
BRIEF REPORT

Dopamine D₂ Receptor Blockade by Haloperidol: ³H-Raclopride Reveals Much Higher Occupancy than EEDQ

Shitij Kapur, M.D., Ph.D., Susan C. Barsoum, M.Sc., and Philip Seeman, M.D., Ph.D.

Two techniques are commonly used to measure antipsychotic induced dopamine D₂ occupancy in animals: competition with a reversible radioligand (³H-raclopride) or with an irreversible receptor inactivator (EEDQ). While both of these techniques have been used in the past, there is no direct and systematic comparison. In the first direct comparison of these two methods we find that the dose of haloperidol required for blocking 50% of the dopamine D₂ receptors was 0.02 mg/kg/sc (95% CI 0.018–0.022 mg/kg) as measured using ³H-raclopride method; but was significantly higher with the EEDQ method 0.14 mg/kg/s.c. (95% CI 0.048–0.224 mg/kg).

The ³H-raclopride method showed significantly lesser variance ($p = 0.02$) despite the higher sensitivity. This seven-fold difference in the sensitivity of the two techniques to measure antipsychotic-induced D₂ occupancy explains discrepancies in the previous studies which have used these two methods and also suggest that for future studies the ³H-raclopride method is a more sensitive and, likely, a more valid reflector of true receptor occupancy.

[Neuropsychopharmacology 23:595–598, 2000] © 2000 American College of Neuropsychopharmacology. Published by Elsevier Science Inc.

KEY WORDS: Dopamine receptors; Occupancy; Raclopride; EEDQ

The blockade of receptors by antipsychotics is a crucial parameter for understanding their pharmacological actions. Slight differences in receptor blockade can have important functional consequences. For example, in clinical studies a change in receptor occupancy from 75% to 85% is associated with a sharp rise in Parkinsonian side effects (Kapur et al. 2000b). Similar findings have been reported in animal studies where a few percent shift in occupancy was associated with a signifi-

cant appearance of catalepsy (Kapur et al. 2000a). Therefore, the determination of receptor occupancy with precision and reliability is of great importance. In animal studies two techniques have been commonly used for the determination of drug-receptor occupancy *in vivo*. One technique (referred to here as the radiotracer method) relies on the competition between a selective reversible radiotracer and the antipsychotic, *in vivo*, to measure occupancy (³H-raclopride or ³H-spiroperone are common choices for the dopamine D₂ receptor) (Kohler et al. 1981; Kohler and Karlsson-Boethius 1988). The other technique relies on the competition between an irreversible ligand which inactivates the receptors, and the antipsychotic, *in vivo*. Alkylating agent N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) is the most common choice for the dopamine D₂ receptor (Meller et al. 1988; Saller et al. 1989), though other inactivating agents (e.g., phenoxybenzamine) have also been used (Hamblin and Creese 1982). There has been no direct comparison of the two, even though the results and inferences from the two methods are of-

From the Schizophrenia Program (SK, SCB, PS) and PET Centre (SK), CAMH, Toronto, Ontario, Canada; Department of Psychiatry (SK) and Department of Pharmacology (PS), University of Toronto, Toronto, Ontario, Canada.

Address correspondence to: Dr. Shitij Kapur, PET Centre, The Clarke Division of the CAMH, 250 College Street, Toronto, ON Canada M5T 1R8. Tel.: 416-535-8501, x4361; fax: 416-979-4656; E-mail: skapur@camhpet.on.ca

Received February 23, 2000; revised April 20, 2000; accepted May 1, 2000.

ten used interchangeably. The purpose of this experiment was to compare these two commonly used methods in their ability to measure haloperidol-induced dopamine D₂ receptor occupancy.

METHODS

Adult male Sprague-Dawley rats (200–225 g) were used for this study. The animals were divided into parallel groups for ³H-raclopride and EEDQ studies. The animals were treated with stratified doses of haloperidol (0.0125; 0.025; 0.0625; 0.1; 0.5; 1 and 2 mg/kg/s.c.; *n* = 3–5 for each dose) and appropriate vehicle controls (saline for haloperidol; 1:1 ethanol:water for EEDQ).

In the radiotracer group, the animals were first given a dose of haloperidol (0.0125–1 mg/kg or saline control) followed by high specific activity ³H-raclopride (78 Ci/mM), 7.5 µCi/animal/i.v., 90 min later. The animals were sacrificed at 2 h after the injection of haloperidol. This sacrifice time was chosen since it has previously been shown that 30 min is sufficient time for the ³H-raclopride specific/non-specific binding to reach a state of stable pseudoequilibrium (Kohler et al. 1985). The striata and the cerebellum were immediately dissected, dissolved in 2 ml of Solvable (an NaOH and alcohol solution from Canberra Packard, Canada) for 24 h at 23°C, thereafter 5 ml of Aquasure (formerly Formula 965, Canberra Packard, Canada) scintillation fluid was added, and the concentration of accumulated radioactivity (cpm/mg) was determined by liquid scintillation spectrometry. The ratio of specific (striatal minus cerebellar) to non-specific binding (cerebellar) at the time of sacrifice was taken as a measure of the available dopamine D₂ receptors. Occupancy, in haloperidol-treated animals, was calculated as: Occupancy (%) = 100 × (1 – D_{2Halo}/D_{2Vehicle}); where D_{2Halo} is the specific/non-specific ratio in haloperidol treated rats, and D_{2Vehicle} is the corresponding value in controls. This approach and the equation is similar to the method used in humans to obtain D₂ occupancy using PET (Kapur et al. 2000b).

Calculating occupancy using EEDQ is based on the principle that EEDQ inactivates any unoccupied D₂ receptors. Thus by measuring the amount of protection from inactivation afforded by an antipsychotic one can obtain a measure of antipsychotic receptor occupancy (Meller et al. 1988; Saller et al. 1989). As in past studies, three groups of animals were needed in this case. One group was treated with vehicles for haloperidol and EEDQ (VV) and provided the number of baseline D₂ receptors. A second group was treated with haloperidol vehicle and EEDQ (VE) and provided the number of D₂ receptors when there was no drug occupancy (i.e., zero protection). The third group was treated with varying doses of haloperidol as well as EEDQ (HE). In each

group haloperidol or vehicle was injected first, 2 h later EEDQ (10 mg/kg/i.p.) or vehicle was injected. The animals were sacrificed 24 h later in keeping with previous studies using this method (Meller et al. 1988; Saller et al. 1989). The striata were dissected in each of the three groups and the B_{max} and K_d of the dopamine D₂ receptors were obtained using ³H-Spiperone and a Scatchard analysis as described previously (Seeman and Van Tol 1995). The D₂ occupancy was obtained using the formula: Occupancy (%) = 100 × (HEB_{max} – VEB_{max})/(VVB_{max} – VEB_{max}). HEB_{max} – VEB_{max} measures the level of protection afforded by a given dose and VVB_{max} – VEB_{max} is the maximum level of protection possible.

RESULTS

There was a substantial difference in the sensitivity of the two techniques as illustrated in Figure 1. The radiotracer method revealed a much higher occupancy by haloperidol at all the doses, with the difference particularly remarkable at lower doses. The dose of haloperidol required to produce a 50% occupancy was 0.02 mg/kg (95% CI 0.018–0.022 mg/kg). The dose of haloperidol required to produce 50% occupancy using the EEDQ method was significantly higher 0.14 mg/kg (95% CI 0.048–0.224 mg/kg). While both methods showed an orderly dose-response, on theoretical grounds one would expect the dose-occupancy data to conform to a saturating hyperbola (i.e., occupancy = dose/(dose + ED₅₀)). The ³H-raclopride occupancies conform to theory more closely than EEDQ data (R² of the fit = 0.95 for radiotracer; 0.79 for EEDQ). Finally, the variance in occupancy at a given dose level was much lower for the radiotracer method than the EEDQ method (t_{df = 4} = 3.74, *p* = .02).

DISCUSSION

The radiotracer method using ³H-raclopride shows a seven-fold higher sensitivity for detecting haloperidol induced D₂ occupancy *in vivo*. This difference in the sensitivity of the two methods explains significantly disparate estimates of drug potencies in previous reports using these two different techniques (Kohler and Karlsson-Boethius 1988; Saller et al. 1989). A higher sensitivity does not by itself mean greater validity, but the ³H-raclopride data show several other features that enhance its validity: (1) despite this increased sensitivity, the technique shows lower variance; (2) the radiotracer data conform very precisely to the predicted relationship between dose and occupancy, while the EEDQ data do not; (3) finally, the ED₅₀ for haloperidol obtained with the radiotracer method (0.02 mg/kg) is

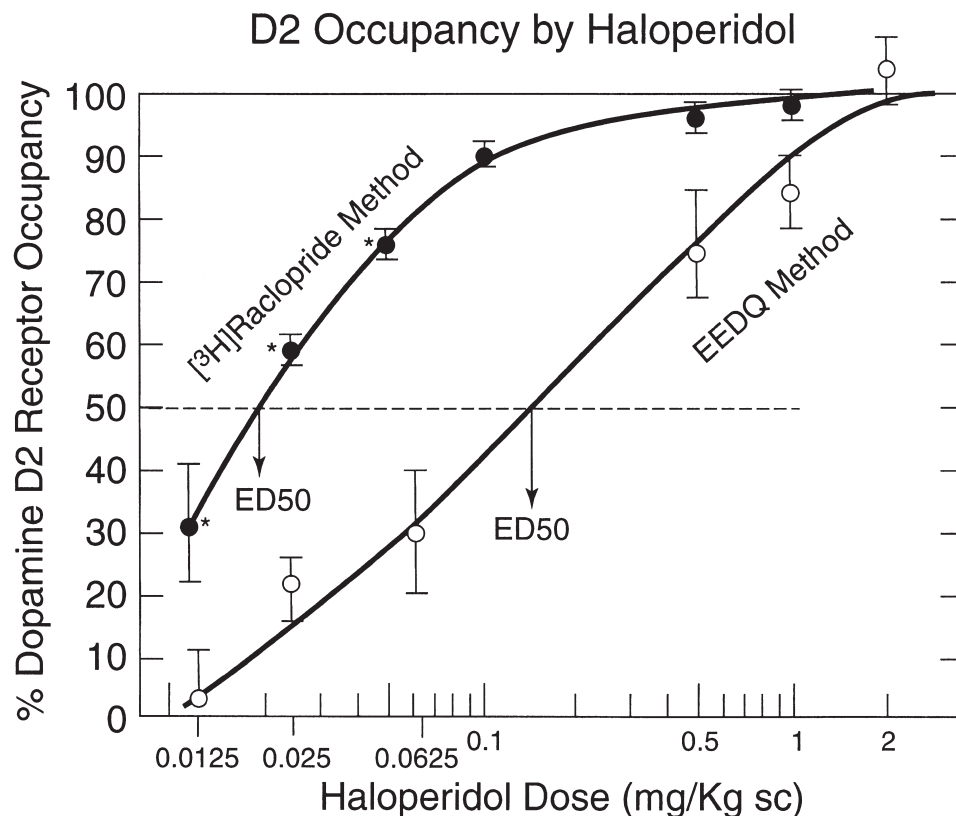


Figure 1. Relationship between dose and D₂ occupancy as determined by the two methods. Error bars represent standard errors; *Significant post-hoc difference ($p < 0.05$).

much closer to the value obtained in patients with PET imaging (0.013 mg/kg) (Kapur et al. 2000b) than the value obtained by the EEDQ method (0.17 mg/kg).

An important limitation of the current study is the use of different radioligands to measure *in vivo* receptor occupancy (raclopride) and the number of receptors protected by EEDQ (spiperone). Our choice of ligands was in keeping with the practice of past studies using these two methods (Meller et al. 1988; Saller et al. 1989) (Kohler et al. 1981; Kohler and Karlsson-Boethius 1988). While differences between these two ligands have been reported before, these differences are unlikely to be an explanation for the observed difference. First, even though spiperone is used in the EEDQ method, the method is based on the competition (protection) between EEDQ and haloperidol, and not spiperone and haloperidol. Second, the design of EEDQ controls for any ligand-related differences by measuring B_{\max} of all the three groups (VV, VE and HE) with the same ligand. Despite these theoretical reasons, a complex three-way interaction (i.e., haloperidol \times EEDQ \times measuring ligand) cannot be ruled out and future studies may empirically control for this possible difference.

The precise reason for this substantial difference cannot be inferred from our results. However, a few possibilities need to be considered. EEDQ is an irreversible inhibitor/inactivator (Meller et al. 1988) of the dopamine D₂ (and other monoamine) receptors while haloperidol/raclo-

pride/spiperone are all competitive antagonists. While it has been shown that haloperidol shows a dose-dependent competition with EEDQ, it has yet to be shown that both haloperidol and EEDQ vie for the same site on the receptor in a simple competitive fashion. If they do not vie for the same site/s, then the EEDQ-haloperidol competition will not follow the simple mass-action laws of bimolecular competition, a situation which will invalidate the use of EEDQ for measuring occupancy.

Furthermore, the determination of protection after EEDQ is done *ex vivo* after a 24-h delay. This is a standard in all previous studies and is observed to make sure that the challenge drug, haloperidol in this study, has exited the system and is not directly competing with the *ex vivo* ligand. Since the receptors have been shown to recover with a half-rate of 95 hours (Pich et al 1987), 24 h may lead to recovery of about 10–20% of the receptors. While this recovery may lead to an error in occupancy determination, the direction of this error will lead to a false assumption of *greater* protection/occupancy with the EEDQ method. Since observed results show a *lesser* occupancy with EEDQ, receptor recovery in the 24 h is unlikely to be an explanation for this difference.

In summary, the *in-vivo* method for determining D₂ occupancy is substantially more sensitive than the EEDQ method. Until the reasons for the observed discrepancy are better understood, caution is advised in using EEDQ-derived dopamine D₂ occupancy estimates

since they may represent a serious underestimate of the true *in vivo* situation.

ACKNOWLEDGMENTS

We thank Corey Jones, Alex Kecojevic, Doug Hussey, Kevin Cheung, and Dr. H-C Guan for their expert technical assistance. This research is supported by grants from the MRC of Canada, Stanley Foundation, USA, and the EJLB Foundation, Canada.

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