Biochemical Pharmacology, Vol. 27, pp. 323–328. Pergamon Press, 1978. Printed in Great Britain.

SPIPERONE: A LIGAND OF CHOICE FOR NEUROLEPTIC RECEPTORS

3. SUBCELLULAR DISTRIBUTION OF NEUROLEPTIC DRUGS AND THEIR RECEPTORS IN VARIOUS RAT BRAIN AREAS

PIERRE M. LADURON, P. F. M. JANSSEN and JOSÉE E. LEYSEN
Department of Biochemical Pharmacology, Jansssen Pharmaceutica, B-2340 Beerse, Belgium

(*Received* 4 *May* 1977; *accepted* 8 *July* 1977)

Abstract—Tissue fractionation was used as an analytical tool to study in various rat brain regions the subcellular distribution of the neuroleptic receptor and labeled neuroleptics previously injected into the animals. In order to assess the composition of the different subcellular fractions, the distribution pattern of various marker enzymes was also determined.

After differential centrifugation of rat striatum homogenate, [^{3}H]spiperone binding receptors together with 5′ nucleotidase were found to be mainly enriched in the microsomal fraction. Similarly, after injection of [^{3}H]spiperone or [^{3}H]pimozide into rats, the radioactivity was specifically recovered in the microsomal fraction in the striatum, the olfactory tubercle and nucleus accumbens and the frontal cortex but not in the cerebellum.

After equilibration through a sucrose density gradient, the distribution pattern of [^{3}H]spiperone revealed a main peak in a gradient region of low density very close to that of 5′ nucleotidase. The present results indicate that the receptor sites of neuroleptic drugs in the brain dopaminergic areas is associated with membrane-like structures but not with mitochondria or nerve terminals containing dopamine.

Besides kinetic analysis and the regional distribution studies, brain fractionation using differential and density gradient centrifugation can provide another way of revealing at the subcellular level the occurrence of specific neuroleptic binding sites in the brain.

There are two possible ways to go about this, depending on whether tissue fractionation is used as a preparative tool or an analytical one [1]. For the latter, numerous criteria which are mainly biochemical must be applied to assess the composition of the different subcellular fractions.

In this regard, attempts were undertaken in our laboratory to make brain fractionation more analytical and sufficiently refined as to allow the study of more complex subcellular localizations such as those of receptors, particle-bound enzymes or drugs [2–5].

We will report in this part of the work the data concerning the subcellular distribution in the rat striatum of neuroleptic receptors when using [^{3}H]spiperone under *in vitro* conditions and the subcellular distribution in various brain areas of two neuroleptic drugs, spiperone and pimozide, which were injected into rats.

MATERIALS AND METHODS

Male Wistar rats (250 g) were used and brain areas were dissected as described in the previous paper.

For *in vivo* experiments, rats were injected intravenously with [^{3}H]spiperone (0.005 mg kg^{-1}; sp. act. 9 Ci/m-mole and [^{3}H]pimozide (0.02 mg kg^{-1}; sp. act. 13 Ci/m-mole for 2 hr.

Tissue fractionation. Brain samples were homogenized in 10 vol. of 0.25 M sucrose with a Dual homogenizer. The total homogenate was centrifuged at low speed (600 g × 10 min) in a Sorvall centrifuge. The pellet so obtained was again homogenized in 5 vol. of 0.25 M succrose and then recentrifuged. Both pooled supernatants corresponding to the cytoplasmic extract (CY) were further fractionated according to the five-fraction scheme already described [2–4]. An aliquot of the fraction CY was kept to determine the recovery. The heavy and light (M and L) mitochondrial fractions were washed once by suspending the sediment in 5 vol. of 0.25 M sucrose. The washing solutions were never discarded but pooled with the obtained supernatant, and were thus incorporated into the following fractions.

Density gradient centrifugation. An aliquot of the cytoplasmic extract was layered on a continuous sucrose gradient which was prepared using a very simple device consisting of two syringes connected to each other and filled with two different sucrose concentrations (0.6 M and 1.6 M). Tubes were centrifuged in a Swinging bucket (SW 40-Rotor) for 2 hr at 30,000 rev/min. Then 16 fractions were collected by means of an automatic device whereby the liquid column was ejected through a piston hermetically sealed to the top of the gradient tube.

Enzyme assays. 5′ nucleotidase activity was estimated in an incubation medium containing 1.5 μmoles adenosine 5′-phosphate, 25 μmoles Tris–HCl buffer pH 7.7, 1 μmole $MgCl_2$ and Triton X-100 (final concentration 0.1%) in a total vol. of 0.5 ml. After incubation at 37° for 20 min, the phosphate was determined by the automatic method of Van Belle [6].

Cytochrome oxidase was assayed according to Cooperstein and Lazarow [7] and *N*-acetyl-β-D-Glucosaminidase according to Sellinger and Hiatt [8].

Lactate dehydrogenase was determined following the automatic fluorimetric method of Brooks and Ollen [9].

Analytical methods. Dopamine was estimated by the COMT method of Coyle and Henry [10]. Proteins were estimated by Lowry's method. Quantitative estimation of unchanged pimozide and its metabolites was carried out by applying the inverse isotope dilution method [11].

Presentation of results. The enzyme activities except for cytochrome oxidase are expressed in units/g of original tissue. One unit of enzymic activity represents the decomposition of 1 μmole of substrate per min under the conditions of the assay. One unit of cytochrome oxidase is defined as the amount of enzyme causing the decadic logarithm of the concentration of reduced cytochrome *c* to decrease by one unit/min/100 ml of incubation mixture [7].

The distribution pattern after differential centrifugation is expressed in relative specific activity, i.e. the percentage of the total activity against the percentage of total proteins recovered [12].

The recovery expressed in per cent is equal to the sum of the activities found in all the fractions divided by the activity in the starting material:

$$(N + M + L + P + S)/(N + CY) \times 100$$

The results of density gradient centrifugation are expressed in terms of relative concentration or activity C/C_0; C is the activity in a given fraction of the gradient after equilibration, whereas C_0 is the initial concentration or the concentration in a fraction if the material was homogeneously distributed through the gradient. For this purpose, the same amount of material that was layered on the gradient was mixed with a volume of sucrose (equal volume of 0.6 M and of 1.6 M) corresponding to the total volume of the gradient.

Materials. Cytochrome *c* from horse heart type III was purchased from Sigma Chemical Co. [^{3}H]*S*-adenosyl-L-methionine (sp. act. 8.82 Ci/m-mole) was obtained from New England Nuclear, Boston, MA.

RESULTS

Fractionation by differential centrifugation. In vitro spiperone binding (cfr. standard assay in methods of the first paper) was determined in various subcellular fractions obtained by differential centrifugation from rat brain striata. Figure 1 shows that the receptor binding was found to be mainly enriched in the P fraction (microsomal) which is known to contain various membrane-like structures as revealed by the high content in 5′ nucleotidase, inosine diphosphatase and ATPase [cf. also ref. 3]. This distribution pattern markedly differed from that of cytochrome oxidase (mitochondrial) and of the particulate-bound lactate dehydrogenase (synaptosomal).

In Table 1 are listed the means of several fractionation experiments carried out from rat brain striatum; 65 per cent of specific spiperone binding was recovered in the P fraction. In most cases the recoveries ranged between 80 and 100 per cent, except for the cytochrome oxidase and spiperone binding assay. For the latter, the addition of supernatant to each particulate fraction for measuring the spiperone receptor binding allowed to obtain a better recovery (110 per cent). It may therefore, be assumed that an inhibitory process took place with the cytoplasmic extract presumably owing to the presence of a high amount of dopamine present in the supernatant [3] or perhaps of other endogenous ligands, able to interfere at the level of neuroleptic receptor. The subcellular localization of neuroleptic drugs was also studied *in vivo* in various rat brain regions. Figures 2 and 3 show the distribution pattern of radioactivity in different subcellular fractions obtained from rats injected with [^{3}H]]spiperone and [^{3}H]pimozide. The radioactivity, at least 90 per cent of which was unchanged drug, was found to be mainly enriched in the P fraction in the striatum and the limbic system and to

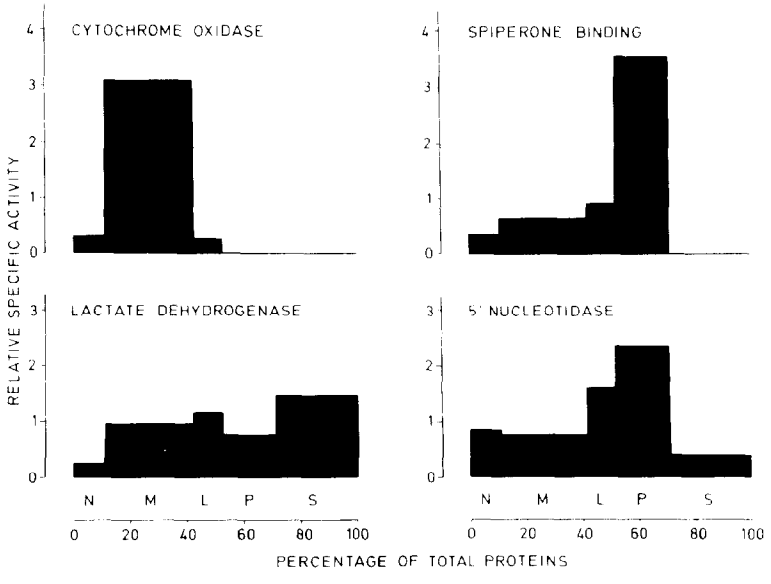

Fig. 1. Distribution pattern of *in vitro* spiperone binding and marker enzymes in subcellular fractions of rat striatum obtained by differential centrifugation (cf. Methods and Table 1).

Table 1. Intracellular distribution of protein, marker enzymes and *in vitro* spiperone binding in rat brain striatum

	No. of expts	Absolute values N + CY	Percentage values N	M	L	P	S	Recovery %
Protein	9	95.8 ± 4.6	12.6 ± 0.9	31.7 ± 1.0	9.2 ± 0.6	15.5 ± 0.9	31.0 ± 0.8	97.4 ± 1.9
Cytochrome oxidase	9	47.7 ± 4.6	12.9 ± 1.9	84.2 ± 1.9	2.6 ± 0.2	0.3 ± 0.1	0	64.9 ± 3.1
Lactate dehydrogenase	9	97.7 ± 3.3	6.2 ± 0.7	26.1 ± 1.2	9.3 ± 0.5	11.6 ± 0.5	46.8 ± 1.7	79.1 ± 3.3
N Acetyl-β-D-glucosaminidase	9	0.77 ± 0.1	17.2 ± 0.5	38.0 ± 1.0	14.9 ± 0.6	18.6 ± 1.2	11.3 ± 0.6	91.1 ± 1.9
5′ Nucleotidase	9	2.4 ± 0.1	11.0 ± 1.2	33.2 ± 2.6	10.6 ± 0.9	38.0 ± 1.6	7.2 ± 1.2	90.0 ± 4.0
Spiperone binding	6	25.9 ± 0.4	3.7 ± 0.3	20.2 ± 0.7	10.4 ± 0.7	65.7 ± 1.0	0	156.8 ± 3.4

Absolute values are in mg g^{-1} for proteins, in units g^{-1} for enzymes and in pmoles g^{-1} for spiperone binding (±S.E.M.); N, nuclear fraction; CY, cytoplasmic extract; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, final supernatant.

a lesser extent in the frontal cortex especially when using [^{3}H]pimozide. In contrast, the cerebellum did not reveal such an enrichment in the P fraction, but did so in the S fraction at least when [^{3}H]spiperone was concerned. Here again the distribution of the labeled drug in the dopaminergic areas markedly differed from that of the marker enzymes, except for 5′ nucleotidase, which is known to be a typical microsomal enzyme.

Control experiments were performed in order to eliminate possible redistribution artefacts throughout the whole fractionation procedure. An amount of [^{3}H]spiperone, equal to that found in the brain of injected animals was added to the brain tissue just before homogenization. Table 2 clearly shows that when using rat striatum, the radioactivity was mainly recovered in the S fraction but not in the P fraction as when labelled spiperone was injected into the animals. In contrast to this, there was practically no difference between both experimental conditions in the cerebellum. It should be noted that [^{3}H]pimozide was found to be much more associated with the particulate fractions from the cerebellum than [^{3}H]spiperone.

In order to characterize the nature of the *in vivo* labeling, the unchanged pimozide and its metabolite were isolated in the different subcellular fractions of brain of rats injected with [^{3}H]pimozide. Table 3 shows that the total radioactivity recovered in the supernatant fraction increased in terms of time. On the contrary, the unchanged pimozide remained quite low in this fraction. Since most of the metabolites were found in the supernatant, the increase of the total radioactivity in this fraction was due to the metabolites.

For spiperone, it has been shown by thin layer chromatography that 2 hr after intravenous injection of [^{3}H]spiperone, more than 90 per cent of the radioactivity consisted of unchanged drug, thus confirming previous results obtained by an inverse dilution procedure [13].

Density gradient centrifugation. Cytoplasmic extracts from brain areas of rats injected with [^{3}H]spiperone were submitted to isopycnic equilib-

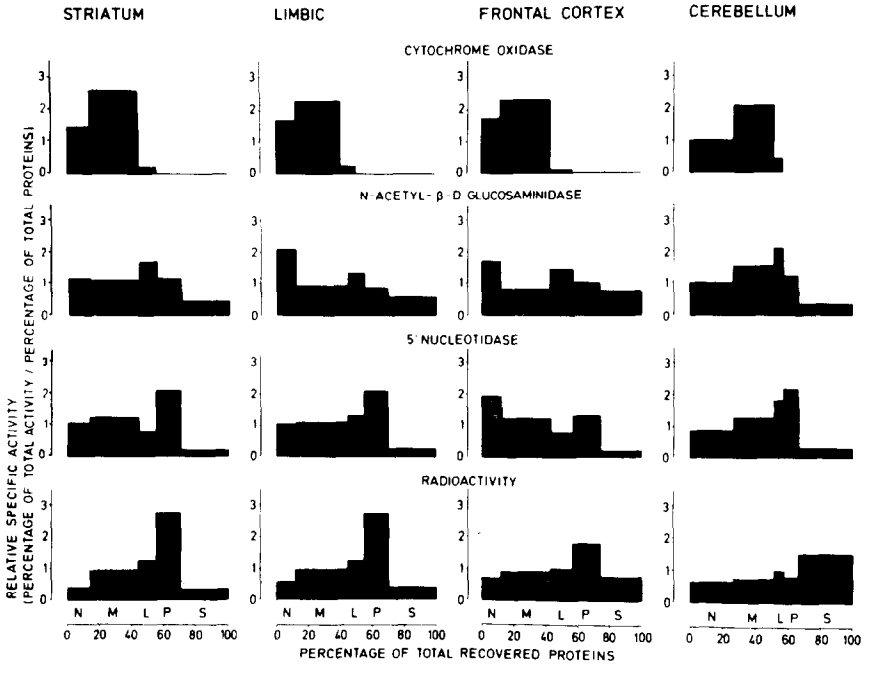

Fig. 2. Distribution pattern of radioactivity and marker enzymes in subcellular fractions of various brain areas 2 hr after intravenous injection of [^{3}H]spiperone (0.005 mg kg^{-1}). Limbic corresponds to the nucleus accumbens together with the olfactory tubercle (cf. Table 1).

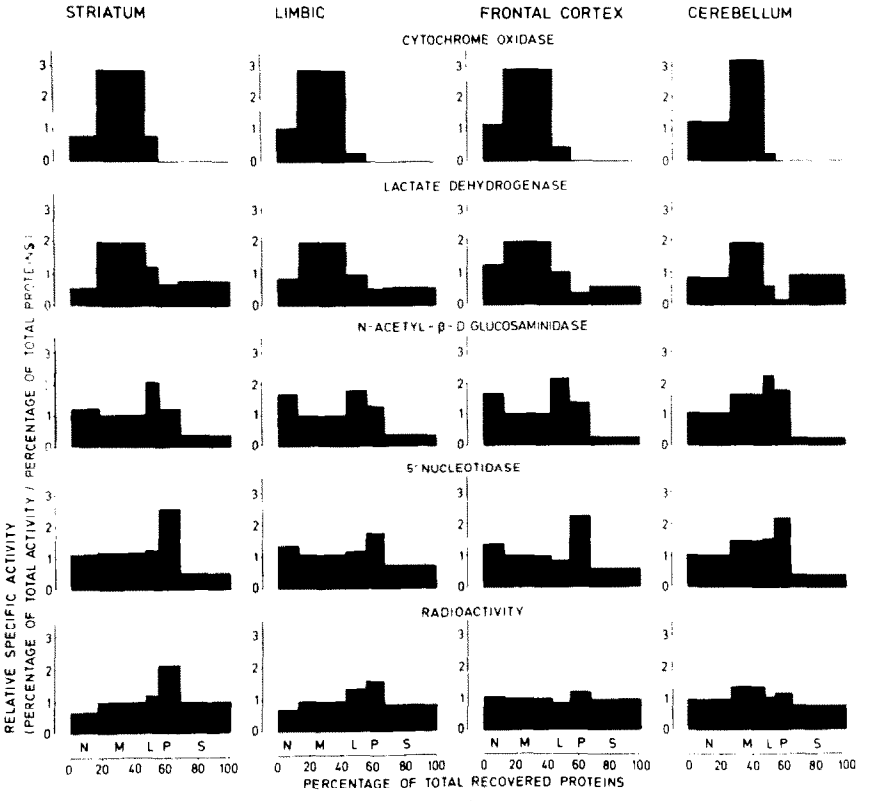

Fig. 3. Distribution pattern of radioactivity and marker enzymes in subcellular fractions of various brain areas 2 hr after intravenous injection of [^{3}H]pimozide (0.02 mg kg^{-1}).

ration through sucrose gradients. In addition to the radioactivity, several marker enzymes and dopamine were determined in order to localize various subcellular structures. In the striatum, the main peak of radioactivity was found in a zone corresponding to a low density of sucrose (0.7 M) close to the peak of 5′ nucleotidase. A second peak though much less pronounced more or less paralleled the peak of LDH but markedly differed from that of dopamine and of cytochrome oxidase. In the limbic system (olfactory tubercle + nucleus accumbens), the distribution pattern of the radioactivity was about the same as in the striatum except that the second peak was less well characterized and paralleled that of 5′ nucleotidase.

Table 2. Subcellular distribution of labeling in striatum and cerebellum of rat brain, after intravenous injection of [^{3}H]spiperone and [^{3}H]pimozide or after directly adding them to the homogenate

	Absolute values ng g^{-1}	Percentage values N	M	L	P	S	Recovery %
[^{3}H]Spiperone injected 0.005 mg kg^{-1} i.v.							
Striatum	2.92	5.8	28.5	13.6	42.1	10.0	85
Cerebellum	0.4	17.5	9.2	6.0	7.2	50.1	95
[^{3}H]Spiperone added to homogenate							
Striatum	1.76	7.4	24.9	7.8	18.1	41.8	100
Cerebellum	1.94	15.8	22.2	4.0	9.6	48.3	109
[^{3}H]Pimozide injected 0.02 mg kg^{-1} i.v.							
Striatum	6.2	10.2	27.3	10.3	26.3	25.9	102
Cerebellum	2.7	22.9	27.6	6.7	15.3	27.6	106

Table 3. Distribution of labeled pimozide and its metabolites in the supernatant obtained by differential centrifugation of brain striata of rats injected with [^{3}H]pimozide (0.31 mg kg^{-1} s.c.)

Hours after injection	Percentage of labeling in the supernatant fraction Total radioactivity	Unchanged pimozide	Pimozide metabolites
1	17.9	4.7	65.2
2	22.1	5.2	63.2
4	19.2	5.3	68.4
8	33.0	3.9	43.2
16	42.9	3.9	49.3
32	58.8	-	-

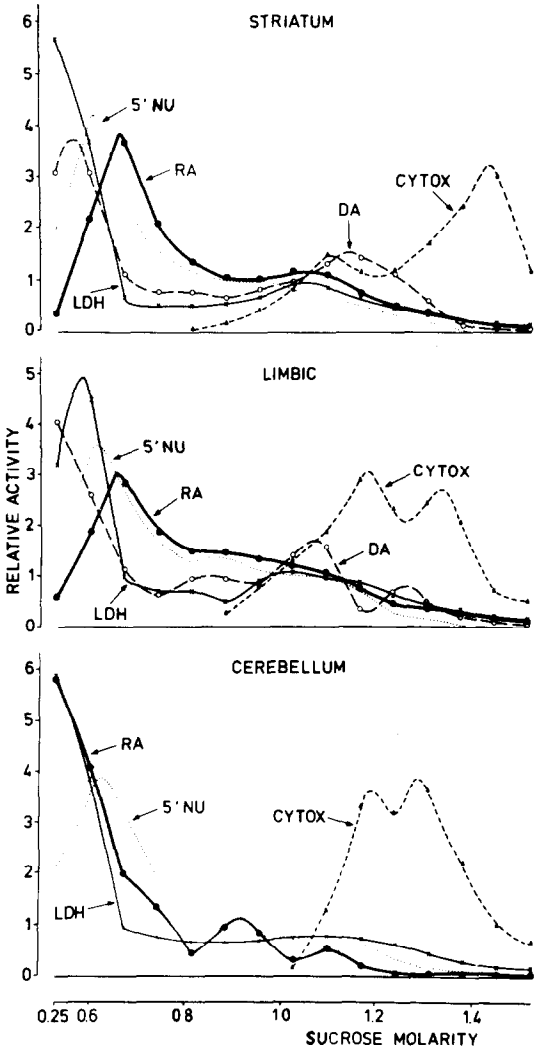

Fig. 4. Subfractionation by isopycnic centrifugation of a cytoplasmic extract (1 ml dilution 1:10) from various brain areas of rats injected with [^{3}H]spiperone (0.005 mg kg^{-1} i.v. 2 hr). Graph shows density distributions of radioactivity (RA) ●——●, cytochrome oxidase (CYTOX) ▲----▲, lactate dehydrogenase (LDH) ×——×, dopamine (DA) O----O, and 5′ nucleotidase (5′ NU) .....

Finally, in the cerebellum the radioactivity displayed a quite different profile in that the largest amount of the radioactivity appeared in the first tube-fraction at the top of the gradient. Moreover there was no typical peak just after that of 5′ nucleotidase although the latter has a distribution pattern comparable to those of other brain areas. In the cerebellum, the profile of lactate dehydrogenase resembled somewhat that of the radioactivity.

DISCUSSION

The present results have shown that the neuroleptic receptor is specifically associated with a membrane-like structure contained in the microsomal fraction, thus confirming our previous observations, when [^{3}H]haloperidol was used as ligand in the binding assay. However, using [^{3}H]spiperone, the enrichment in the P fraction was still more pronounced (65 per cent against 53 per cent with [^{3}H]haloperidol) a fact which may be attributed to the higher affinity of spiperone for specific binding and its lower affinity for the aspecific one.

The main finding of this part of the work concerns the intracellular localization in the brain of neuroleptic drugs injected into rats. Here again, only the brain dopaminergic regions were found to possess a retention mechanism for specifically taking up neuroleptic drugs in the microsomal fraction, where the neuroleptic receptors were found when measured in *in vitro* conditions. This retention capacity evaluated by the level of drug enrichment in the P fraction appears to depend on the number of receptors (striatum > limbic > frontal cortex). It should be noted that the enrichment of [^{3}H]pimozide in the frontal cortex was very modest presumably due to the relatively high affinity of this drug for aspecific binding (unpublished results). In contrast, there was a very large amount of [^{3}H]spiperone in the microsomal fraction of the frontal cortex which further support our suggestion that the frontal cortex may be a major site for the antipsychotic activity of neuroleptic agents [14].

The lack of specific *in vivo* binding of [^{3}H]spiperone in the microsomal fraction from cerebellum provides more support to the conclusion that an enrichment of neuroleptic drugs in this subcellular fraction from dopaminergic areas represents a specific *in vivo* binding to receptor sites. Therefore we believe that all the subcellular distribution studies of drug should have to include a parallel study in a tissue or a region which does not contain specific receptors. This is of major importance especially in *in vivo* experiments where the specific binding must be distinguished from the aspecific one. In our experiments, at the regional level as well as the subcellular one, the results obtained from the cerebellum represent such control or blank values.

The foregoing results provide also a better understanding of the precise intracellular localization of neuroleptic drugs in the brain. We could not confirm the previous finding that a significant shift appeared in the subcellular distribution of [^{3}H]pimozide in the caudate nucleus from the mitochondrial fraction to the fraction consisting mainly of submicroscopic nerve endings [15]. Neither the fractionation by differential centrifugation nor that by density gradient could localize labeled neuroleptic drugs associated with mitochondria. In isopycnic centrifugation, the main peak of [^{3}H]spiperone, was found to equilibrate in a gradient region of low density very close to where 5′ nucleotidase was detected. This suggests that neuroleptic receptors are associated with membrane-like structures, thus not with mitochondria and nerve endings. However, a second peak of radioactivity, though very small, was also detected in a zone where LDH was detected. This peak was quite distinct from that of dopamine, a fact which suggests that neuroleptic drugs are not associated with presynaptic receptors located on dopaminergic nerve terminals [16]. In agreement with this view, is the fact that the second peak did not disappear in the striatum even after lesion on the nigrostriatal pathway (unpublished results). The physiological significance and the exact nature of this second peak are not yet known; this

still remains open for speculations (distribution artefact, aspecific binding or specific binding on nerve endings containing GABA, serotonin, acetylcholine or peptides...). Further experimental results are needed to solve this problem.

Throughout the present work, we have provided evidence from *in vitro* as well as *in vivo* experiments that the neuroleptic receptor is mainly a membrane-like structure and does not seem to be associated with dopaminergic nerve terminals. Moreover its intracellular localization markedly differs from that of dopamine-sensitive adenylate cyclase [3–4]. Our results support the idea that the "neuroleptic receptor" characterized by binding studies is the main target for the neuroleptic drugs.

From this series of papers, it is obvious that spiperone may become a very useful tool for studying the mechanism of action of neuroleptic agents.

Acknowledgements—We would like to thank J. Heykants for his help in the identification of pimozide and its metabolites. Part of this work was supported by a grant from IWONL. The technical assistance of M. Verwimp, W. Gommeren, A. Aerts and P. Van Gompel is greatly appreciated.

REFERENCES

1. P. Laduron, *Int. Rev. Neurobiol.* **20**, 251 (1977).
2. P. M. Laduron, M. F. Verwimp, P. F. M. Janssen and W. K. Gommeren, *Biochimie* **57**, 253 (1975).
3. P. Laduron, M. Verwimp, P. F. M. Janssen and J. Leysen, *Life Sci.* **18**, 433 (1976).
4. J. Leysen and P. Laduron, *Life Sci.* **20**, 281 (1977).
5. P. Laduron and J. Leysen, *Biochem. Pharmac.* **26**, 1003 (1977).
6. H. Van Belle, *Analyt. Biochem.* **33**, 132 (1970).
7. S. J. Cooperstein and A. Lazarow, *J. biol. Chem.* **189**, 665 (1951).
8. O. T. Sellinger and R. A. Hiatt, *Brain Res.* **7**, 191 (1968).
9. L. Brooks and H. Ollen, *Clin. Chem.* **8**, 748 (1965).
10. J. T. Coyle and D. Henry, *J. Neurochem.* **21**, 61 (1973).
11. P. A. J. Janssen, W. Soudijn, I. van Wijngaarrden and A. Dresse, *Arzneimittel-Forsch.* **18**, 282 (1968).
12. C. de Duve, B. C. Pressman, R. Gianetto, R. Wattiaux and F. Appelmans, *Biochem. J.* **60**, 604 (1955).
13. J. Heykants (unpublished results).
14. P. Laduron, K. De Bie and J. Leysen, *Naunyn-Schmiedeberg's Arch. Pharmak.* **296**, 183 (1977).
15. W. Soudijn and I. Van Wijngaarden, *J. Pharm. Pharmac.* **24**, 773 (1972).
16. A. Carlsson, in *Pre- and Postsynaptic Receptors* (Eds Usdin and Bunney), p. 49. Marcel Dekker Inc., New York (1975).