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ORIGINAL INVESTIGATION

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Drug-induced receptor occupancy: substantial differences in measurements made in vivo vs ex vivo

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Abstract *Rationale:* The number of receptors occupied by a given drug is a central construct in understanding drug action in the brain. Two techniques have been commonly used to measure drug receptor occupancy. In one method, the drug and the radioligand used to measure occupancy compete in vivo while in the other method, the drug is injected into the living animal, the animal killed and the radioligand competes for available receptors ex vivo. While these methods are often used interchangeably, there has been no systematic comparison of their sensitivities and consistency. *Objectives:* In this study, we performed a systematic within-animal comparison of drug-induced receptor occupancy as measured by the in vivo vs the ex vivo methods. *Methods:* We examined the occupancy of dopamine D_2 receptors by different doses of the drug raclopride using the in vivo and ex vivo autoradiographic methods in the same rat with ^{11}C-raclopride and ^{3}H-raclopride as radioligands, respectively. *Results:* The in vivo method showed a significantly greater sensitivity and internal consistency while the ex vivo method was less sensitive, and increasingly so as a function of longer incubation times. The lack of sensitivity was accounted for by the unidirectional dissociation of the drug from the receptors in the incubation medium. *Conclusions:* Our data suggest that these two methods are not interchangeable; the ex vivo method is much less sensitive, lacks internal consistency and hence is best avoided.

Keywords Receptor occupancy · In vivo · Ex vivo · Raclopride

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Introduction

The blockade of receptors by neuropsychiatric drugs is a crucial parameter for understanding their pharmacological actions (Kenakin 1997). Clinical studies suggest that slight differences in receptor blockade can have significantly different consequences. For example, a 10% change in dopamine D_2 receptor occupancy (from 75% to 85%) is associated with a sharp increase in extrapyramidal side-effects (Farde et al. 1992; Kapur et al. 2000). Therefore the determination of receptor occupancy with precision and reliability is of considerable importance in understanding drug action and in constructing valid animal models. Ideally, one would use the same technique for imaging humans as well as animals. However, PET imaging in small animals is, as yet, not a viable option due a lack of sufficient resolution, expense and limited availability.

Most previous studies that have attempted to measure drug receptor occupancy in animals have relied on radioligand competition as an index of drug occupancy, but have operationalised it in two different ways. In one method, the drug of interest and the competing radioligand are injected into the live animal and the degree to which the drug interferes with the radioligand binding in vivo is taken as an index of receptor occupancy (herein called the "in vivo method"). This method has been extensively applied to the study of antipsychotics (Stockmeier et al. 1993), antidepressants (Scheffel et al. 1994) and anxiolytics (Paul et al. 1979). With the other method, the drug is injected and the animal is killed after a given interval. Subsequently, brain sections from these drug-treated animals are mounted on slides, and the radioligand competition is carried out in a 'bath' ex vivo to obtain an index of occupancy (herein called the "ex vivo

method"). This method has also been applied to the study of antipsychotics (Kaichi et al. 2000; Schotte et al. 1996), antidepressants (Owens et al. 2000) and benzodiazepines (Funakoshi et al. 1999). The fundamental difference between the two techniques is the milieu in which the drug and the radioligand compete. In the in vivo method, the drug and the radioligand compete in the brain, which has ambient levels of both drug and radioligand. In the ex vivo method, the drug and the radioligand compete in an experimental bath which has a fixed level of the radioligand but no ambient drug levels. Therefore, in the in vivo method, any dissociation of the drug during the experiment is likely to be replenished by the ambient brain levels. However, in the ex vivo condition, there is a high likelihood that the drug of interest will dissociate with a negligible likelihood of any replenishment, a process likely to lead to an underestimation of the drug occupancy.

While both of these methods continue to be applied for assessing drug occupancy (Funakoshi et al. 1999; Kaichi et al. 2000; Owens et al. 2000; Paul et al. 1979; Scheffel et al. 1994; Schotte et al. 1996; Stockmeier et al. 1993), there has never been a systematic comparison of their sensitivity and validity. The objective of this study was to undertake a systematic within-animal comparison of drug-induced receptor occupancy as measured by the in vivo vs the ex vivo methods. As we observed a major discrepancy, we undertook further studies to examine the effects of drug dissociation ex vivo as they might contribute to the discrepancy between the methods.

Materials and methods

Adult male Sprague-Dawley rats (200–275 g) were used for the study. There were two sets of experiments. In the first experiment, we compared the receptor occupancy measurements obtained by the in vivo vs the ex vivo methods in the same rat. When the results of the first experiment suggested a significant discrepancy between the methods, and because the ex vivo method was very sensitive to the time of incubation in the bath, we undertook a second experiment to examine the rate of drug dissociation in the bath as an explanation for this discrepancy.

Experiment 1

Thirty-four animals were assigned to a vehicle control group (n=6) or a drug treatment group (n=28) which received varying doses of the drug (raclopride) whose occupancy was to be measured (0.01, 0.02, 0.03, 0.06, 0.10, 0.15, 0.2, 1 and 2 mg/kg s.c.; n=3–4/dose). Thirty minutes after the drug, tracer quantities of high specific activity ^{11}C-raclopride (specific activity ca 1000–1500 Ci/mmol; average injection 1300 µCi) was injected into the tail vein. Thirty minutes after the tracer injection, that is, 1 h after the injection of the unlabeled drug, the animals were killed by decapitation. The brain was rapidly removed and hemisected into right and left hemispheres, which were randomly assigned to measurement with the in vivo or the ex vivo method. A particular innovation in this study was the use of the short-lived radioligand ^{11}C-raclopride (half-life=20.4 min) which made it possible to measure occupancy using the in vivo method first and then, a few days later (when the signal had decayed), to measure ex vivo occupancy in tissue from the same animal.

Those tissue samples that were assigned to the in vivo method were then further dissected. Striatal and cerebellar tissue samples were immediately put in a gamma-emission well-counter (Cobra II; Packard Instruments, USA) and the amount of radiotracer accumulation in the striatum and the cerebellum was measured as cpm/mg of tissue. The striatal count density was taken as an index of specific plus non-specific tracer binding to dopamine D_2 receptors, while the cerebellar count density was taken as an index of non-specific binding. The ratio of specific to non-specific binding was used as an index of available D_2 receptors (D_2BP), as has been previously validated in animal and human studies (Kohler et al. 1985; Lammertsma et al. 1996). The index of dopamine receptor availability in each of the drug-treated animals was compared to the index obtained in the control animals to attain a measure of occupancy using the equation:

$$\% \text{ Occupancy} = 100 \times (D_2BP_{control} - D_2BP_{drug}/D_2BP_{control}) \quad (1)$$

The tissue assigned to the ex vivo condition was protected in aluminium foil, immediately frozen over dry ice and transferred to –80°C storage until further processed. Twenty-micron sections were cut throughout the striatum and cerebellum on a Hacker Bright cryostat and mounted onto subbed microscope slides. For autoradiographic analyses, slides were first allowed to equilibrate to room temperature, then incubated in 50 mM TRIS-HCl buffer containing 2 nM [^{3}H]raclopride (NEN Dupont, USA; 78.4 Ci/mmol) for different durations (10, 30, 120 min). In each case, adjacent sections were incubated with the same radioligand in the presence of 1 µM sulpiride to define non-specific binding. Sections were then washed in two quick rinses of fresh buffer, followed by one dip in ice-cold distilled water before drying under a stream of cold air. Sections were then apposed to 3H-Hyperfilm (Amersham) for 3 weeks in the presence of calibrated slides. Autoradiograms were analysed by an observer blind to treatment conditions using an AISC system (Imaging Research St. Catharines, Canada). For each brain, measurements were taken at 16 rostrocaudal levels within the striatum and 8 levels within the cerebellum.

Under these assay conditions cerebellum binding as well as non-specific binding images were indistinguishable from film background. Therefore, it is reasonable to assume that the striatal counts represent binding to the dopamine D_2 receptors. Occupancy was thus obtained by comparing the average striatal count density (D_2CD) in drug-treated animals to data from vehicle-treated controls using an equation similar to that used in the in vivo method:

$$\% \text{ Occupancy} = 100 \times (D_2CD_{control} - D_2CD_{indiv}/D_2CD_{control}) \quad (2)$$

As predicted by bimolecular competition theory, one would expect the dose of the drug to give rise to occupancy as a saturating hyperbola determined by the equation (Kenakin 1997):

$$\% \text{ Occupancy} = 100 \times (\text{Dose}/(\text{Dose}+ED_{50})) \quad (3)$$

To examine how the data conformed to this theoretical expectation the observed values were fit to this equation, and the best estimate of ED_{50} as well as its confidence interval was obtained for the different methods.

Experiment 2

The pattern of results from experiment 1 suggested that the dissociation of drug from receptor during the process of incubation in the ex vivo method might be contributing to the significant underestimation of drug occupancy by the ex vivo method. To study this process explicitly, two rats were injected i.v. with 30 µCi [^{3}H]raclopride (78.4 Ci/mmol) and were killed 15 min later. The brains were frozen and sectioned as described in experiment 1. Adjacent sections from the same brain were then exposed to varying degrees of buffer incubation (0–120 min) with buffer changes every 15–30 min. These washes/incubations reflected the procedures followed in experiment 1, and were emulated to provide an idea of the degree of drug dissociation during the measurements being made in experiment 1. The amount of drug attached to the tissue sections was measured using autoradiography as described above.

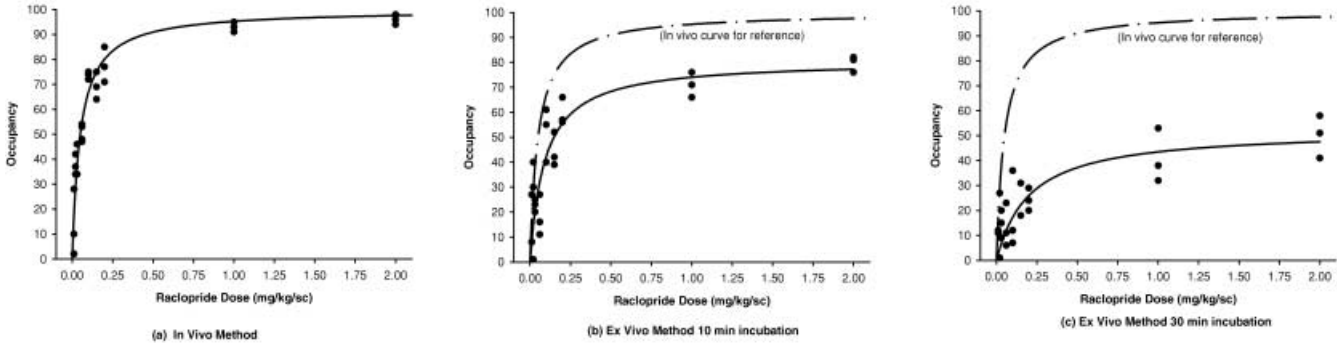

Fig. 1a–c The relationship between dose and occupancy for each of the three methods for determining occupancy. The ex vivo method with a 2-h incubation gave no evidence of any drug or dose effect on occupancy (data not shown). The *solid curves* in each case represent the best-fit hyperbola curve to the data; in **b** and **c** the *broken-line curves* show the in vivo method curve for reference

Results

The detailed data, relating dose to occupancy measured by the different methods, are presented in Fig. 1. A repeated-measures ANOVA, with different methods (in vivo vs ex vivo) as a within-subject factor and dose as a between-subject factor, showed a significant main effect of dose on occupancy [$F(1,8)=15.9$, $P<0.001$], as well as a significant main effect of method [$F(1,19)=97.9$, $P<0.001$]. As is evident in Fig. 1, a post hoc analysis showed that all three ex vivo occupancy protocols resulted in a significantly lower level of receptor occupancy as compared to the in vivo method [all comparisons $F(1,19)>50$; $P<0.001$].

Since the relationship between dose and occupancy is not a linear one, the differences are better assessed by modelling the data to the theoretically expected saturating hyperbola equation. Using that method, the dose of raclopride that gives rise to 50% occupancy, i.e. the parameter ED_{50} in Eq. (2) was 0.049 mg/kg (95% CI 0.042–0.057; r^2 for the fit 0.93) by the in vivo method, 0.15 mg/kg (95% CI 0.10–0.19; $r^2=0.72$) by the ex vivo 10-min method, and 1.2 mg/kg (95% CI 0.72–1.7; $r^2=0.50$) by the ex vivo 30-min method, with no detectable drug occupancy being seen with the ex vivo 2-h method.

Figure 2 shows the dissociation of the drug from the receptor when it is exposed to a series of washes. As would be predicted by a model of bimolecular dissociation with no re-association the amount of drug bound to receptors shows a very consistent ($r^2=0.91$) monoexponential decline (bound drug=4.7×exp(-0.034×time)), suggesting that the occupancy declines by 50% every 15 min that the tissue is exposed to the wash. As would be expected from this observed dissociation, the longer the incubation the lower the occupancy detected [$F(1,19)=56.4$; $P<0.001$]. As an illustration, for animals that received 0.2 mg/kg raclopride the determined occupancy was 77±7% for the in vivo method, 59±6% with the ex vivo 10-min method, 24±4% in the ex vivo 30-min method and no detectable occupancy with the ex vivo 2-h method.

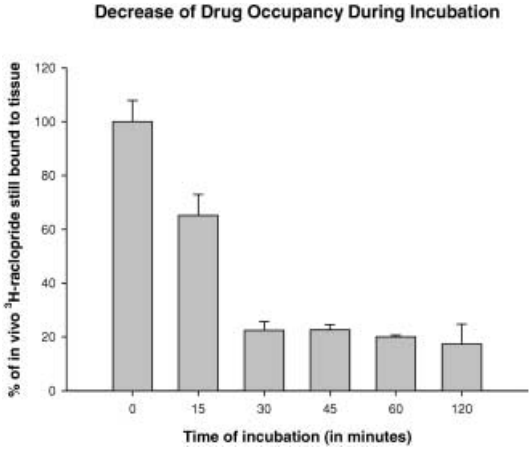

Fig. 2 The decline in receptor occupancy as a function of time in the incubation buffer

Discussion

Our data clearly show that the two methods of measuring drug occupancy, which are used commonly and often interpreted interchangeably, give significantly different estimates of occupancy. The in vivo method is significantly more sensitive and shows a much greater internal validity. One of the main contributors to this discrepancy seems to be dissociation of the drug in the process of ex vivo incubations.

These different measures of occupancy can have significant consequences for interpretation of functional effects. For example, in a recent study examining the threshold for the onset of catalepsy in animals treated with raclopride we found that the threshold for catalepsy was about 0.5 mg/kg s.c. (Wadenberg et al. 2000). Depending on the technique chosen to determine occupancy, one would infer that catalepsy occurs with 91% D_2 blockade (in vivo method), or 68% D_2 blockade (ex vivo 10-min method), or 37% D_2 blockade (ex vivo 30-min method), or in the absence of D_2 blockade as per the ex vivo 2-h method. While higher sensitivity does not, by itself, mean greater validity, the in vivo method

shows several other features that support its validity: (1) despite the increased sensitivity, the in vivo technique shows lower variance at each dose (Fig. 1), (2) the in vivo data conform very precisely to the predicted relationship between dose and occupancy (r^2=0.93) while the ex vivo data show successively poor fits (r^2=0.72 for ex vivo 10-min; r^2=0.50 for ex vivo 30-min; no significant fit for the 2-h incubation) and, finally, (3) an extrapolation of the data (Fig. 1) shows that only the in vivo method leads to complete occupancy, and even the best ex vivo method tends to reach a ceiling at 80% occupancy, significantly different (P<0.05) from the expected 100%. Thus, in the absence of an external gold standard, the in vivo method appears to be more valid than the ex vivo methods.

How generalisable are these findings to the occupancy of other drugs? It seems that a major reason for the discrepancy between the two methods is drug dissociation during measurement. Therefore, drugs that dissociate faster than raclopride would be subject to an even greater underestimation of occupancy while drugs which show only a limited dissociation in the incubation time would be relatively resistant to this discrepancy. In a recently published report we examined the rate of dissociation of a series of antipsychotics from D_2 receptors in striatal homogenates in vitro, and found that quetiapine, clozapine and olanzapine had even faster dissociation rates than raclopride, while haloperidol and spiperone were considerably slower (Kapur and Seeman 2000). As a result one would predict that not only would the in vivo–ex vivo discrepancy be seen with other antipsychotics (and for that matter, most other drugs) but that the degree of discrepancy would depend upon on the k_{off} (dissociation constant) of the drug, and thus may differ from one drug to the other.

Since the discrepancy in occupancy results from dissociation of the drug during incubation, shortening the incubation could decrease this discrepancy. Inspection of the results suggests that this is indeed the case (for example, occupancy induced by 0.5 mg/kg s.c. would seem to be 0% if incubated for 2 h, 37% if incubated for 30 min, 68% if incubated for 10 min vs 91% in the in vivo method). This may suggest that shortening the time further, to 5 min or even less, may actually resolve the sensitivity problem. However, while a short incubation may address one problem, it would introduce another – the radioligand will then not have time to reach equilibrium. The problem is further compounded by the fact that the in vivo competition proceeds under tracer conditions (less than 1% of the receptors are occupied by the radioligand) wherein the equilibrium level of radioligand binding can be assumed to linearly reflect drug occupancy (Farde et al. 1992). On the other hand, ex vivo competitions proceed under concentrations where the radioligand itself occupies 50% receptors (at or near K_d). As a result of this, the equilibrium level of radioligand binding reflects drug occupancy in a non-linear fashion (Limbird 1986), further complicating simple inferences or comparisons.

In summary, our data suggest that the in vivo and ex vivo methods of determining occupancy are not equivalent. The ex vivo occupancy measurement is confounded by significant dissociation of the drug during the process of occupancy measurement. Future studies relating occupancy to the pharmacological and behavioural effects of drugs, would do well to avoid the ex vivo methods.

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