Visualization of Antipsychotic Drug Binding to Living Mesolimbic Neurons Reveals D2 Receptor, Acidotropic, and Lipophilic Components

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Abstract: To examine the binding of antipsychotic drugs to living neurons, we applied fluoroprobe derivatives of the D2 antagonist spiperone to mesolimbic system neurons in postnatal culture. We found that rhodamine-N- (p-aminophenethyl) spiperone (rhodamine-NAPS) stereospecifically labeled the plasma membranes of 38 ± 9% of ventral tegmental area neurons, 22 ± 7% of which were dopaminergic, and 50 ± 6% of medium-sized putatively GABAergic nucleus accumbens neurons, with a time constant of ~8 min. In contrast, the BODIPY derivative of NAPS rapidly labeled intracellular sites in all neurons in a punctate pattern, consistent with acidotropic uptake. Native antipsychotics also show acidotropic uptake, which we visualized by their displacement of the fluorescent weak base, a vital dye, acridine orange from acidic intracellular compartments. We found that acidotropic uptake correlated best with the partition coefficients of the drugs. With a time constant of 23 min, rhodamine-NAPS labeled all neurons in a pattern suggestive of lipophilic solvation. Thus, initially rhodamine-NAPS makes possible visualization of D2 receptors on living neurons; however, acidotropic uptake and lipophilic solvation obscure receptor labeling and may account for time-dependent actions in the action of antipsychotic drugs, as well as their use as radioreceptor ligands.

Key Words: Dopamine—Fluorescent label—Spiperone—Raclopride—Neuroleptics—Weak base.


The therapeutic efficacy of antipsychotic drugs correlates with their affinity for D2-like dopamine (DA) receptors (Javitch and Kaufmann, 1991), but also with their brain-to-serum ratios (Tsuneizumi et al., 1992), suggesting that the physicochemical properties of the drugs play a critical role in their action. Indeed, receptor binding is far more rapid than therapeutic action, indicating that factors beyond receptor binding contribute importantly to antipsychotic drug action. It is striking that most antipsychotic drugs are detectable in rat brain for days to weeks following a single administration, far longer than their receptor affinity would dictate (Cohen et al., 1992). In positron emission tomography (PET) of D2 receptors in humans, 18F-spiperone behaves as an irreversible D2 ligand (Wong et al., 1986). These observations suggest that enduring binding of antipsychotic drugs in the living brain may explain in part the time dependence of their therapeutic action.

Two ideas have been advanced to explain the prolonged binding of the butyrophenone antipsychotic spiperone in vivo. Synaptic or ambient DA could trigger agonist-mediated receptor internalization (Chugani et al., 1988) and convey receptor-bound antipsychotics into endocytic compartments. Although this may be operative in PET studies that use tracer levels of antipsychotics, therapeutic antipsychotic drug levels lead to near-complete D2 receptor occupancy (Farde et al., 1992), which should block agonist-mediated receptor internalization. An alternate possibility is that antipsychotic drugs, which are almost without exception highly lipophilic weak bases (Seeman, 1980), partition across membranes and are trapped in acidic intracellular compartments (Maloteaux et al., 1983; Dannies et al., 1984), principally endosomes, lysosomes, and synaptic vesicles (de Duve et al., 1974; Sulzer and Holtzman, 1989).

Laduron (1984) highlighted the importance of trapping in acidic intracellular compartments as an artifact in studies of spiperone binding in lymphocytes, which although they show saturable binding do not express...
D2-like receptors. Indeed, living cells may show both specific receptor binding and acidotropic uptake (Gosuin et al., 1984). Acidotropic uptake may dramatically increase the concentrations the drugs reach intracellularly. For example, haloperidol is concentrated >600-fold by synaptosomes (Schmalzing, 1988); ~70% of this uptake is due to acidotropic uptake (Schmalzing, 1988), whereas the remainder is probably due to binding to intravesicular anionic groups (Sulzer et al., 1987) and lipophilic solvation. Antipsychotic drugs trapped in synaptic vesicles can be released exocytotically (Schmalzing, 1988) and thus could in effect be recycled at synapses, possibly accounting for the persistence of the drugs in the brain.

The advent of fluorescent spiperone analogues as fluoroprobes for dopamine receptors (Ariano et al., 1989; Monsma et al., 1989) and the ability to culture postnatal mesolimbic system neurons (Rayport et al., 1992b; Shi and Rayport, 1994) that robustly express D2-like receptors (Shi and Rayport, 1991) now make possible visualization of the interaction of antipsychotic drugs with the cells that are their putative sites of action. We have thus been able to evaluate the relative importance of specific receptor binding, acidotropic uptake, and lipophilic solvation in antipsychotic drug action. A preliminary report of this work has appeared in abstract form (Rayport et al., 1992a).

**EXPERIMENTAL PROCEDURES**

**Cell culture and identification**

Three sets of mesolimbic system neurons were cultured from postnatal day 2 rats as previously described (Rayport et al., 1992b; Cubells et al., 1994; Shi and Rayport, 1994). All animal procedures complied with the NIH Guide for the Care and Use of Laboratory Animals of the U.S. Public Health Service and were conducted under protocols approved by the Institutional Animal Care and Use Committees of both Columbia Health Sciences and the New York State Psychiatric Institute. Ventral midbrain (VM) neurons were obtained from the ventral portion of coronal slices through the midbrain, cut at the dorsal margins of the ventral tegmental area (VTA) and substantia nigra. VTA neurons were obtained from a further dissection of the ventral midbrain slice, cut at the lateral margins of the VTA. Nucleus accumbens (nAcc) neurons were obtained from horizontal slices taken at the level of the anterior commissure after removal of adjacent areas. DA neurons in ventral midbrain cultures were identified by tyrosine hydroxylase (TH) immunohistochemistry (Rayport et al., 1992b). In brief, cultures were fixed with a combination of 2% paraformaldehyde and 0.1% glutaraldehyde, permeabilized with 0.1% Triton-X-100 (Sigma), incubated at 4°C overnight in a 1:10,000 dilution of a polyclonal anti-TH antiserum (Eugene Tech or Chemicon), and stained with the Vectastain Elite kit and diaminobenzidine.

**Imaging**

Before fluoroprobe labeling, cultures were washed twice with room temperature, oxygenated physiological saline, containing 135 mM NaCl, 3 mM KCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, 10 mM glucose, and 10 mM HEPES (pH 7.35). Autofluorescence in selected fields of interest was bleached by 60 s of epifluorescence illumination (neighboring fields were always examined to rule out effects due to photodynamic damage). Cells were imaged with a 40× Plan-Neofluar 1.30 numerical aperture oil-immersion lens under Nomarski differential interference contrast (DIC) optics or epifluorescence (100 W DC Hg-arc source) and low-light, chilled-CCD video images taken (Zeiss Axiosvert; Photometrics Star 1; Star 1 Camera Controller software running on a Macintosh IIfx). Standard fluorescein (emission band-pass) and rhodamine filter sets (Zeiss) were used.

In each fluorescence series, illumination conditions were held constant, the field iris was stopped down so as to excite only the region of interest, 1–5 s exposures were made with a camera gain of 4, and the resulting digital images were opened as a set in NIH-Image (written by Wayne Rasband, National Institutes of Health; available on the Internet by anonymous ftp from zippy.nimh.nih.gov in the directory/pub/nih-image) using fixed import parameters. Confocal images were obtained by digital deconvolution (MicroTome; VayTek) of stacks of images taken at 0.5-μm focus increments (IP-Lab; Signal Analytics). Montages were assembled in NIH-Image and lettered in Canvas (Deneba) or Freehand (Aldus), and plates were made by high-resolution linotronic output or dye-sublimation printing.

For quantification of rhodamine-N-(p-aminophenethyl)-spiperone (rhodamine-NAPS) binding over time, relative intensity was determined for regions of interest (defined by outlining the membrane or the cytoplasm of individual cells) by taking the mean of all nonzero pixels in successive images and determining the time constants by peeling of exponentials. Fluorescence intensity at r = 0 was measured in the image taken just before fluoroprobe application after adding a constant equal to the increase in background fluorescence due to the fluoroprobe to the entire image. To show relative intensity in the stereospecificity experiment, pseudocolor images were made using the NIH-Image 32-color look-up table. For the quantification of specific binding, background fluorescence was measured in an area of the field devoid of cells and then subtracted from the entire image; then mean membrane fluorescence of all neurons in the presence of the active butaclamol enantiomer ($M_{\text{dissolved}}$; which is nonspecific labeling) and mean membrane fluorescence of the labeled cells in the presence of the inactive enantiomer ($M_{\text{labelled}}$) which comprises specific and nonspecific labeling) were measured. Specific labeling ($M_{\text{specific}}$) was then

$$M_{\text{specific}} = M_{\text{labelled}} - M_{\text{dissolved}}$$

For counts of rhodamine-NAPS-positive cells, cultures were scanned before labeling under DIC optics, and up to 10 fields each containing several neurons were selected; the coordinates were noted, the DIC images were saved to disk, and the fields were bleached with a 60-s epifluorescence illumination. Starting after a 10-min incubation in rhodamine-NAPS, the fields were relocated and imaged under rhodamine epifluorescence. Cells were counted as positive if they showed continuous labeling of the soma membrane. To compare the incidence of rhodamine-NAPS labeling with the incidence of DA neurons, the imaged fields were circled with a diamond object marker (Zeiss), the fields were relocated after TH immunohistochemistry, and the numbers of DA neurons were counted. The difference in the incidence
of rhodamine-NAPS labeling versus TH staining was evaluated by a paired, two-tailed t test.

**Fluoroprobes**

Stock solutions of rhodamine-NAPS and BODIPY-NAPS (5,7-dimethylbodipy-1-propionic acid-NAPS; Molecular Probes) were prepared in dimethyl sulfoxide (DMSO) at 1 mg/ml and stored in 5-μl aliquots at −75°C (under these conditions, we have not observed any deterioration in labeling strength over 3 years). On each experimental day, a 10 μM working stock was prepared in saline and kept in the dark; this was diluted 1,000-fold to make a 10 nM fluorochrome labeling solution. Thus, the final DMSO concentration was 0.001%, which is far below the concentration that appears to affect neurotransmitter receptor function, e.g., 0.3% at the GABA receptor (Nakaihiko et al., 1992). Glass tubes were used for fluorochrome dilutions to minimize adsorption (M. Ariano, personal communication). For labeling, the saline in the culture dish was suctioned off, leaving 100 μl covering the cells, and the dish was flooded with the fluorochrome solution (2.5 ml), which was left on during subsequent imaging; at the 10 nM concentration used, free fluorochrome caused only a modest increase in background fluorescence. For tests of pharmacological specificity, the competitor was prepared in saline and used for a third wash and as the saline for the final fluorochrome dilution, assuring a constant competitor concentration.

Acidic intracellular compartments were visualized with the weak base vital dye acidine orange (AO) under fluorescent epifluorescence. AO was made up as a 1 mM stock in water and diluted to 100 nM in saline for staining. After allowing 20 min for AO staining to stabilize, drugs were added in an ascending concentration series, allowing ~10 min for stabilization of AO staining after each change. The fluoroprobes showed minimal bleaching after 10 successive exposures, but AO was much more sensitive to bleaching, so we attenuated the fluorescence excitation beam with a 10% neutral density filter. To enhance contrast, AO images were shadowed (NIH-Image, Enhance menu) so that brighter areas appear more raised. Cells showing morphological changes (under DIC optics) during the course of the experiment were excluded from the analysis.

Drugs were obtained from the following sources: Clozapine was a gift from Sandoz, and raclopride was a gift from Astra. Butaclamol, haloperidol, spiperone, and sulpiride were obtained from Research Biochemicals. All other chemicals were obtained from Sigma. Stock solutions of the butyrophenones and clozapine were made up in DMSO at 10 mM; raclopride and sulpiride stocks were made up in distilled water at 100 mM. DMSO at the maximum concentration used (1%) did not affect AO staining. Free BODIPY was provided by Hee Chol Kang (Molecular Probes), and a 10 mM stock was made up in DMSO. All were stored in aliquots at −75°C.

**Partition coefficient measurements**

We measured the octanol:water partition coefficients of the fluoroprobes with a spectrophotometer (Varian) set at λ<sub>ex</sub> = 496 nm and λ<sub>em</sub> = 516 nm for BODIPY-NAPS and λ<sub>ex</sub> = 551 nm and λ<sub>em</sub> = 578 nm for rhodamine-NAPS (slit width, 5 nm). Because the fluoroprobes are hydrophobic (BODIPY-NAPS more so than rhodamine-NAPS), we initially added them to the octanol and measured the fluorescence in octanol before and after mixing with water; however, rhodamine-NAPS fluorescence increased with time in octanol, precluding reliable measurements. Therefore, for these determinations, we added 2.5 μl of 1 mM solutions of the fluoroprobes in DMSO to 1.300 μl of sodium phosphate buffer (0.2 M, pH 7.4, saturated with octanol), and measured the initial fluorescence F<sub>i</sub>. We then added 200 μl of octanol (saturated with phosphate buffer) and vigorously mixed the two phases by sonication, vortex-mixing, and multiple inversions. Finally, we centrifuged the mixture at 2,000 g for 15 min, removed the overlying octanol phase, and measured the fluorescence in the aqueous phase F<sub>f</sub>. We determined the partition coefficient log P from the formula (Leo et al., 1971)

\[
P = \frac{F_f - F_i}{F_f r}
\]

where r is the ratio of the volume of the organic to the aqueous phase. Similar results were obtained with either half the concentration or double the concentration of the fluoroprobes, indicating that we were within the linear range. Because of its extreme hydrophilicity [log P = −2.03 (Leo et al., 1971)], DMSO should not partition appreciably into the octanol and so should not affect the measurements; indeed, repeating the experiment using 10 times the concentration of DMSO did not change the measured log P.

**RESULTS**

The K<sub>i</sub> of NAPS at D2-like receptors is 0.64 nM; conjugation to rhodamine yields a fluorochrome with a K<sub>i</sub> of 3.8 nM (Barton et al., 1991), which we confirmed by <sup>3</sup>H]spiperone displacement in rat brain membrane homogenates. To test whether rhodamine-NAPS would make it possible to visualize D2 receptors on living cells, we examined a human embryonic kidney (HEK) cell line (HEK 293 cells) transfected with human D2 receptors (Javitch et al., 1994) and native HEK 293 cells as controls on a blinded basis. When we applied 10 nM rhodamine-NAPS to HEK 293 cells (Fig. 1), many cells in the transfected cultures (n = 2)
showed robust membrane labeling, seen as a ring outlining the cell perimeter. In untransfected cultures \( (n = 2) \), no cells showed membrane labeling. Labeling intensity began to plateau after \( \sim 10 \) min (however, the constant motion of the cells precluded quantitative determinations).

**Rhodamine-NAPS binding to mesolimbic neurons**

In cultures of mesolimbic system neurons, we found that 10 nM rhodamine-NAPS produced a time-dependent increase in labeling of both membranes and cytoplasm of a subset of neurons (Fig. 2) that we putatively identified as receptor-mediated. In contrast, 100 nM rhodamine-NAPS diffusely labeled all cells in the culture, whereas 1 nM produced only very weak labeling. In neurons showing putative receptor labeling, the most rapid increase in fluorescence was seen on the cell surface, followed closely by an increase in cytoplasmic fluorescence (Fig. 3A), which appeared initially in a punctate cytoplasmic distribution. The intensity of labeling of these components increased exponentially with an initial time constant \( (\tau) \) of \( \sim 8 \) min (Fig. 3B). With longer incubation, a more diffuse labeling of the cell surface and cytosol of all cells developed with a \( \tau \) of \( \sim 23 \) min. By 1 h, this slower developing fluorescence obscured all putative specific labeling. These results were representative of three experiments in which we quantified the time course of labeling. Acutely dissociated cells, cultures younger than 1 week in vitro, or unhealthy cultures with significant ongoing cell death did not show selective labeling of subsets of cells. Glia never showed selective labeling, although diffuse labeling of the glial feeder layer increased in parallel with the slower labeling of neurons.

After digital deconvolution to obtain confocal images, putative specific labeling appeared to be spatially continuous on the membrane at a lower intensity with superimposed hot spots of more intense labeling, extending \( \sim 1 \) \( \mu \)m along the membrane (Fig. 4). Punctate cytoplasmic labeling was also evident. Membrane labeling could be discerned on the processes of labeled cells (Fig. 4C), which were most likely dendrites based on morphological criteria (Rayport et al., 1992b) and appeared to be more discretely arranged in hot spots. With longer incubation times, membrane labeling became more homogeneous, and hot spots were no longer seen (data not shown).

**Pharmacological specificity.** To test whether puta-
In nAcc cultures (a field from one culture is shown in Fig. 6), we found that rhodamine-NAPS specifically labeled 50 ± 6% (mean ± SEM) of the medium-sized neurons (n = 9 cultures; 220 cells total). Rhodamine-NAPS never strongly labeled larger-sized neurons (n = 3 cultures; 12 cells total); however, fainter membrane labeling of larger-sized neurons could be discerned after digital deconvolution. In VTA cultures (a field from one culture is shown in Fig. 4), rhodamine-NAPS labeled 38 ± 7% of the neurons (n = 8 cultures; 114 cells total), whereas in the same fields, 22 ± 7% of the neurons were dopaminergic by immunostaining for TH; these results were significantly different (p < 0.05 by paired two-tailed t test). In VM cultures, 36 ± 7% (n = 4 cultures; 17 cells total) cells were rhodamine-NAPS labeled. To rule out binding to serotonin 5-HT2 receptors, we determined the incidence of rhodamine-NAPS-labeled cells in VM and nAcc cultures in the presence of 100 nM mianserin (n = 2 cultures; 25 cells in each); we found that 36% of VM cells and 51% of medium-sized nAcc cells were labeled.

**BODIPY-NAPS binding**

BODIPY-NAPS has a Kd of 1.0 nM, which is almost the same as that of the parent NAPS and more than threefold better than that of rhodamine-NAPS (Barton et al., 1991). However, when VTA or nAcc cells were incubated with BODIPY-NAPS (1, 10, or 100 nM), all neurons were labeled in a punctate pattern that appeared in <1 min (n = 10 cultures; Fig. 7C). Glia showed a similar increase in punctate labeling but at a reduced intensity. The pattern of labeling with BODIPY-NAPS closely resembled that seen with the weak base vital dye AO (cf. Sulzer and Rayport, 1990), suggestive of uptake into acidic intracellular compartments. Consistent with this, punctate BODIPY-NAPS labeling was seen in cells as young as 1 day in vitro (data not shown), did not occur in fixed cultures (data not shown), and was abolished by the weak base amonia (added as NH4Cl), which collapses acidic intracellular gradients (Fig. 7C). To test whether blocking acidotropic uptake would make it possible to visualize BODIPY-NAPS receptor labeling, we preincubated cultures in 10 or 50 mM NH4Cl and then added BODIPY-NAPS. We saw only diffuse labeling of the cytoplasm, which reached a plateau in <1 min. However, after digital deconvolution, we were able to discern faint BODIPY-NAPS membrane labeling in NH4Cl-treated cultures (data not shown). Punctate BODIPY-NAPS labeling was not due to contamination with free BODIPY because it is neither a weak base nor does it show differential cellular labeling (Fig. 7B); moreover, a thin-layer chromatogram (provided to us by H. C. Kang, Molecular Probes) of the commercial BODIPY-NAPS showed only a single fluorescent compound. Similar diffuse labeling was seen with fluorescein-NAPS (data not shown). NH4Cl did not affect rhodamine-NAPS labeling (data not shown).
FIG. 4. Spatial distribution of rhodamine-NAPS membrane labeling. A: Three neurons in a VM culture (one extending beyond the top of the frame) 8 days in vitro are shown under DIC optics. B: Subsequent processing of the culture for TH immunohistochemistry shows that the middle-right cell is a DA neuron. C: A stack of digitally deconvoluted confocal fluorescence slices taken in 0.5-μm focus increments 15 min after addition of rhodamine-NAPS (10 nM) to the living culture is shown. Relative intensity is indicated in the NIH-Image 32 color pseudocolor look up table in C6, where warmer colors indicate stronger labeling. C1: Starting just above the culture substrate, rhodamine-NAPS labels the soma membrane and proximal neurites (most likely dendrites) of the DA neuron at low intensity with superimposed hot spots (arrows). C2 and C3: Moving up, other hot spots become evident (arrows), as well as some punctate cytoplasmic fluorescence. C4 and C5: The membranes of the other two cells, which are also in these planes of focus, are negligibly labeled. C6 and C7: Focusing up further shows more hot spots. C8: In this slice, which is almost tangential to the top of the cell, continuous lower intensity membrane labeling is seen to be relatively homogeneous in the plane of the membrane.

FIG. 5. Stereospecificity of rhodamine-NAPS labeling. A pair of sister nAcc cultures 15 days in vitro were incubated in either the inactive (-) or active (+) enantiomer of butaclamol (100 nM, 5 min) and then in rhodamine-NAPS (10 nM) in the same butaclamol solutions. Relative intensity is displayed using the same pseudocolor scale as Fig. 4. A1: A DIC image of six cells is shown. A2: In (-)-butaclamol, the two middle cells show strong membrane labeling; there is a spot of intense rhodamine-NAPS labeling in the nearby neuropil. B1: A DIC image of four cells in the paired culture is shown. B2: In the presence of (+)-butaclamol, rhodamine-NAPS labeling is about the same intensity as that of unlabeled cells in A2.
Partition coefficients of the fluoroprobes

Because both fluorprobes are weak bases, the preferential acidic uptake of BODIPY-NAPS as compared with rhodamine-NAPS could be due to differences in their membrane permeability. To test this, we determined the octanol:water partition coefficients of the fluoroprobes. We found a log P of 0.79 ± 0.08 for rhodamine-NAPS (n = 3) and 1.12 ± 0.05 for BODIPY-NAPS (n = 3). Rhodamine-NAPS is thus about twice as hydrophilic as BODIPY-NAPS.

Acidotropic uptake of native antipsychotics

To test native antipsychotics for acidic uptake, we examined their ability to dissipate AO staining of acidic intracellular compartments. We applied spiperone (n = 3), haloperidol (n = 3), (+)-butaclamol (n = 1), (−)-butaclamol (n = 1), clozapine (n = 3), raclopride (n = 3), and sulpiride (n = 3) starting at a concentration of 0.01–1 μM and then in increasing orders of magnitude until punctate AO staining was dissipated. In each experiment, the fields imaged included several cells, and other fields in the culture were surveyed to verify that the observed changes were representative. In this way we determined the threshold concentration for each drug where AO staining shifted from a punctate pattern to a more diffuse cytoplasmic pattern, reflecting displacement of AO out of acidic intracellular compartments to the cytoplasm. This threshold correlated better with the partition coefficient (r = 0.92, p < 0.01) than with the pK(r = 0.74, p < 0.1) of the compounds (Table 1). The butyrophenones (spiperone, haloperidol, and butaclamol) were most potent (images for spiperone are shown in Fig. 8), clozapine was intermediate (data not shown), and the substituted benzamides (sulpiride and raclopride) were least potent, only reducing AO staining at considerably higher concentrations (images for sulpiride are shown in Fig. 9). We also used AO staining to assess
TABLE 1. Correlation of AO threshold with amine pK and log P

<table>
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<tr>
<th></th>
<th>Bataclamol</th>
<th>Spiperone</th>
<th>Haloperidol</th>
<th>Clozapine</th>
<th>Raclopride</th>
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<td>Threshold (µM)</td>
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<td>1</td>
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<td>10</td>
<td>100</td>
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<td>8.66</td>
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<td>log P</td>
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<td>3.36</td>
<td>3.93</td>
<td>1.33</td>
<td>0.58</td>
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Values listed vertically are differing determinations by the research groups noted; the top values were used for the correlation in Discussion.

a Identical results were obtained with the (+) and (-) enantiomers.

b Chranowski et al. (1985).


d Pauwels et al. (1986).

e el Tayar et al. (1985) and data cited therein.

f Tsai et al. (1993); raclopride has an additional weak acid pK of 5.81 due to its phenolic hydroxyl group.

g Sulpiride has an additional pK in the very acidic range due to its amino sulfonyl group.

acidotropic uptake of rhodamine-NAPS; 10 µM rhodamine-NAPS dissipated punctate AO staining without itself showing significant uptake (data not shown).

**DISCUSSION**

Visualization of three modes of antipsychotic binding

_D2-like receptor binding_. In cultured mesolimbic system neurons, the rhodamine-NAPS labeling we have identified as being due to receptor binding is D2 mediated based on the following criteria: (a) The pattern of labeling is similar to the pattern of specific labeling of HEK 293 cells transfected with functional D2 receptors. (b) The kinetics of labeling are similar to the kinetics of N-[3H]methylspiperone binding in membrane preparations (Hall et al., 1990). (c) The frequencies of labeled VTA and nAcc cells are similar to the frequency that these cells express D2 receptors in studies in brain sections (see below). (d) Labeling is seen only in older cultures, consistent with the typical increase in neurotransmitter receptor expression in ventral mesencephalic neurons over the first 2 weeks in culture (see, e.g., Dana et al., 1991; Van Muiswinkel et al., 1993). (e) Labeling can be stereospecifically blocked.

It is striking, however, that we could not displace rhodamine-NAPS labeling. This does not appear to be due to toxicity or photodynamic damage as electrophysiological measures are unaffected by fluoroprobe labeling and fluorescence excitation, and imaged cells generally show no reduction in viability after 24 h (S. R., unpublished data). More likely, the inability to displace rhodamine-NAPS labeling is due to lipophilic solvation, which places a time window on the ability to observe specific labeling. When time is allowed for the intensity of specific membrane labeling to build up (observed τon of 8 min) and then for displacement to

**FIG. 8.** Visualization of acidotropic uptake of spiperone. A: A single VM neuron 43 days in vitro is shown. The cytoplasm has a granular appearance under DIC optics, whereas the nucleus appears smooth except for a prominent nucleolus. There was no change in cell morphology during the course of the experiment. B: Acidic intracellular sites are stained with AO (100 nM); images are digitally shadowed to enhance the visualization of punctate cytoplasmic staining. Consistent with its use as a DNA stain, AO also prominently stains the nucleolus (this staining increases as AO is displaced from acidic intracellular compartments, most likely reflecting transiently elevated intracellular free AO concentrations). B1: Spiperone (0.01 µM) for 5 min has no effect on control AO staining (control image not shown). B2: Similarly, 0.1 µM spiperone does not change the pattern of punctate AO staining. B3: At 1 µM, spiperone begins to shift AO staining from punctate to diffuse cytoplasmic. B4: At 10 µM, spiperone has eliminated discernable punctate staining. B5: At 100 µM, spiperone further increases diffuse cytoplasmic staining. Thus, 1 µM is identified as the threshold concentration for acidotropic uptake of spiperone (see Table 1).
FIG. 9. Lack of acidotropic uptake of sulpiride. A: Two nAcc cells 15 days in vitro are shown under DIC optics. B1: Sulpiride (1 μM) for 5 min has no effect on acidic intracellular sites stained with 100 nM AO (control image not shown). B2: Sulpiride at 10 μM (5 min) has no effect on AO staining. B3: Sulpiride at 100 μM (5 min) begins to attenuate AO staining in the lower cell slightly. B4: Sulpiride at 1 mM (5 min) further attenuates but does not abolish AO staining. C: As a control, 10 mM NH4Cl completely dissipates punctate AO staining; cytoplasmic staining became quite intense so the image has been rescanned to reduce its intensity. Thus, 1 mM sulpiride is identified as the threshold concentration for acidotropic uptake of sulpiride (see Table 1).

occur (assuming that the \( \tau_{off} \) is minimally twice the \( \tau_{on} \)), generalized lipophilic solvation has increased significantly (\( \tau \) of 23 min), raising the fluorescence of all membranes and impeding the resolution of a diminution in membrane labeling. Consistent with this, Vincent et al. (1993) found a similar time window for optimal visualization of receptor labeling in fresh frozen sections. Because rhodamine-NAPS is very lipophilic, it is also possible that it is displaced into the nearby membrane; if so, cells would continue to show the pattern of specific labeling even though the rhodamine-NAPS was no longer receptor bound. This is supported by the observation that hot spots of membrane labeling become less evident with time. If so, putative specific receptor labeling may comprise rhodamine-NAPS that is receptor bound and rhodamine-NAPS that has undergone what may be termed receptor-enhanced lipophilic solvation. Thus, the inability to displace rhodamine-NAPS membrane labeling is not inconsistent with mediation by specific receptor binding.

Acidotropic uptake. BODIPY-NAPS labeling is paradoxic. In membrane preparations, the compound shows a \( K_d \) over threefold stronger than that of rhodamine-NAPS, close to that of NAPS itself (Barton et al., 1991). Yet, when applied to neuronal cultures, BODIPY-NAPS shows a rapidly appearing punctate pattern of cytoplasmic labeling, resembling that seen with weak base vital dyes such as AO, which accumulates in acidic intracellular compartments (de Duve et al., 1974; Sulzer and Holtzman, 1989). Consistent with acidotropic uptake, punctate BODIPY-NAPS labeling is abolished by weak bases and is blocked by fixation of the cells. The labeling is not D2 mediated because it occurs in all neurons, as well as glia (although like AO staining, it is far more prominent in neurons because they are more three dimensional), and, unlike rhodamine-NAPS receptor labeling, which is seen only in more mature cultures, punctate labeling with BODIPY-NAPS is seen in cultures as young as 1 day in vitro. The rapid onset of this BODIPY-NAPS labeling argues against endocytosis, which is likely to be much slower. Furthermore, NAPS is a pure antagonist, so it should block agonist-mediated receptor internalization (e.g., von Zastrow and Kobilka, 1992).

Because antipsychotic drugs are thought to bind to D2 receptors in part via their positively charged amine moieties (Harrold et al., 1989), antipsychotics should be weak bases. Indeed, all have pK values in the range of 7.0–9.5 (see references in Table 1), indicating that some antipsychotic molecules will be neutral at physiological pH (or zwitterions forming an internal salt, as is the case for rasopride (Tsai et al., 1993)) and will partition across membranes. Once in acidic intracellular compartments they will be protonated and trapped (de Duve et al., 1974; Okuma and Poole, 1981; Maloteaux et al., 1983; Schmalzing, 1988). Trapping may be enhanced by complexation with fixed anionic sites on intraluminal membranes (Sulzer et al., 1987). The better correlation of the threshold for dissipation of AO staining with the partition coefficient than the pK suggests that lipophilicity plays the more important role in regulating the entry of antipsychotic drugs into acidic intracellular compartments.

Lipophilic solvation. Both rhodamine-NAPS and BODIPY-NAPS appear to show lipophilic solvation. This is based on the following arguments: (a) The extreme lipophilicity of spiperone and most other antipsychotics is well known (Seeman, 1980). (b) The time course is slower as compared with receptor labeling (at least for rhodamine-NAPS, where we can see receptor labeling well), consistent with lower affinity binding. (c) The pattern of labeling coincides with the distribution of lipid membranes in cells. This is most evident in the retina where the highest nonspecific BODIPY-NAPS labeling is seen in photoreceptor outer segments (Muresan and Besharse, 1993). (d) Neither weak bases nor competitors/displacers affect this labeling, and it can be seen in fixed cells; it appears also to be a factor in labeling of frozen sections (Vincent...
et al., 1993). Because most native antipsychotics, with the exception of the substituted benzamides, are far more lipophilic than the fluoroprobe, the lipophilic solvation we have visualized with the fluoroprobe is likely to be an underestimate of the magnitude of the effect in the binding of unmodified butyrophenones and other lipophilic antipsychotic drugs.

**Rhodamine-NAPS labeling and D2 receptor distribution**

*Subcellular distribution.* We have shown that rhodamine-NAPS labeling of somatodendritic membranes for up to 30 min mainly reflects stereospecific D2 binding. Based on the similar time course, rhodamine-NAPS probably also labels intracellular D2 receptors. Initially, cytoplasmic fluorescence in cells showing specific labeling is more punctate, distinct from the diffuse cytoplasmic fluorescence associated with lipophilic solvation, arguing that rhodamine-NAPS labels intracellular D2 receptors on a subset of organelles. On the cell surface, the distribution of rhodamine-NAPS D2 labeling is relatively homogeneous with superimposed hot spots. We have found that hot spots are often associated with neurites making close contact with specifically labeled cells (Rayport and Sulzer, 1993), which could represent labeling of pre- or postsynaptic receptors. In favorable cultures, rhodamine-NAPS D2 labeling can be followed for some distance on putative dendritic processes before it becomes indistinguishable from glial labeling, which based on its subcellular distribution is probably due to lipophilic solvation—although we cannot rule out that it may also be due to low levels of D2 receptor expression in glial cells (Hösl and Hösl, 1987).

In the intact brain at the light level, D2-specific antipeptide antisera predominantly label the membranes of striatal cells (Ariano et al., 1993), in a pattern very similar to what we observe in culture. However, immunocytochemical localization of D2 receptors at the electron microscopic level shows that the receptors tend to be more densely expressed on distal dendrites and spines both in the VM and striatum (Levey et al., 1993; Sesack et al., 1994). Further work will be required to assess whether D2 receptor labeling can be discerned on more distal processes of living cells in culture. Possibly coculturing VTA neurons with their nAcc targets will favor greater receptor expression on neuronal processes. Consistent with this, the hot spots we have seen so far have been only on VTA neurons and not on nAcc neurons, which might be an in vitro correlate of the DA synapses formed by DA neurons onto other DA neurons in the intact VTA (Bayer and Pickel, 1990), whereas nAcc cultures devoid of DA neurons lack DA synapses.

*Cellular distribution.* In the dorsal striatal complex, in situ hybridization shows that about half (Gerfen et al., 1990; Le Moine et al., 1991) to two-thirds (Meador-Woodruff et al., 1991; Lester et al., 1993) of the medium-spiny GABAergic neurons and most of the larger cholinergic interneurons (Le Moine et al., 1990) express D2 receptors. In postnatal nAcc cultures, these two classes of neurons can be distinguished by size (Shi and Rayport, 1994; L. Lin and S. Rayport, unpublished data). We find that about half of medium-sized, putative medium-spiny GABA neurons show D2 labeling and that the larger, putative cholinergic neurons are labeled at a lesser intensity, which is only obvious after digital deconvolution. This pattern is also consistent with rhodamine-NAPS labeling in the intact striatum (Ariano et al., 1991).

In the VTA, although D2 receptors are principally expressed by the intrinsic DA neurons (Schalling et al., 1990; Le Moine and Bloch, 1991; Meador-Woodruff et al., 1991), recent work suggests that there may be non-DA VTA neurons that also express D2 receptors (Sesack et al., 1994). In the cultures, we find that the incidence of cells showing rhodamine-NAPS labeling significantly exceeds the incidence of DA neurons. Although siperone also binds to 5-HT₂ receptors with moderately high affinity \( K_i = 100 \text{ nM} \) for rhodamine-NAPS (Barton et al., 1991), blocking 5-HT₂ receptors with mianserin did not affect the incidence of labeled cells. Moreover, 5-HT₂ receptor expression in the VM is very low (Palacios and Dietl, 1989), and blocking 5-HT₂ receptors does not alter fluoroprobe labeling in VM or striatum (Ariano et al., 1991) or only slightly reduces labeling intensity slightly in prefrontal cortex without altering the pattern of labeled cells (Vincent et al., 1993). The excess of rhodamine-NAPS-labeled cells may also be due in part to rhodamine-NAPS labeling of D2 receptors on DA neuron varicosities, which envelope non-DA neurons in nest-like contacts (Rayport et al., 1992b), producing a pattern of labeling that mimics soma membrane labeling (Rayport and Sulzer, 1993).

**Difference between rhodamine-NAPS and BODIPY-NAPS**

Despite the higher affinity of BODIPY-NAPS as compared with rhodamine-NAPS for D2 receptors, BODIPY-NAPS fails to label D2 receptors clearly. Because the fluoroprobe share the same parent NAPS, the differences in their properties must be due to differences in the properties of the fluorochromes. BODIPY is uncharged but polar owing to its fluorines (Fig. 10), so that it increases the hydrophilicity of NAPS but not as much as rhodamine, which introduces a resonant positive charge on its tertiary amine groups and a weak acid carboxyl group, together accounting for its greater hydrophilicity and the latter for the lack of accumulation. Because BODIPY-NAPS accumulates in acidic intracellular compartments, it can be classified as a vacuologenic weak base, whereas rhodamine-NAPS dissipates AO staining without being accumulated, making it a nonvacuologenic weak base (Okuma and Poole, 1981). Thus, the lack of rhodamine-NAPS accumulation and its lesser lipophilicity appear to favor receptor visualization, whereas greater acidotropic uptake and
rapid lipophilic solvation of BODIPY-NAPS likely obscures receptor labeling. After dissipation of acidic intracellular gradients, BODIPY-NAPS shows very faint D2 labeling, arguing that lipophilic solvation alone obscures a significant portion of D2 labeling. Another factor that is likely to obscure D2 labeling with BODIPY-NAPS (as well as with fluorescein-NAPS) is the greater tissue autofluorescence in the green. It is also possible that rhodamine-NAPS is concentrated near receptors owing to receptor-enhanced lipophilic solvation, thereby intensifying labeling.

Fluoroprobe binding as an in vitro correlate of PET DA receptor labeling

We find that spiperone dissipates A0 staining at 1 μM concentrations, whereas raclopride dissipates A0 staining at 100 μM. Like rhodamine-NAPS, spiperone more readily enters cells than raclopride and is likely to label intracellular receptors as well as surface receptors. Like BODIPY-NAPS, spiperone is also more likely than raclopride to be sequestered in acidic intracellular compartments. Also, based on its greater lipophilicity, spiperone should show more lipophilic solvation than raclopride. Taken together these factors may account for the surprising persistence of spiperone (and of other butyrophenones) in the living brain.

This persistence may account in part for the divergent results found with radiolabeled spiperone derivatives and raclopride in PET studies that have sought to assess whether D2 receptor numbers are elevated in patients with schizophrenia (Sedvall, 1992). The differences in physiochemical properties may impact in two ways: (a) If DA receptor turnover is greater in schizophrenia, the intracellular receptor pool might be larger, which would be detected with spiperone but not with raclopride. (b) Because synaptic vesicles apparently acidify during recycling (Sulzer and Holtzman, 1989) and synaptic vesicles require acidic gradients for monoamine accumulation (Johnson, 1988), if DA release is elevated in schizophrenia (Sedvall, 1992), this might selectively enhance acidotropic uptake of spiperone and not of raclopride. Thus, the physiochemical differences we have highlighted—along with differing receptor affinities [accounting for selective interference by endogenous DA in raclopride binding (Young et al., 1991)] and differential binding of spiperone to D4 receptors [which may be selectively elevated in schizophrenia (Seeman et al., 1993)]—may account for the divergent results obtained with spiperone and raclopride.

Therapeutic implications

Acidotropic uptake of antipsychotic drugs appears to reduce DA accumulation in synaptic vesicles (Moriyama et al., 1993), and once in synaptic vesicles the drugs show K⁺-dependent release (Schmalzing, 1988). This suggests that antipsychotic drugs may be recycled at DA synapses. If so, part of the delay in the onset of the therapeutic action characteristic of these drugs could reflect the time required for their concentration to build up at DA synapses. To the extent that antipsychotic drugs dissipate acidic intracellular gradients, chronic use may also impair acidification-dependent cellular metabolism (Kodavanti and Mehendale, 1990) with subtle toxic effects.

Acidotropic uptake of antipsychotic drugs may determine in part when rebound symptoms occur during drug withdrawal. For instance, fluphenazine washes out of the brain ~10 times faster than haloperidol, paralleling the recovery of the behavioral response to apomorphine challenge, and this does not appear to be due to differences in receptor affinity or partition coefficient (Cohen et al., 1992) but might be due to less acidotropic uptake of fluphenazine. Like fluphenazine, clozapine also shows rapid wash out from the brain (Baldessarini et al., 1993), and this may account for clinical observations of rapid relapse following withdrawal of clozapine but not of haloperidol (Alphs and Lee, 1991). Consistent with its more rapid wash out, we found that clozapine showed less acidotropic uptake than the butyrophenones. This suggests that acidotropic uptake may regulate the concentration of antipsychotic drugs at DA synapses.

Conclusions

Fluorescent derivatives of spiperone can be used to follow the interaction of antipsychotic drugs with living mesolimbic neurons. In this way we have visualized D2 receptors on living mesolimbic system neurons for the first time. Rhodamine-NAPS binding for up to 30 min
principally labels D2-like receptors, whereas BODIPY-NAPS shows rapid acidotropic uptake and lipophilic solvation. Because acidotropic uptake closely mimics patchy receptor distribution, acidotropic uptake of fluoro- probes must be ruled out in visualization of receptors on living cells. The differences between BODIPY-NAPS, which is more representative of spiperone, and rhodamine-NAPS, which is more representative of raclopride, illustrate a lipophilic to hydrophilic spectrum in antipsychotic drug binding and make possible discerning the relative impact of these different modes of binding. Depending on the relative importance of receptor binding, acidotropic uptake, and lipophilic solvation, different antipsychotic drugs may have strikingly different properties as PET tracers and as therapeutic agents.

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