In Vitro Receptor Binding and In Vivo Receptor Occupancy in Rat and Guinea Pig Brain: Risperidone Compared with Antipsychotics Hitherto Used

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ABSTRACT—Risperidone was compared with antipsychotics hitherto used for in vitro receptor binding using animal brain or cloned (human) receptors and in vivo receptor occupancy in rat and guinea pig brain following acute treatment. Both in vitro and in vivo, risperidone, 9-OH-risperidone, SM-9018, clozapine and clozapine showed higher affinity for 5-HT_{2A} receptors than for D_{2}-receptors, whereas mosapramine, haloperidol, bromperidol and nemonapride had a slight to strong preference for D_{2}-receptors compared to 5-HT_{2A}-receptors. In vivo, risperidone showed the highest potency for 5-HT_{2A}-receptor occupancy; To obtain the same extent of D_{2}-receptor occupancy, a 19-times higher dosage was required. 9-OH-Risperidone, the principal active metabolite of risperidone, showed a receptor occupancy profile comparable to that of risperidone. No regional selectivity for D_{2}-receptor occupancy in mesolimbic vs nigrostriatal areas was detected for any of the compounds. Risperidone differed from the other compounds by the remarkably shallow slope of its D_{2}-receptor dose-occupancy curve. A greater predominance of 5-HT_{2A}-receptor vs D_{2}-receptor occupancy and a more gradual occupancy of D_{2}-receptors differentiate risperidone from the other compounds. Both properties probably assist in preventing an extensive blockade of D_{2}-receptors, the cause for extrapyramidal symptoms (EPS). The predominant 5-HT_{2A}-receptor occupancy most likely underlies risperidone's beneficial effects on the negative symptoms of schizophrenia and an adequately low D_{2}-receptor occupancy adds to the treatment of positive symptoms with a low liability of EPS.

Keywords: Receptor binding, Autoradiography, Risperidone, Antipsychotic, Schizophrenia

Schizophrenia is a devastating psychiatric disorder often with onset in puberty and lasting throughout life. It means a dramatic burden for the patient and others in his close environment. In addition to the mental suffering, the significant cost of the disease to society is to be considered (1, 2). Although the disease can be manifested in several ways, schizophrenia is often characterized by the appearance of positive (hallucinations, delusions, disorganized speech, disorganized behavior) and negative symptoms (alogia, affective blunting, avolition, anhedonia/asociality, attention impairment); both symptoms, although independent, may coexist in the same patient (3–5). The etiology of schizophrenia is still not understood. Conventional neuroleptics have been widely used since the early 1960s for treatment of the positive symptoms of schizophrenia, and a correlation was established between the average daily therapeutic dose and their binding affinities to dopamine D_{2}-receptors (6). Although representing a breakthrough in the treatment of schizophrenic disorders, conventional neuroleptics displayed major shortcomings: the induction of neurological side-effects (dystonia, parkinsonism, akathisia, tardive dyskinesia) and a lack of efficacy for the treatment of the negative symptoms of schizophrenia (6, 7). With the recent discovery of D_{3}- and D_{4}-receptors, the new subtypes of dopamine receptors (8, 9), hypotheses were raised about possible involvement of these receptors in the pathophysiology of schizophrenia (10); they have been proposed as potential targets for antipsychotic drugs.

Recently, renewed attention was paid to the role of serotonin in schizophrenia. Upon the discovery of 5-HT_{2} (now termed 5-HT_{2A})-receptors in 1978 (11), we noted that several neuroleptics bound to both 5-HT_{2A} and D_{2}-receptors. Pipamperone (a butyrophenone derivative) stood out because of its predominant 5-HT_{2A}-receptor interaction. This compound was noted in the clinic for its beneficial effect in socially withdrawn and agitated psy-
chiatric patients and for normalizing disturbed sleep rhythms (12). It suggested a beneficial role of 5-HT2A-receptor blockade for the treatment of schizophrenia. In subsequent studies, the potent and long acting 5-HT2A/2c-receptor antagonist ritanserin was found to alleviate negative symptoms in schizophrenics (13) and to reduce extrapyramidal symptoms (EPS) induced by neuroleptic treatment (14). In animal studies it was shown that 5-HT2A antagonists reduced neuroleptic-induced catalepsy (15-17), that they were involved in the restoration of burst firing of dopaminergic neurons (18, 19) and that they would attenuate the effects of D2-receptor blockade in the striatum (20). 5-HT2A antagonism was suggested to play a major role in the distinction between "atypical" and conventional neuroleptics (21). With the introduction of risperidone (Risperdal®), it appeared that potent 5-HT2A antagonism combined with a milder D2 antagonism (22, 23) resulted in remarkably improved clinical properties. In addition to the treatment of the positive symptoms, risperidone was also capable of treating the negative symptoms of schizophrenia with a very low liability of extrapyramidal symptoms (24-26).

In the present study, risperidone was compared to antipsychotics hitherto used to estimate the benefits of this new compound versus the available medication for the treatment of schizophrenia. For this purpose, in vitro receptor binding assays using tissue or cell membrane preparations were performed to detect the target neurotransmitter receptors of selected antipsychotic drugs. Secondly, the occupancy of these target receptors by the test-compounds was investigated in rat brain, 2 hr after s.c. administration. In vivo occupancy of the receptors was revealed by subsequent ex vivo radioligand binding in brain sections and quantified by autoradiography, according to a technique developed in this laboratory (27).

Fig. 1. Chemical structures of the test compounds.
MATERIALS AND METHODS

In vitro receptor binding

Risperidone, 9-OH-risperidone, haloperidol, bromperidol, nemonapride, SM-9018, clozapine, clocapramine and mosapramine (chemical structures are shown in Fig. 1) were screened for inhibition of radioligand binding in vitro to neurotransmitter-, peptide- and lipid-derived factor receptors, ion channels and neurotransmitter transporters. Systems, radioligands and assay conditions are summarized in Table 1.

Neurotransmitter receptors that are targets for the compounds were further assayed in vitro using homogenates from animal brain or cloned (human) receptors stably expressed in mammalian cell lines. Compounds were incubated under the assay conditions described in Table 1, at 10 to 12 concentrations between 10^{-11} to 10^{-5} M. Incubations were stopped by rapid filtration of the binding mixture over glass fiber filters (manual filtration manifold or Tomtec semi-automatic filtration); The filters were then rapidly rinsed, and membrane-bound radioactivity collected on the filters was determined by scintillation counting for tritiated ligands (Packard Tricarb or Wallac scintillation counter) or by using a gamma counter for iodinated ligands (Packard Cobra). Counts were directly captured in a computer. Data were further calculated and analyzed using automated procedures. Radioligand binding in the presence of non-labelled compound was expressed as a percent of the total binding and plotted against the log of the concentration of the compound. Hence, sigmoidal curves were generated. The curve of best fit was calculated by computerized curve fitting using equations as described by Oestreicher and Pinto (56). pIC_{50}-values (−log of the concentration producing 50% inhibition of specific radioligand binding) were derived from the curves. K_i-values were calculated according to Cheng and Prusoff (57). Experiments were repeated 2–5 times independently.

Drug treatment

Male Wistar rats (200 g) and male Dunkin-Hartley guinea pigs (300 g), both bred in own facilities, were treated with subcutaneous injections of saline or test compounds at 6 or 7 dose levels ranging from 0.0025 to 40 mg/kg body weight. Six to twelve animals were used per dose. The animals were sacrificed by decapitation 2 hr after drug administration. Brains were immediately removed from the skull and rapidly frozen in dry-ice cooled 2-methylbutane (−40°C). Twenty-micron-thick frontal sections were cut out of these brains using a Reichert-Jung 2800E cryostat-microtome (Cambridge Instruments, Cambridge, UK) and thaw-mounted on adhesive microscope slides (Star Frost, Knittel Gläser, Germany). The sections were then kept at −20°C until use.

Ex vivo radioligand binding in brain sections and quantitative autoradiography

The receptors for which the compounds revealed K_i-values below 100 nM were studied for in vivo occupancy after acute treatment: where appropriate, occupancy of 5-HT_1A, 5-HT_2A, 5-HT_2C, D_1, D_2, D_3, α_1, α_2, cholinergic muscarinic- or H_1-receptors was measured. The in vivo occupancy of D_4-receptors could not be investigated since no reliable model was available for D_4-binding in brain sections. Receptor occupancies were quantified in brain areas showing high receptor density, which were selected after regional distribution studies performed by ourselves and by others (58–62). The selected brain regions and their precise localization are listed in Table 2. Occupancy of all selected receptors was quantified in each individual brain (6–12 animals per dose, 6–8 dosages).

Neurotransmitter receptors were labelled in brain sections, and autoradiograms were generated according to the protocols summarized in Table 2. The following general procedure was applied: after thawing, sections were dried under a cold air stream. The sections were not washed prior to incubations in order to avoid dissociation of the drug-receptor complex. D_2-receptors, however, cannot be labelled in unwashed brain sections, due to occlusion by an endogenous substance likely to be dopamine (69). A minimal preincubation (30 sec) was therefore applied to render D_2-receptors partially accessible for [3H]7-OH-DPAT. Radioligand solution (200 µl) was applied on each section; incubation was restricted to 10 min at room temperature in order to minimize dissociation of the drug from the receptor. Brain sections of drug-treated and saline-treated animals were incubated in parallel, and the incubation time was rigorously controlled. Full association of the radioligand to the receptor was, in general, not achieved after 10 min of incubation. As a consequence, radioactive labelling of the receptors remained below its maximal level. This loss in radioactive signal was compensated for by using longer exposure times to generate the autoradiograms. Non-specific binding was measured in adjacent sections, in the presence of an excess of an unlabelled competitor with chemical structure different from the radioligand (Table 3). After the incubation, the excess of radioactivity was washed-off in consecutive baths of ice-cold buffer, followed by a quick rinse in ice-cold water. The sections were then dried under a cold-air stream, placed in a light-tight cassette and covered with a light-sensitive film. Ektascan GRL films (Kodak) were used for the generation of autoradiograms by iodinated ligands. After the exposure time, these films were deve-
Table 1. Assay conditions for radioligand binding and neurotransmitter uptake

<table>
<thead>
<tr>
<th>Receptor site</th>
<th>Species, area, membrane preparation, mg tissue per assay</th>
<th>Assay conditions</th>
<th>Labelled ligand</th>
<th>Blank</th>
<th>References</th>
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<tbody>
<tr>
<td>Serotonin-5-HT&lt;sub&gt;1A&lt;/sub&gt;</td>
<td>Rat, hippocampus, TP, 10</td>
<td>A, pH 7.7, 1.1 ml, 37°C, 30 min</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]8-OH-DPAT, 0.5 nM, (0.84 nM)</td>
<td>Spiroxatrine, 1 μM</td>
<td>28</td>
</tr>
<tr>
<td>Serotonin-5-HT&lt;sub&gt;1A&lt;/sub&gt;</td>
<td>Cloned human 5-HT&lt;sub&gt;1A&lt;/sub&gt;, HEK-293 cells</td>
<td>B, pH 7.7, 0.5 ml, 37°C, 30 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]8-OH-DPAT, 0.5 nM, (1.5 nM)</td>
<td>Spiroxatrine, 1 μM</td>
<td>29</td>
</tr>
<tr>
<td>Serotonin-5-HT&lt;sub&gt;1B&lt;/sub&gt;</td>
<td>Rat, striatum, TP, 4</td>
<td>I, pH 7.7, 0.5 ml, 37°C, 30 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]-5-HT + 8-OH-DPAT 30 nM + Mesulergine 30 nM, 4 nM, (2.6 nM)</td>
<td>5-HT, 10 μM</td>
<td>30</td>
</tr>
<tr>
<td>Serotonin-5-HT&lt;sub&gt;2A&lt;/sub&gt;</td>
<td>Rat, frontal cortex, TP, 2</td>
<td>A, pH 7.7, 0.5 ml, 37°C, 30 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Ketanserin, 1 nM, (0.42 nM)</td>
<td>Methysgeride, 1 μM</td>
<td>31</td>
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<tr>
<td>Serotonin-5-HT&lt;sub&gt;2C&lt;/sub&gt;</td>
<td>Pig, chorioid plexus, M + L + P, 4</td>
<td>B, pH 7.7, 0.5 ml, 37°C, 30 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Mesulergine, 1 nM, (2.1 nM)</td>
<td>Ritalazine, 1 μM</td>
<td>32</td>
</tr>
<tr>
<td>Serotonin-5-HT&lt;sub&gt;3&lt;/sub&gt;</td>
<td>N × G 108CC15 cells, ± 200,000 cells</td>
<td>J, pH 7.4, 0.5 ml, 37°C, 60 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]IGR 65630, 2 nM, (1.7 nM)</td>
<td>MCPP, 1 μM</td>
<td>33</td>
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<tr>
<td>Dopamine-D&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Rat, striatum, TP, 2</td>
<td>C, pH 7.7, 0.5 ml, 37°C, 30 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]SCH23390, 0.25 nM, (0.63 nM)</td>
<td>Pfluftuxol, 1 μM</td>
<td>34</td>
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<tr>
<td>Dopamine-D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Rat, striatum, TP, 5</td>
<td>C, pH 7.7, 0.5 ml, 37°C, 30 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Haloperidol, 2 nM, (1.3 nM)</td>
<td>(+)butaclanol, 10 μM</td>
<td>35</td>
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<tr>
<td>Dopamine-D&lt;sub&gt;2L&lt;/sub&gt;</td>
<td>Cloned human D&lt;sub&gt;2L&lt;/sub&gt;, CHO cells</td>
<td>C, pH 7.7, 0.5 ml, 37°C, 30 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Spiperone, 0.2 nM, (0.09 nM)</td>
<td>(+)butaclanol, 1 μM</td>
<td>36</td>
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<tr>
<td>Dopamine-D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Cloned rat D&lt;sub&gt;3&lt;/sub&gt;, COS-7 cells, TP</td>
<td>C, pH 7.4, 0.25 ml, 37°C, 60 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Iodosulpiride, 0.2 nM, (1.1 nM)</td>
<td>TL99, 1 μM</td>
<td>37</td>
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<tr>
<td>Dopamine-D&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Cloned human D&lt;sub&gt;4&lt;/sub&gt;, CHO cells</td>
<td>C, pH 7.4, 0.25 ml, 37°C, 60 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Iodosulpiride, 0.2 nM, (1.1 nM)</td>
<td>TL99, 1 μM</td>
<td>38</td>
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<tr>
<td>Dopamine-D&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Cloned human D&lt;sub&gt;4&lt;/sub&gt; CHO cells, TP</td>
<td>C, pH 7.4, 0.5 ml, 37°C, 30 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Spiperone, 0.5 nM, (0.35 nM)</td>
<td>Haloperidol, 1 μM</td>
<td>39</td>
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<tr>
<td>α&lt;sub&gt;1&lt;/sub&gt;-Adrenergic</td>
<td>Rat, cortex, TP, 2</td>
<td>C, pH 7.7, 0.5 ml, 25°C, 30 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Prazosine, 0.25 nM, (0.179 nM)</td>
<td>Aceperone, 1 μM</td>
<td>40</td>
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<tr>
<td>α&lt;sub&gt;2&lt;/sub&gt;-Adrenergic</td>
<td>Rat, cortex, TP, 10</td>
<td>A, pH 7.1, 1.1 ml, 25°C, 30 min</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Clonidine, 3 nM, (1.8 nM)</td>
<td>Norpazephrine, 2 μM</td>
<td>41</td>
</tr>
<tr>
<td>β&lt;sub&gt;1&lt;/sub&gt;-Adrenergic</td>
<td>Cloned human-β&lt;sub&gt;1&lt;/sub&gt;, E. coli</td>
<td>K, pH 7.4, 0.25 ml, 37°C, 60 min, AF</td>
<td>[&lt;sup&gt;125&lt;/sup&gt;I]iodocyanopindolol, 0.025 nM, (0.029 nM)</td>
<td>Propranolol, 0.25 μM</td>
<td>42</td>
</tr>
<tr>
<td>β&lt;sub&gt;2&lt;/sub&gt;-Adrenergic</td>
<td>Cloned human-β&lt;sub&gt;2&lt;/sub&gt;, E. coli</td>
<td>K, pH 7.7, 0.5 ml, 37°C, 60 min, AF</td>
<td>[&lt;sup&gt;125&lt;/sup&gt;I]iodocyanopindolol, 0.025 nM, (0.017 nM)</td>
<td>Propranolol, 0.25 μM</td>
<td>43</td>
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<tr>
<td>Histamine-H&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Guinea pig, cerebellum, TP, 4</td>
<td>D, pH 7.5, 0.5 ml, 25°C, 30 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Pyrilamine, 1 nM, (0.8 nM)</td>
<td>Astemizole, 1 μM</td>
<td>44</td>
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<tr>
<td>Cholinergic-muscarinic</td>
<td>Rat, striatum, TP, 2</td>
<td>D, pH 7.5, 0.5 ml, 37°C, 30 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Dextetimide, 2 nM, (0.65 nM)</td>
<td>Dextetimide, 1 μM</td>
<td>45</td>
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<tr>
<td>μ-Opiate</td>
<td>Rat, forebrain, M + L + P, 2</td>
<td>A, pH 7.4, 0.5 ml, 37°C, 30 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Sufentanil, 0.5 nM, (0.13 nM)</td>
<td>Dextetomoramide, 0.5 μM</td>
<td>46</td>
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<tr>
<td>δ-Opiate</td>
<td>N × G 108CC15 cells, ± 200,000 cells</td>
<td>A, pH 7.4, 0.5 ml, 25°C, 30 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]DPDPDE, 2 nM, (1.47 nM)</td>
<td>Naltrindole, 0.1 μM</td>
<td>47</td>
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<tr>
<td>κ-Opiate</td>
<td>Guinea pig, cerebellum, TP, 4</td>
<td>A, pH 7.4, 0.4 ml, 37°C, 60 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]U69593, 2 nM, (1.53 nM)</td>
<td>Spiradoline, 1 μM</td>
<td>48</td>
</tr>
<tr>
<td>Haloperidol-sensitive σ&lt;sub&gt;1&lt;/sub&gt; sites</td>
<td>Guinea pig, medulla oblongata, TP, 2</td>
<td>A, pH 7.7, 0.5 ml, 25°C, 60 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Hologenopindol, 1 nM, (0.58 nM)</td>
<td>(+) 3-PPP, 10 μM</td>
<td>49</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt; channel</td>
<td>Rat, cortex, M + L + P, 2</td>
<td>A, pH 7.7, 0.5 ml, 37°C, 30 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Nitrendipine, 0.1 nM, (0.23 nM)</td>
<td>Nifedipine, 1 μM</td>
<td>50</td>
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<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt; channel</td>
<td>Rat, cortex, M + L, 4</td>
<td>H, pH 7.4, 0.5 ml, 25°C, 30 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Batrachotoxin-B, 1 nM, (5000 nM)</td>
<td>Penfluridol, 1 μM</td>
<td>51</td>
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<tr>
<td>Benzodiazepine</td>
<td>Rat, forebrain, TP, 4</td>
<td>A, pH 7.4, 0.5 ml, 0°C, 30 min, AF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Flunitrazepam, 0.3 nM, (2.4 nM)</td>
<td>Clonazepam, 1 μM</td>
<td>52</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>Guinea pig, forebrain, TP, 10</td>
<td>F, pH 7.4, 1.1 ml, 25°C, 20 min, MF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Neurotensin, 1.0 nM, (7.1 μM)</td>
<td>Neurotensin, 1 μM</td>
<td>53</td>
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<tr>
<td>Substance P</td>
<td>Cloned human SP, CHO cells, TP</td>
<td>E, pH 7.4, 0.5 ml, 25°C, 30 min, MF</td>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]Substance P, 0.5 nM, (0.1 nM)</td>
<td>Substance P, 0.1 μM</td>
<td>54</td>
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Table 1. (continued)

<table>
<thead>
<tr>
<th>Receptor site</th>
<th>Tissue</th>
<th>Assay conditions</th>
<th>Labelled ligand, $(K_D$-value)</th>
<th>Blank</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholecystokinin</td>
<td>Rat, pancreas, TP, 1</td>
<td>L, pH 7.4, 0.5 ml, 25 °C, 30 min, MF</td>
<td>$[^3]H$CCK8, 2 nM, (1.68 nM)</td>
<td>CCK8, 1 µM</td>
<td>50</td>
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<tr>
<td>CCK-A</td>
<td></td>
<td></td>
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<tr>
<td>Cholecystokinin</td>
<td>Guinea pig, total cortex, TP, 4</td>
<td>Q, pH 7.4, 0.5 ml, 0°C, 60 min, AF</td>
<td>$[^3]H$CCK8, 1 nM, (0.57 nM)</td>
<td>CCK8, 1 µM</td>
<td>50</td>
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<tr>
<td>CCK-B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Dopamine-transporter</td>
<td>Rat, striatum, TP, 4</td>
<td>Q, pH 7.4, 0.5 ml, 0°C, 60 min, AF</td>
<td>$[^3]H$WIN35428, 2 nM, (1.14 nM)</td>
<td>Mazindol, 1 µM</td>
<td>51</td>
</tr>
<tr>
<td>Norepinephrine-transporter</td>
<td>Rat, cortex, TP, 4</td>
<td>Q, pH 7.4, 0.5 ml, 0°C, 60 min, AF</td>
<td>$[^3]H$Nisoxetine, 2 nM, (1.75 nM)</td>
<td>Mazindol, 1 µM</td>
<td>52</td>
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<tr>
<td>Serotonin-transporter</td>
<td>Human platelets, TP, 5 x 10$^5$ platelets</td>
<td>R, pH 7.4, 0.5 ml, 25°C, 60 min, AF</td>
<td>$[^3]H$Paroxetine, 0.5 nM, (0.15 nM)</td>
<td>Imipramine, 1 µM</td>
<td>53</td>
</tr>
<tr>
<td>Platelet activating factor (PAF)</td>
<td>Rabbit platelets, TP, 5 x 10$^5$ platelets</td>
<td>N, pH 7.4, 0.25 ml, 25°C, 30 min, MF</td>
<td>$[^3]H$PAF, 0.5 nM, (0.55 nM)</td>
<td>PAF, 1 µM</td>
<td>54</td>
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<td>Leukotriene D$_4$</td>
<td>Guinea pig, lung, M+L+P, 20</td>
<td>O, pH 7.4, 0.25 ml, 25°C, 30 min, MF</td>
<td>$[^3]H$Leukotriene D$_4$, 0.25 nM, (0.24 nM)</td>
<td>ICI 198615, 10 µM</td>
<td>55</td>
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</table>

*Buffers:
A. Tris-HCl 50 mM
B. Tris-HCl 50 mM, CaCl$_2$ 4 mM
C. Tris-HCl 50 mM, NaCl 120 mM, KCl 5 mM, MgCl$_2$ 1 mM, CaCl$_2$ 2 mM (ascorbic acid 0.1%, pargyline 1 µM for dopamine-D$_2$ only, BSA 0.2% for dopamine-D$_3$ only)
D. Na-K phosphate 50 mM
E. Tris-HCl 50 mM, bovine serum albumin 0.1%, bacitracin 0.2 mM, KCl 5 mM, MnCl$_2$ 3 mM
H. HEPES-Tris 50 mM, choline chloride 130 mM, KCl 5.4 mM, MgSO$_4$ 0.8 mM, glucose 5.5 mM
J. Tris-HCl 20 mM, NaCl 154 mM
K. Tris-HCl 10 mM, NaCl 100 mM
L. Tris HCl 10 mM, NaCl 120 mM, MgCl$_2$ 10 mM, bovine serum albumin 0.1%, EGTA 1 mM, soybean trypsin inhibitor 50 µg/ml, bacitracin 0.14 mM, phenyl methan sulfonyl fluoride 10 µM
N. Tris-HCl 10 mM, NaCl 120 mM, MgCl$_2$ 10 mM, bovine serum albumin 0.25%
O. Tris-HCl 10 mM, MgCl$_2$ 10 mM, CaCl$_2$ 10 mM, cysteine 5 mM, glycine 5 mM, bovine serum albumin 0.25%
Q. Tris-HCl 50 mM, NaCl 300 mM, KCl 5 mM
R. Tris-HCl 50 mM, NaCl 120 mM, KCl 5 mM


Table 2. Location of selected receptors

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<tr>
<th>Receptor</th>
<th>Selected brain area for ex vivo autoradiography</th>
<th>Coordinates</th>
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<td>dentate gyrus</td>
<td>IL + 5.20 mm</td>
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<tr>
<td>5-HT$_{2A}$</td>
<td>frontal cortex (fourth layer)</td>
<td>IL + 12.7 mm</td>
</tr>
<tr>
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<td>IL + 8.6 mm</td>
</tr>
<tr>
<td>D$_1$</td>
<td>caudate-putamen</td>
<td>IL + 10.0 mm</td>
</tr>
<tr>
<td>D$_2$</td>
<td>caudate-putamen</td>
<td>IL + 10.0 mm</td>
</tr>
<tr>
<td>D$_2$</td>
<td>nucleus accumbens</td>
<td>IL + 10.0 mm</td>
</tr>
<tr>
<td>D$_2$</td>
<td>olfactory tubercle</td>
<td>IL + 10.0 mm</td>
</tr>
<tr>
<td>D$_2$</td>
<td>substantia nigra (compacta)</td>
<td>IL + 3.40 mm</td>
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<tr>
<td>D$_3$</td>
<td>islands of Calleja</td>
<td>IL + 10.0 mm</td>
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<tr>
<td>a$_1$</td>
<td>frontal cortex (fourth layer)</td>
<td>IL + 12.7 mm</td>
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<td>a$_1$</td>
<td>thalamic nuclei (cluster)</td>
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<td>IL + 3.7 mm</td>
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<td>H$_1$</td>
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<td>CBm (gp)</td>
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Coordinates according to Paxinos and Watson (63). IL + 10.0 mm: 10 mm anterior to the inter-aural line.
Table 3. Autoradiography protocols

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<th>Incubation: 10 min at 20°C in medium</th>
<th>Rineses at 4°C (min)</th>
<th>Rinse medium</th>
<th>Film</th>
<th>Exposure time (weeks)</th>
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<td>BW501, 1 µM</td>
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<td>BW501, 1 µM</td>
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<td>2 × 10</td>
<td>B</td>
<td>Y</td>
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<td>58</td>
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<td>Piflutixol, 1 µM</td>
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<td>B</td>
<td>Y</td>
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<tr>
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<td>Domperidone, 1 µM</td>
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<td>Prazosin, 0.1 µM</td>
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<td>Y</td>
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<td>66</td>
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<tr>
<td>ACh musc.</td>
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<td>Atropine, 1 µM</td>
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<td>2 × 5</td>
<td>B</td>
<td>Y</td>
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<td>67</td>
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<tr>
<td>H1</td>
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<td>Asimetizole, 1 µM</td>
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<td>2 × 2</td>
<td>B</td>
<td>Y</td>
<td>4</td>
<td>68</td>
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</table>

Incubation/Rinse media:
A. Tris-HCl buffer (50 mM, pH 7.4), 5 mM CaCl2, 0.1% BSA
B. Tris-HCl buffer (50 mM, pH 7.4)
C. Tris-HCl buffer (50 mM, pH 7.4), 0.1 µM prazosin, 1 µM tetrabenazine, 0.1% BSA
D. Tris-HCl buffer (50 mM, pH 7.4), 120 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 0.1% BSA
E. Tris-HCl buffer (50 mM, pH 7.4), 1 mM EDTA 1, 0.1% BSA
F. Tris-HCl buffer (50 mM, pH 7.4), 1 µM BW501, 1 µM tetrabenazine, 0.1% BSA
G. Na2HPO4 phosphate buffer (50 mM, pH 7.4).

No precubing of the sections except for D3-receptors [i.e. 30 sec in Tris-HCl buffer (50 mM, pH 7.4)]

Films:
Y. 3H-Hyperfilm (Amersham) Z. Ektascan GRL (Kodak)

loped in a Kodak X-Omat processor. 3H-Hyperfilms (Amersham, Little Chalfont, UK) were used for the generation of autoradiograms by tritiated ligands. They were developed manually in Kodak D19 developer for 2 min and fixed with Kodak Readymatic for 3 min.

Data analyses

Autoradiograms were quantified by an MCID image analyzer (Imaging Research, St-Catharines, Ontario, Canada) (70). Optical densities were transformed into levels of bound radioactivity after calibration of the image analyzer with grey-values generated by coexposed commercially available polymer standards ([125I]Micro-scales and [3H]Micro-scales from Amersham). Specific binding was given as the difference between total binding and non-specific binding measured in adjacent sections.

Ex vivo receptor labelling by the radioligand in brain sections of drug-treated animals was expressed as the percentage of receptor labelling in corresponding brain sections of saline-treated animals. Percent receptor occupancy by the drug administered to the animal was given by 100% minus the percent receptor labelling in the treated animal. Percent receptor occupancies were plotted vs dose. For each of the 6–8 doses per compound, individual values from 6–12 animals (mostly 6) were used. Each individual value was the mean of measurements performed in 3 consecutive sections. The sigmoid log dose-effect curve of best fit was calculated by non-linear regression analysis by a computer program using equations as described by Oestreicher and Pinto (56). From these dose-response curves, the ED25-, ED50- and ED75-values (the drug dose producing 25%, 50% and 75% receptor occupancy, respectively) and the slopes of the curves for receptor occupancy were calculated. A non-overlap of the 95% confidence limits was considered to reflect biologically relevant differences.

Materials

[3H]7-OH-DPAT (4.22 TBq/mmol or 114 Ci/mmol), [3H]CCCK8 (2.85 TBq/mmol or 77.0 Ci/mmol), [3H]mesulergine (3.15 TBq/mmol or 85.0 Ci/mmol), [3H]spiperone (3.59 TBq/mmol or 97.0 Ci/mmol), [3H]U69593 (2.29 TBq/mmol or 62.0 Ci/mmol), [125I]7-amino-8-iodo-ketanserin ([125I]AMIK (74 TBq/mmol or 2000 Ci/mmol), [125I]iodosulpride (74 TBq/mmol or 2000 Ci/mmol) and Autoradiographic Micro-scales were purchased from Amersham.

[3H]8-OH-DPAT (4.79 TBq/mmol or 129.5 Ci/mmol), [3H]batrachotoxin-B (1.87 TBq/mmol or 50.5 Ci/mmol), [3H]clonidine (2.3 TBq/mmol or 63.5 Ci/mmol), [3H]DPDPE (1.22 TBq/mmol or 33.0 Ci/mmol), [3H]flunitrazepam (3.12 TBq/mmol or 84.3 Ci/mmol), [3H]GR 65630 (2.27 TBq/mmol or 61.4 Ci/mmol), [3H]haloperidol (0.55 TBq/mmol or 15 Ci/mmol), [3H]leuko-
triene D₄ (4.73 TBq/mmol or 128 Ci/mmol), [³H]nisoxetine (2.74 TBq/mmol or 74.0 Ci/mmol), [³H]nitrendipine (2.72 TBq/mmol or 73.0 Ci/mmol), [³H]PAF (5.55 TBq/mmol or 150 Ci/mmol), [³H]paroxetine (0.61 TBq/mmol or 16.6 Ci/mmol), [³H]prazosin (0.64 TBq/mmol or 17.4 Ci/mmol), [³H]pyrilamine (0.918 TBq/mmol or 24.8 Ci/mmol), [³H]QNB (1.59 TBq/mmol or 43.0 Ci/mmol), [³H]SCH-23390 (2.6 TBq/mmol or 71.1 Ci/mmol), [³H]-substance P (1.15 TBq/mmol or 31.0 Ci/mmol), [³H]-WIN35428 (3.13 TBq/mmol or 84.5 Ci/mmol), [¹²⁵I]iodocyanopindolol (81.4 TBq/mmol or 2000 Ci/mmol) were purchased from New England Nuclear (Du Pont, Dreieich, Germany).

[³H]Dexetimide (0.36 TBq/mmol or 9.78 Ci/mmol), [³H]ketanserin (1.18 TBq/mmol or 32 Ci/mmol), and [³H]sufentanil (0.85 TBq/mmol or 23.0 Ci/mmol) were synthesized by the Janssen Research Foundation, Beerse, Belgium (C. Janssen).

The drugs were obtained from the companies of origin. Risperidone, 9-OH-risperidone, haloperidol, bromperidol were from Janssen Pharmaceutica (Beerse, Belgium). Nemonapride was from Yamanouchi (Tokyo), SM-9018 was from Sumitomo (Osaka), clozapine was from Sandoz (Basel, Switzerland), clocapramine and mosapramine were from Yoshitomi (Osaka).

RESULTS

In vitro competition binding of risperidone, 9-OH-risperidone, haloperidol, bromperidol, clozapine, nemonapride, SM-9018, clocapramine and mosapramine to neurotransmitter receptors or neurotransmitter transporters, as listed in Table 1, was investigated using animal brain or cloned (human) receptors stably expressed in

<table>
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<tr>
<th>Receptor</th>
<th>Species</th>
<th>Brain area/cells</th>
<th>RIS</th>
<th>9-OH-R</th>
<th>HAL</th>
<th>BROM</th>
<th>NEMO</th>
<th>SM-9018</th>
<th>CZP</th>
<th>CLOCA</th>
<th>MOSA</th>
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</table>

Affinity ratio 5-HT₂A/D₂ (rat) 21 16 1/18 1/39 1/136 5.9 46 6.8 1.3

mammalian cell lines. Table 4 contains the in vitro receptor binding profiles (K<sub>i</sub>-values) of the compounds tested in this study. Receptors for which a compound displayed a K<sub>i</sub>-value below 100 nM were considered as potential targets for the compound and were selected for the investigation of in vivo receptor occupancy.

 Autoradiograms representing the various binding models used, obtained after labelling of brain sections of saline-treated animals (maximal amount of available binding sites), were similar to those published previously (27).

Figure 2 shows the dose-occupancy curves for the test-compounds at the D<sub>2</sub> receptor in the caudate-putamen and 5-HT<sub>2A</sub> receptors in the frontal cortex. For each dose-level median values are plotted (n=6–12). The ED<sub>50</sub>-values (derived from the dose-occupancy curves) for the occupancy of the various target receptors by the test-compounds are listed in Table 5. The slopes of the D<sub>2</sub>-5-
HT2A- and adrenergic α1-receptor occupancy curves are listed in Table 6; also included are the ED25- and ED75-values for the occupancy of D2-receptors in the caudate-putamen (striatum). Data on D2-receptor occupancy in the caudate-putamen (striatum), substantia nigra and in the mesolimbic areas (nucleus accumbens and olfactory tubercle) are listed in Table 7.

### DISCUSSION

#### Potency of interactions with dopamine receptors

In accordance with their antipsychotic properties, all compounds displayed affinity in vitro D2-receptors, but with a wide range of potency (Table 4): nemonapride showed the highest affinity (subnanomolar, 5 times higher...
than haloperidol) and clozapine showed the lowest affinity for D2-receptors (100 times lower than haloperidol).

The affinities of risperidone and 9-OH-risperidone for D2-receptors were two to three times lower than that of haloperidol. Clozapine was the only drug to show higher affinity in vitro for D4-receptors than D2-receptors. Its D4 affinity, however, was not very high. Nemonapride and SM-9018 had almost equivalent affinity for D4- and D2-receptors. Mosapramine had the lowest ratio, showing a 25-times lower affinity for D4-receptors than D2-receptors. D1 affinities were at least 100 times lower than the drug's D2 affinities, except for clocapramine (20 times lower) and clozapine (4 times lower). In vivo, mosapramine, haloperidol, bromperidol and nemonapride were confirmed to predominantly occupy D2-receptors, nemonapride being extremely potent, i.e., 27 times more potent than haloperidol (Table 5). The rank order of potencies for D2-receptor occupancy was in good agreement with the in vitro binding affinities. Clocapramine, however, which had still a moderate affinity for D2-receptors in vitro, showed the lowest potency for D2-occupancy in vivo. Fifty percent occupancy of D2-receptors was not entirely reached by clocapramine at its highest dosage (40 mg/kg, s.c., 2 hr), indicating probably a more rapid clearance/metabolization of the compound. Its ED50 dose was estimated, under the present conditions, to be around 65 mg/kg in the rat caudate-putamen.

For most compounds, the ratio of D2- vs D3-occupancy potency in vivo was reduced as compared to the in vitro affinity ratio. This may be due to a competition with endogenous dopamine (35), which shows nanomolar affinity for the D3-receptor (8) and could hinder the access of the antipsychotics to the D3-receptors in the islands of Calleja. Nemonapride, which is the only test compound with subnanomolar affinity in vitro for D3-receptors (slightly more potent than its D2 affinity), also showed a sixfold decrease in ratio of D2 vs D3 interaction potency as compared to its in vitro ratio.

D1-receptor occupancy could be demonstrated for clozapine, with a two times lower potency than its D2-receptor occupancy.

The measurement of D2-receptor occupancy in the rat brain still awaits the validation of a reliable model for D2-receptor labelling, which has not been achieved to date despite numerous attempts.

### Predominance of interaction with 5-HT2A-receptors vs D2-receptors

The predominance of 5-HT2A- vs D2-antagonism is believed to be an important factor for obtaining the therapeutic properties of “atypical neuroleptics”; tenfold higher affinity in vitro for 5-HT2A-receptors vs D2-receptors being the minimal requirement (21). Among the compounds analyzed here, only risperidone, 9-OH-risperidone and clozapine fulfil this requirement. However, the present study goes a step further by also analyzing the ratios of the in vivo occupancy of receptors at 2 hr after s.c. administration. Peak occupancy of receptors usually occurs within 1 hr after s.c. administration.

Risperidone, 9-OH-risperidone, SM-9018, clozapine and clocapramine showed higher affinity for 5-HT2A- than for D2-receptors in vitro. Mosapramine, haloperidol, bromperidol and nemonapride showed a slight to high predominance of D2-receptor compared to 5-HT2A-receptor affinity. In vivo data confirmed the in vitro observations (Tables 4 and 5). Both in vitro and in vivo, risperidone displayed the highest predominance for 5-HT2A-receptor vs D2-receptor interaction (17- and 19-fold, respectively). For 9-OH-risperidone predominance of 5-
the restored dopaminergic transmission. This condition
occupied by the antipsychotic drug to receive the signal of
a milder D2 antagonism, leaving enough D2-receptors un-
benefit from these regulating properties, an extensive
(20), supporting the idea that 5-HT2A antagonism has an
striatum but not on mesolimbic dopaminergic neurons
of D2 blockade on dopaminergic transmission in rat
and patients (14-17). It was demonstrated in this respect
reduce the neuroleptic-induced side effects both in rats
and only a fivefold preference in vivo.
Possible significance of predominant 5-HT2A-receptor
antagonism vs D2-receptor antagonism
A complex interaction between the serotonergic and
dopaminergic systems seems to exist. It has been shown
that the 5-HT2A antagonist ritanserin increased both burst
firing and firing rate of midbrain dopaminergic neurons
(18), indicating that 5-HT exerts (probably indirectly) an
inhibitory control of midbrain dopaminergic neurons. In
an animal model of hypofrontality, a phenomenon that
has been associated with negative symptoms of
schizophrenia, the activity of midbrain dopaminergic
neurons projecting to the frontal cortex is deadened to
pacemaker activity by local cooling of the frontal cortex.
5-HT2A antagonists can restore the dopaminergic burst
firing in these conditions (19). Similarly, in animals treated
with low doses of the dopamine antagonist raclopride,
co-treatment with ritanserin could restore burst firing
(=signal) and reduce tonic activity (=noise) of midbrain
dopaminergic neurons. However, restoration of burst
firing by the 5-HT2A antagonist could no longer be
achieved when higher doses of raclopride were used (19).
This suggests the importance of the ratio between 5-
HT2A- and D2-receptor occupancy for obtaining the op-
timal regulation of the dopaminergic neurotransmission
by 5-HT2 antagonism. The restoration of regular burst
firing of midbrain dopaminergic neurons is likely to play
a role in the treatment of the negative symptoms of
schizophrenia. 5-HT2A antagonism is also known to
reduce the neuroleptic-induced side effects both in rats
and patients (14–17). It was demonstrated in this respect
that blockade of 5-HT2A-receptors attenuated the effects
of D2 blockade on dopaminergic transmission in rat
striatum but not on mesolimbic dopaminergic neurons
(20), supporting the idea that 5-HT2A antagonism has an
important role in the reduction of EPS. However, to
benefit from these regulating properties, an extensive
blockade of 5-HT2A-receptors needs to be combined with
a milder D2 antagonism, leaving enough D2-receptors un-
occupied by the antipsychotic drug to receive the signal of
the restored dopaminergic transmission. This condition
can be achieved with risperidone better than with any of
the other compounds analyzed in this study.
Interaction with various neurotransmitter receptors
Besides affinity for 5-HT2A- and D2-receptors, the com-
pounds also showed moderate to high occupancy of α1-
receptors, except for clozapine and nemonapride.
Yet, the significance of central adrenergic α1-receptor oc-
cupancy is not quite clear. Only risperidone and clozapine
occupied adrenergic α2-receptors. Clozapine's primary
affinity in vitro is for H1-receptors, which was 30
times higher than its D4 affinity and 70 times higher
than its D2 affinity. The in vivo data confirmed both its
predominant occupancy of H1-receptors (47 times more
potent than D2-receptor occupancy) and potent oc-
cupancy of cholinergic-receptors (equivalent to D2-receptor
occupancy). The occupancy of muscarinic receptors by
clozapine may contribute to its particular clinical profile
but may also be responsible for side effects like hypersali-
vation. The latter effect may in part be explained by
clozapine's selective agonism for muscarinic M4-receptors
(although being an antagonist at the other muscarinic
subtypes) (71). The extensive sedation caused by cloza-
pine (72) is most likely due to the H1-receptor occupancy.
Risperidone, and to a lesser extend 9-OH-risperidone,
also showed an occupancy of H1-receptors in the guinea
pig cerebellum. However, from in vitro binding experi-
ments using cloned human H1-receptors, it appeared that
risperidone shows a 6-times lower affinity for the human
H1-receptor than for the guinea pig one, whereas cloza-
pine shows the same affinity for both receptors (K. De
Loore, manuscript in preparation). In vitro affinity for
α1-receptors was reported for haloperidol, bromperidol,
logenapride and SM-9018, but since a participation of
this receptor in the therapeutic properties of antipsychotic
compounds seems unlikely, occupancy of the latter site
was not investigated in vivo.
Course of interaction and regional interaction with do-
pamine D2-receptors
For increasing dosages, risperidone displayed a more
gradual D2-receptor occupancy than the other drugs. 9-
OH-Risperidone showed similarly a tendency towards
more gradual occupancy of D2-receptors in the striatum.
The drugs classified as typical neuroleptics like
haloperidol and bromperidol showed steeper curves for
the occupancy of D2-receptors in the rat brain; the slopes
of the curves were significantly different from that of
risperidone (Table 6). The more gradual occupancy curve
of risperidone is in line with previous behavioral observa-
tions which showed that a much wider dose-range was re-
quired between the inhibition of evoked dopaminergic
responses (low D2-receptor blockade) and the occurrence
of catalepsy in rats (high D₂-receptor blockade) with risperidone than with haloperidol (22). In other experiments it was demonstrated in rats that the disinhibition by risperidone of locomotor activity induced by a high dose of amphetamine was maintained over a wider dose range before reaching immobility (high D₂-receptor blockade) (73) than with haloperidol. The slopes of the occupancy curves of adrenergic α₁-receptors were never significantly different from the slopes for D₂-receptor occupancy curves in the caudate-putamen. The slopes of the 5-HT₂A-receptor occupancy curves were not different from those of D₂-receptor occupancy in the caudate-putamen except for risperidone that showed a steeper 5-HT₂A-receptor occupancy and clozapine that displayed a more gradual 5-HT₂A-receptor occupancy than their respective D₂-receptor occupancies. The reason for these two discrepancies is not understood. In both cases however, slopes of the 5-HT₂A occupancy curves were not different from slopes for the α₁-receptor occupancy.

Further experiments are required to determine if risperidone exhibits a more gradual occupancy of D₂-receptors also in humans. Such a property could effectively contribute to risperidone's ability to avoid extrapyramidal side effects at therapeutic dosages. Indeed, a compound with a more shallow curve for D₂-receptor occupancy could benefit from this property by better avoiding the extensive occupancy of striatal D₂-receptors, which is believed to cause extrapyramidal side effects.

An advantage of ex vivo autoradiography is certainly its high anatomical resolution allowing regional measurement of receptor occupancy in vivo, which is of particular interest for the dopaminergic pathways. It is accepted that occupancy of D₂-receptors located in the mesolimbic system plays an important role in the therapeutic properties of antipsychotic drugs, while an extensive occupancy of striatal D₂-receptors is believed to produce the neurological side-effects. One can therefore suppose that a preferential D₂-receptor occupancy in the mesolimbic system may be propitious. However, none of the compounds investigated in this study showed any regional preference for D₂-receptor occupancy in mesolimbic vs nigrostriatal areas. Each of them showed ED₅₀ doses with overlapping 95% confidence limits among the four dopaminergic areas analyzed.

Conclusions

In view of the above results, it can be concluded that risperidone is different from the other compounds in this study. The predominance of 5-HT₂A-receptor vs D₂-receptor occupancy and the more gradual occupancy of D₂-receptors differentiate risperidone from the other antipsychotic compounds. These properties probably underlie the excellent clinical results obtained with risperi-
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