is moved strongly towards Paroxetine $\cdot R_1$.

The processes

$$\text{Paroxetine} \cdot R_1 \leftrightharpoons \text{Paroxetine} \cdot R_2 \leftrightharpoons \text{Paroxetine} + R_2$$

and

$$R_2 \leftrightharpoons R_1$$

thus become dominant under the experimental conditions used in binding curve experiments. This process, however, is also the dominant process above 19°C, thus leading to the straight Van't Hoff plot (Fig. 5) under these special conditions.

As mentioned previously, this model is speculative, but the experimental data seem to fit reasonably well with the proposed model. Confirmation or rejection of the model must, however, wait until further experimental data have been gathered.

Acknowledgement

This study was supported by Axel Thomsen and hustrus Fund, Jacob Madsens Fund and the Danish Research Council. The excellent assistance of Bente Bennike is appreciated.

References

1 Langer, S.Z., Briley, M.S., Raisman, R. and Henry, J.F. (1980) Naunyn Schmiedebergs Arch. Pharmacol. 313, 189–194

2 Paul, S.M., Rehavi, M., Skolnick, P. and Goodwin, F.K. (1980) Life Sci. 26, 953–959

3 Raisman, R., Briley, M.S. and Langer, S.Z. (1979) Nature 281, 148–149

4 Ahtee, L., Briley, M., Raisman, R., Lebrec, D. and Langer, S.Z. (1981) Life Sci. 29, 2323–2329

5 Mellerup, E.T., Plenge, P. and Engelstoft, M. (1983) Eur. J. Pharmacol. 96, 303–309

6 Braestrup, C. and Squires, R.F. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3805–3809

7 Speth, R.C., Wastek, G.J. and Yamamura, H.I. (1979) Life Sci. 24, 351–358

8 Peterson, G.L. (1977) Anal. Biochem. 83, 346–356

9 Mahler, H.R. and Cordes, E.H. (1966) Biological Chemistry, pp. 267–270, Harper International Edition, New York and London

10 Langer, S.Z., Moret, C., Raisman, R. and Rubocovich, M.L. (1980) Science 210, 1133–1135

11 Paul, S.M., Rehavi, M., Rice, K.C., Ittah, Y. and Skolnick, P. (1981) Life Sci. 28, 2753–2760

12 Raisman, R., Briley, M.S., Bouchami, F., Sechter, D., Zarifian, E. and Langer, S.Z. (1982) Psychopharmacology 77, 332–335

13 Wennogle, L.P. and Meyerson, L.R. (1983) Eur. J. Pharmacol. 86, 303–307

14 Braestrup, C., Schmiechen, R., Neef, G., Nielsen, M. and Petersen, E.N. (1982) Science 216, 1241–1243

15 Sette, M., Briley, M.S. and Langer, S.Z. (1983) J. Neurochem. 40, 622–628

16 Rehavi, M., Ittah, Y., Skolnick, P., Rice, K.C. and Paul, S.M. (1982) Naunyn Schmiedebergs Arch. Pharmacol. 320, 43–49

17 Rehavi, M., Tracer, H., Rice, K., Skolnick, P. and Paul, S.M. (1983) Life Sci. 32, 645–653

18 Keyer, S.R. and Rudnick, G.R. (1982) J. Biol. Chem. 257, 1172

19 Nelson, P.J. and Rudnick, G. (1979) J. Biol. Chem. 254, 10084–10089

20 Klingenberg, M. (1981) Nature 290, 449–454

21 Bennett, J.P. (1978) in Neurotransmitter Receptor Binding (Yamamura, H.I., Enna, S.J. and Kuhar, M.J., eds.), pp. 57–90, Raven Press, New York