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COMMENTARY

HIGH-AFFINITY BINDING SITES FOR NEUROLEPTIC DRUGS IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES AND THEIR RELATION TO DOPAMINE RECEPTORS

A LONG-STANDING CONTROVERSY

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In the early 1980s, Le Fur and co-workers [1] reported the existence of high-affinity binding sites for the neuroleptic [^{3}H]spiperone in the PBL† of both rats and humans. [^{3}H]Spiperone has been used extensively to label D_2 dopamine receptors in brain (see, for example, Ref. 2), and Le Fur interpreted the binding sites for this radioligand on PBL as dopamine receptors. Since then, there has been a continuing controversy as to the nature of these binding sites for [^{3}H]spiperone on human PBL, and what their relevance to neurological and psychiatric diseases might be.

More recently, other groups have used separate approaches to this problem (other radiolabelled ligands, molecular biological techniques) and re-opened the debate. In this review, we shall trace the history of the "high-affinity spiperone" site and assess its current status in view of these new reports.

Potential clinical significance

There are several very important diseases where a disorder of central dopamine function is postulated but where the diagnosis is almost entirely clinical, i.e. no reliable laboratory tests are available. These disorders include schizophrenia and Parkinson's disease (reviewed in Ref. 3). A peripheral marker of central dopamine function would be valuable in understanding the pathology of these disorders, in diagnosis and assessment of treatment, and potentially in the assessment of vulnerability to the disorders.

Multiple dopamine receptors

Dopamine receptors were originally subclassified into D_1 and D_2 subclasses on the basis of pharmacological and biochemical differences [4]. [^{3}H]Spiperone was introduced as a radioligand for labelling the D_2 subclass, but although it has a high affinity for this receptor class, it has subsequently been recognized that it will also label 5-HT_2 (serotonin) and α_1-adrenergic receptors and an acceptor site for the spirodecanone structure [5]. Therefore, careful pharmacological analysis of any [^{3}H]spiperone binding sites is required to validate their nature. The picture has been further complicated by the application of molecular biological techniques to dopamine receptors, which has identified five or more receptor subtypes [6]. It seems that there are three D_2-like dopamine receptors (D_2, D_3, and D_4), each of which can bind [^{3}H]spiperone with high affinity.

Radioligand-binding methodology

Figure 1 shows the major steps in ligand binding studies of PBL. Each assay requires approximately 50 mL of blood from the subject, and the requirement for fresh blood for each assay makes it a time-consuming procedure and extensive replication difficult. In the procedure, each step is a potential source of error and variation between laboratories. Bondy *et al.* [7] and Wodarz *et al.* [8] have addressed these methodological issues in detail (see below). It is important, however, to consider one aspect of the radioligand-binding procedure in detail here, namely the definition of "specific" as opposed to "non-specific" binding of the radioligand. This definition is central to the validation of any binding sites detected, and there are three major methods for defining specific radioligand binding:

(1) Use of a high concentration of the non-radioactive version of the labelled ligand. This is assumed to saturate the specific binding of the radioligand to receptors but not to inhibit interaction of the radioligand with the non-specific sites, which are assumed to be far from saturation at this concentration. A problem with this definition is that a high concentration of the non-radioactive ligand may alter interaction with the non-specific sites, but, more importantly, the definition provides no specificity between different receptors for which the radioligand has high affinity and includes acceptor sites for the ligand. For [^{3}H]spiperone, as outlined above, D_2-like (D_2, D_3, D_4) receptors will be labelled as well as 5-HT_2 (serotonin) and α_1-adrenergic receptors and the spirodecanone acceptor site.

(2) Use of a chemically distinct compound to inhibit radioligand binding to the "specific" sites such that

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† Abbreviations: PBL, peripheral blood lymphocytes; and 5-HT, 5-hydroxytryptamine.

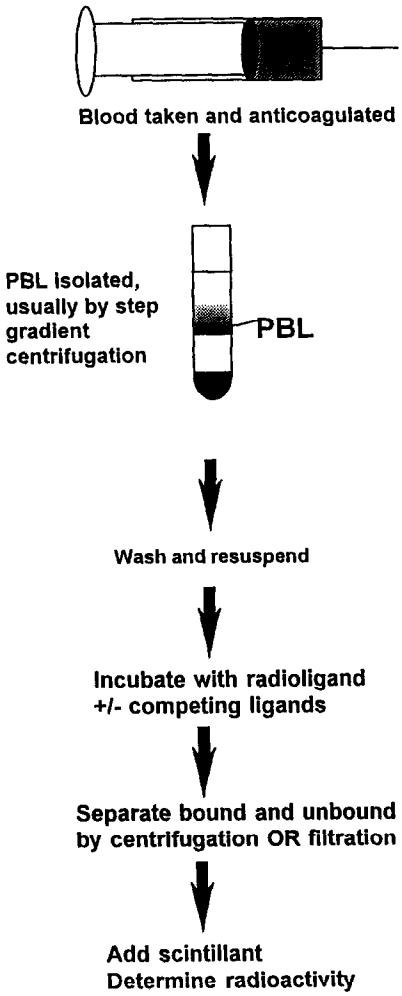

Fig. 1. Outline of methodology for radioligand binding studies of peripheral blood lymphocytes (PBL).

any binding that remains is assumed to be non-specific. This is a more rigorous definition as it combines the specificity of both radioligand and the competitor in defining the sites labelled. Nevertheless, it is desirable to perform a pharmacological analysis of the radioligand binding with several specific competitors to validate the nature of the sites labelled. In the case of [^{3}H]spiperone and PBL, haloperidol has been used to define specific binding (see below). Depending on the concentration of haloperidol used, this may provide a reasonable definition of specific binding to dopamine receptors, but it will still be necessary to establish in competition analyses the precise nature of the specific radioligand binding.

(3) The most rigorous test is to use two optical isomers of a competing ligand, one of which is believed to occupy the specific sites under investigation and the other of which does not. Comparison of the binding of the radioligand in parallel assays in the presence of the different isomers defines specific binding, and the use of the inactive isomer allows for any competition between the radioligand and the active isomer at non-specific sites. In the case of [^{3}H]spiperone binding, this protocol has been used effectively with the (+)- and (−)-isomers of butaclamol [9]. This provides an excellent definition of [^{3}H]spiperone binding to D_2-like dopamine receptors in brain, providing blockade of 5-HT_2 receptors has been achieved. This emphasizes again the need for proper pharmacological definition of radioligand binding.

Development of the concept of a high-affinity [^{3}H]-*spiperone binding site in PBL*

In the original study of Le Fur *et al.* [1], specific [^{3}H]spiperone binding to rat and human PBL was defined using 10 μM haloperidol, and saturation analysis of radioligand binding showed two components: a higher affinity site (K_D 5.4 nM, B_{max} 103 fmol/10^6 cells for human PBL; K_D 1.9 nM for rat PBL) and a lower affinity site (K_D 36.2 nM for rat PBL).

Attempts to replicate the results of Le Fur *et al.* [1] met with varied success. Several groups were able to show a biphasic saturation curve for [^{3}H]-spiperone binding. For example, Maloteaux *et al.* [10] demonstrated a biphasic curve using 1 μM haloperidol to define specific [^{3}H]spiperone binding. However, they observed that the "binding" of [^{3}H]spiperone was blocked most effectively by chloroquine, and the rank order of competition by neuroleptics did not correspond to that expected for dopamine receptors in brain. They concluded that the [^{3}H]spiperone was being taken up into lysosomes in a non-specific way rather than interacting with specific receptors.

Two studies that were mainly concerned with a comparison of [^{3}H]spiperone binding in different patient populations also reported high-affinity sites for the radioligand. Grodzicki *et al.* [11] used 10 μM haloperidol to define specific [^{3}H]spiperone binding, and Halbach and Henning [12] used 10 μM (+)-butaclamol to define specific [^{3}H]spiperone binding. Further details of both of these studies are given in the section entitled "Clinical studies of [^{3}H]spiperone binding to PBL".

Bondy's group has spent several years refining the methodology to study [^{3}H]spiperone binding to PBL [7, 13–16]. The latest version of their technique is described in detail in Ref. 7. Using 1 μM (+)-butaclamol to define specific [^{3}H]spiperone binding, a biphasic binding saturation curve is obtained. This can be analysed using non-linear least squares curve-fitting into contributions from a high-affinity saturable site (B_{max} 1.47 fmol/10^6 cells, K_D 0.08 nM) and a non-saturable set of sites. If a higher concentration of (+)-butaclamol is used, it becomes impossible to define the characteristics of the specific [^{3}H]spiperone binding because there is a "massive" displacement of [^{3}H]spiperone that swamps the high-affinity site. Bondy *et al.* [7] also reported that several other factors are important variables in obtaining reproducible results with the assay, e.g. the use of anticoagulant (EDTA) causes less clumping of platelets that might otherwise contaminate the PBL preparation; the forcefulness needed to resuspend the PBL after washing; and the duration of shaking

of filters before counting the scintillation. It is clear that they have gone to great lengths to optimize the conditions, including having tested the background absorption of all the brands of 1.5-mL tubes available in Germany!

The need for such accuracy can be understood when the nature of the calculation involved in the derivation of B_{max} and K_D is examined. For example, for figures taken from Bondy *et al.* [7]: for a [^{3}H]-spiperone concentration of 0.47 nM (the concentration of ligand that will give the best estimate of B_{max} in this example), the total dpm added is 36,811 and of this the total bound [^{3}H]spiperone is approximately 1600 dpm. The non-specific binding is about 1000 dpm, making the specific binding about 600 dpm. Of these 600 dpm, approximately half are contributed by the high-affinity component. Therefore, approximately 300/1600 = 19% of the total bound [^{3}H]spiperone is due to the high-affinity component. Any part of the procedure that introduces error will make the results less reliable, and it is clear that great care will be required to obtain reproducible data. Given such technical details, it is remarkable that such consistent results are produced by this group and understandable that others have failed to replicate the results. Bondy's group consistently has reported K_D values between 0.08 and 0.4 nM and B_{max} values between 0.5 and 4.0 fmol/10^6 cells for [^{3}H]spiperone binding to PBL of normal controls.

Wodarz *et al.* [8, 17] have also looked very carefully at the methodological aspects of the assay. They have highlighted the variable filter binding of [^{3}H]spiperone (up to 70% of total binding) and the fact that (+)-butaclamol can increase this non-specific filter binding of [^{3}H]spiperone. They recommend pretreating filters with polyethylenimine to reduce this effect. Controlling for these factors, Wodarz *et al.* [8, 17] reported a biphasic saturation curve for [^{3}H]spiperone binding similar to that seen by Bondy *et al.* [7] with a K_D for the high-affinity site of 1.5 nM but a significantly higher B_{max} (25 fmol/10^6 cells).

Studies unable to demonstrate the high-affinity [^{3}H]-*spiperone binding site*

In contrast to these reports of a high-affinity binding site for [^{3}H]spiperone on PBL, several groups have been unable to replicate these results. As discussed above, Maloteaux *et al.* [10] obtained a biphasic saturation curve but interpreted it as accumulation in lysosomes. Fleminger *et al.* [18] used 10 μM haloperidol to define specific [^{3}H]-spiperone binding in an attempt to replicate the results of Le Fur *et al.* [1]. They observed no high-affinity saturable site but instead reported non-saturable sites for [^{3}H]spiperone. They also reported that disruption of the cells abolished the [^{3}H]-spiperone binding with no specific binding to isolated membranes. A range of substances was used to compete for [^{3}H]spiperone binding to intact PBL, and the most potent competitor was chloroquine, in agreement with the work of Maloteaux *et al.* [10]. It should be noted, however, that Fleminger *et al.* [18] used a high concentration of [^{3}H]spiperone (4 nM) and, under these conditions, the high-affinity saturable sites seen by Bondy *et al.* [7] might be obscured by the non-saturable [^{3}H]spiperone binding sites reported by these workers. Feenstra *et al.* [19] and Rao *et al.* [20, 21] also attempted to replicate Bondy's results of a high-affinity saturable [^{3}H]-spiperone binding site on PBL without success. However, direct comparison with Bondy's results is difficult in view of the strict conditions Bondy reports as essential and which these other workers did not attempt to copy exactly.

Although much work has been done to identify the ideal conditions for [^{3}H]spiperone binding to PBL, none of the groups who report being able to identify a high-affinity binding site for [^{3}H]spiperone has reported a full pharmacological characterization of these sites. This is inevitably due, in part, to the labour-intensive nature of such an enterprise with human blood cells. Coccini *et al.* [22] attempted this using 10 μM haloperidol to define specific [^{3}H]-spiperone binding. Their results do not fit neatly into either the pro or anti high-affinity site category. They derived B_{max} and K_D values in the region of 10 nM and 100 fmol/10^6 cells, respectively. In competition experiments versus [^{3}H]spiperone (4 nM), however, they observed a reversed stereoselectivity for the isomers of butaclamol (with an unmeasurable IC_{50} in rat PBL) and a pharmacological profile that they interpreted as being closer to a sigma receptor than a dopamine receptor; they were unable to detect any competition by dopamine for [^{3}H]spiperone binding, whereas sigma-selective compounds, such as pentazocine and 1,3-di-*o*-tolylguanidine (DTG) were effective at inhibiting [^{3}H]-spiperone binding.

Zaitsev *et al.* [23] provide yet another interpretation of the spiperone binding to human PBL. They carried out binding at 37°, reporting saturation occurring within 10 min. They analysed their saturation and kinetic data in terms of two sites (with K_D values of 3 and 20 nM). They carried out a limited pharmacological characterization of the spiperone binding. (+)- and (−)-butaclamol and ketanserin competed with the spiperone with approximately equal potency, but haloperidol and sulpiride were ineffective as competitors, even at 500 nM. Zaitsev *et al.* [23] concluded that the binding is to 5-HT_2 receptors. However, it is difficult to reconcile this to their observation of two different populations of receptor binding to spiperone. Again, the incomplete nature of the characterization leaves many questions unanswered.

Assessment of high-affinity [^{3}H]*spiperone binding sites on PBL*

To assess the validity of the reports of high-affinity [^{3}H]spiperone binding sites on PBL (see, for example, Refs. 7 and 8) and the reports of other groups unable to replicate these results, it is very important to clarify what Bondy's model of [^{3}H]-spiperone binding actually entails. The model requires that there are two components to the specific [^{3}H]spiperone binding. One component is a non-saturable site and does not exhibit stereoselectivity for binding the isomers of butaclamol. The second smaller component of specific [^{3}H]-spiperone binding, as defined by 1 μM (+)-butaclamol [7], is

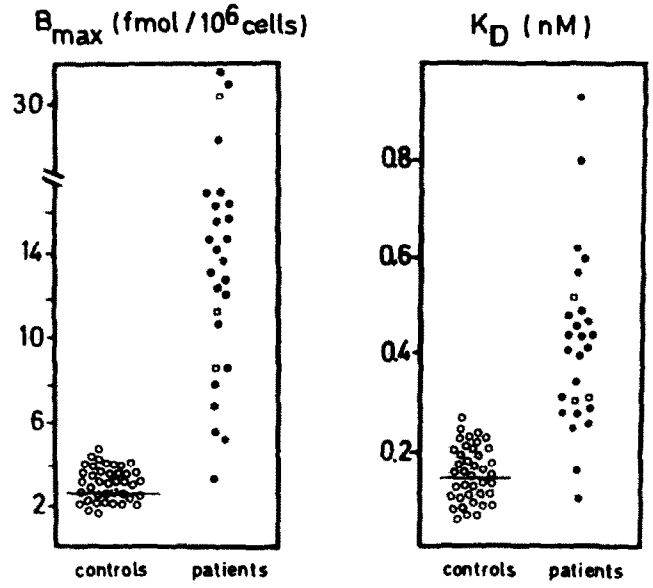

Fig. 2. Specific binding of [^{3}H]spiperone to lymphocytes of controls and acute, unmedicated schizophrenic patients (disorder classification is from ICD-9). The part of the [^{3}H]spiperone binding that was displaced by 1 μM (+)-butaclamol was analysed into saturable and non-saturable components. The saturable component was then defined as the specific binding. Taken with permission from *Psychiatry Res* **15**: 41–48, 1985. Copyright (1985) Elsevier Science Ltd [Ref. 15].

saturable, shows some stereoselectivity for binding the isomers of butaclamol, and has a maximum capacity of about 2 fmol/10^6 cells and a K_D of about 0.2 nM. As argued above, only 20% of the total [^{3}H] spiperone binding represents the high-affinity site at concentrations around the K_D. We would argue that experimental errors in these techniques are too great either to be certain of a true effect or to discount the model because others are unable to replicate the results. Nevertheless, the consistency of the results that Bondy *et al.* [7] report are quite striking, especially when results for different clinically defined groups are determined with the clinical allocation being blind to biochemical results (see, for example, Fig. 2 taken from Ref. 15). Such differences between clinically defined groups do suggest that something of interest is being measured.

Once the details of the model are clear, possible interpretations can be discussed. All the groups who have assayed [^{3}H]spiperone binding to membranes of PBL have reported a dramatic reduction in [^{3}H]-spiperone binding compared with that seen to intact PBL [10, 18–20]. This should be contrasted with data for brain where [^{3}H]spiperone binding to D_2 dopamine receptors in membrane preparations is the routine assay system (see, for example, Ref. 24).

This suggested to Maloteaux *et al.* [10] that the uptake was by diffusion and trapping in lysosomes, and the effects of the lysosomotropic agent chloroquine [10, 18] support this conclusion. Since these latter authors were unable to detect the high-affinity sites, then the accumulation in lysosomes must correspond to the non-saturable [^{3}H]spiperone binding.

Concerning the high-affinity site described by Bondy *et al.* [7], its significance is considerably less clear. As stated above, it is far from proven that it is a valid entity, most notably owing to the inability of some groups to replicate the findings. It has certainly never been demonstrated in membrane preparations, which is the most rigorous test of a classical receptor. Assuming that the phenomenon of an apparently saturable component to [^{3}H]-spiperone binding does occur at low [^{3}H]spiperone concentrations, what might this represent? Bondy *et al.* [7] suggest that it might be a transport protein based on the effects of protein-modifying agents and the effects of metabolic inhibitors to prevent this component of [^{3}H]spiperone binding. It seems clear that the high-affinity [^{3}H]spiperone binding site is not a dopamine receptor with the properties seen in the brain, and the claims that it represents some other recognized receptor (sigma or 5-HT) have severely limited experimental support.

Clinical studies of [^{3}H]*spiperone binding to PBL*

Whatever the detailed biochemical basis for the [^{3}H]spiperone binding to PBL, it is possible to examine the question of its use as a clinical marker. There are two separate approaches to this. Several groups, able to derive binding parameters for a high-affinity site, have compared these parameters in different patients and control groups. Others, either not willing or not able to enter the controversy over the nature of the [^{3}H]spiperone binding sites, have chosen to compare the overall binding at a single [^{3}H]spiperone concentration.

Le Fur *et al.* [25] reported data from a limited

series of patients and controls and observed an increase in [^{3}H]spiperone binding to PBL from schizophrenics and a decrease in binding to PBL from patients with Parkinson's disease [26].

Rotstein *et al.* [27] reported "preliminary findings" of increased [^{3}H]spiperone binding to PBL of schizophrenics, but with increased inter-subject variability compared with controls.

Grodzicki *et al.* [11] reported data from a limited series of patients and controls, suggesting that schizophrenic patients who had responded to medication had increased [^{3}H]spiperone binding to PBL, whereas controls and non-responders did not. The drug status of the non-responders at the time of the assays was unclear, so the data may be difficult to interpret.

Halbach and Henning [12] used a method very similar to that of Bondy *et al.* [7], and they observed no difference in [^{3}H]spiperone binding to PBL between controls and schizophrenics until the PBL were incubated in the presence of cortisol. Such incubation produced a change in B_{max} from 1–2 fmol/10^6 cells to ~15 fmol/10^6 cells.

The most extensive clinical studies have been carried out by Bondy and her group. The earlier studies (e.g. Ref. 15) reported increases in B_{max} values for high-affinity [^{3}H]spiperone binding to PBL for schizophrenics (~2.5 fmol/10^6 cells to ~10 fmol/10^6 cells with increased inter-subject variation in the patient group) (see Fig. 2 above). These increases seemed to be independent of drug treatment. These studies were extended to first- and second-degree relatives [13]. It was shown that all patients with schizophrenia had elevated [^{3}H]spiperone binding in PBL, as did some of the well relatives. The authors felt that this increase in [^{3}H]spiperone binding to PBL from well relatives was associated with an increased tendency to transmit schizophrenia, though this can only be an impression as the numbers were rather small.

A further family study [28] extended this work. This study had no normal controls, but assumed a threshold of 4 fmol/10^6 cells as pathological. It was shown that 4 of 17 schizophrenic probands did not have elevated [^{3}H]spiperone binding, but that within families the association of schizophrenia or schizoaffective disorder with or without an increase in [^{3}H]spiperone binding bred true. Comparison with previous studies is difficult, because full results are not shown, but it is clear that in families exhibiting elevated [^{3}H]spiperone binding, this elevated binding was not specific to schizophrenia or schizoaffective disorder but was seen in major depression in contrast to previous reports where psychiatric controls had normal [^{3}H]spiperone binding. It is not clear if this is because major depression in families with schizophrenia is etiologically different from other cases. The presence of controls would have helped clarify this point.

Several studies examining high-affinity [^{3}H]-spiperone binding to PBL failed to show any difference between control and patient populations. Some, such as Rao *et al.* [20], were unable to detect high-affinity [^{3}H]spiperone binding to either group. Others, such as Wodarz *et al.* [29], were able to detect a high-affinity [^{3}H]spiperone binding site in PBL, but could detect no difference between patients and controls. For patients with Parkinson's disease, recent attempts to replicate the work of Le Fur *et al.* [26] by Bondy's group have been unsuccessful, with no difference in [^{3}H]spiperone binding to PBL detected unless the patients were being treated with L-DOPA; this effect was not restricted to Parkinson's patients (Arnold *et al.* [30]).

Itzchaky *et al.* [31] and Griffiths *et al.* [32] both attempted to use a single concentration of [^{3}H]-spiperone to compare [^{3}H]spiperone "binding" to PBL without differentiating between the high- and low-affinity components and reported no difference between controls and schizophrenics.

The results of these clinical studies therefore suggest that in the hands of Bondy's group, some facet of human PBL (detectable as differences in [^{3}H]spiperone binding) differs in some schizophrenics and their families, relative to controls, but that other groups have failed to replicate these findings. As suggested above, we feel that without further characterization of the system, it will be impossible to interpret these results. Certainly, the usefulness of a clinical marker that only one laboratory can detect will be severely limited.

Other approaches

In recent years, several studies of the binding characteristics of PBL, which have not concerned themselves with the controversy concerning [^{3}H]-spiperone binding, have taken place. Ovadia and Abramsky [33] showed that the isolated membranes of rat thymocytes and spleen cells bind [^{3}H]dopamine with high affinity, but this binding was not sensitive to sulpiride or haloperidol so that this [^{3}H]dopamine binding does not correspond to a D_2-like receptor. Santambrogio *et al.* [34] attempted to assay dopamine receptors on PBL using [^{3}H]sulpiride as radioligand with a saturating concentration of non-radioactive sulpiride to define non-specific binding. Their data suggest a single saturable site for sulpiride with a K_D ~0.9 nM and a B_{max} ~10 fmol/10^6 cells for human PBL. [^{3}H]Sulpiride binding to PBL was characterized pharmacologically in competition studies using a series of dopamine receptor agonists and antagonists. The data were consistent with the labelling by [^{3}H]sulpiride of a D_2-like receptor, which resembled the D_2 and D_4 species, identified by molecular biology studies, more than the D_3 species. The study did not include an assessment of the ability of spiperone or the isomers of butaclamol to compete with [^{3}H]sulpiride binding to PBL, but otherwise the results seem robust. The study went on to show that dopamine was able to reduce the cAMP levels in PBL stimulated by forskolin.

Two studies have used the techniques of molecular biology to study the expression of dopamine receptors in PBL. Takahashi *et al.* [35] used reverse transcription of RNA from PBL followed by polymerase chain reaction using primers specific for the D_5 receptor to show that D_5 mRNA is expressed in human PBL preparations. The study then went on to use the D_1/D_5 specific radioligand [^{3}H]SCH-23390 to assay for radioligand binding in PBL preparations, reporting a B_{max} of 3 fmol/mg protein or approximately 0.5 fmol/10^6 cells.

The same group [36] repeated the procedure for the D_3 receptor and showed the expression of D_3 mRNA. They used [^{3}H]7-hydroxy-*N*,*N*-di-*n*-propyl-2-aminotetralin as a specific radioligand for D_3 receptors and derived a B_{max} value of 30 fmol/10^6 cells and a K_D of 12 nM, which is between the K_D values reported for this radioligand in brain by Lévesque *et al.* [37] of 0.78 and 61 nM for D_3 and D_2 dopamine receptors, respectively.

Both of these studies using reverse transcription-polymerase chain reaction and specific ligands are interesting but not conclusive. The technique of polymerase chain reaction is so sensitive that very low levels of expression of the mRNA in other cell types would be detected. For the D_5 study [35], the level of expression of D_5 receptors reported in PBL is very low, and for both studies there is insufficient pharmacological characterization to be certain of the nature of the receptors that are being detected.

Conclusions

It should be clear from the studies outlined above that the use of [^{3}H]spiperone binding to study PBL has been very confusing. Several groups report a non-saturable high-capacity uptake of [^{3}H]spiperone into PBL, that may be related to accumulation in lysosomes. Two groups have provided consistent evidence of a high-affinity [^{3}H]spiperone binding site on PBL, which may have clinical relevance as a marker in psychiatric disorders. However, this component is so small compared with other components of [^{3}H]spiperone binding that in the absence of further "dissecting out" of the components, for example specific inhibition of the non-saturable component of [^{3}H]spiperone binding, it will be difficult to use this as a routine marker for analysis of patients. Recent studies using other radioligands and molecular biological studies have suggested the presence of classical dopamine receptors or their related mRNA in PBL, and these studies need to be extended and replicated to assess their significance. It seems very possible that there is a system that is binding [^{3}H]spiperone and a separate system that binds [^{3}H]sulpiride and other neuroleptics, and the relationship of these two systems is not clear.

Most of the studies discussed in this review assess binding to whole cells. Although there is no coherent picture yet of the details of the binding of dopamine antagonists to PBL, perhaps that is because we are glimpsing the complexities of the surfaces of living cells and the reactions of those cells to the experimental procedures to which they are subjected.

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