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The High Affinity Dopamine D₂ Receptor Agonist MCL-536: A New Tool for Studying Dopaminergic Contribution to Neurological Disorders

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Abstract

The dopamine D₂ receptor exists in two different states, D₂^{high} and D₂^{low}; the former is the functional form of the D₂ receptor and associates with intracellular G-proteins. The D₂ agonist [³H]MCL-536 has high affinity for the D₂ receptor (K_d 0.8 nM) and potently displaces the binding of (*R*)-(-)-*N*-*n*-propylnorapomorphine (NPA; K_i 0.16 nM) and raclopride (K_i 0.9 nM) in competition binding assays. Here, we further characterize [³H]MCL-536. [³H]MCL-536 was metabolically stable, with about 75% of the compound remaining intact after 1 h incubation with human liver microsomes. Blood–brain barrier penetration in rats was good, attaining at 15 min a % injected dose per gram of wet tissue (%ID/g) of 0.28 in males versus 0.42 in females in the striatum. Specific uptake ratios ([%ID/g striatum]/[%ID/g cerebellum]) were stable in males during the first 60 min and in females up to 15–30 min. The D₂-rich striatum exhibited the highest uptake and slowest washout compared to D₂-poor cortex or cerebellum. In peripheral

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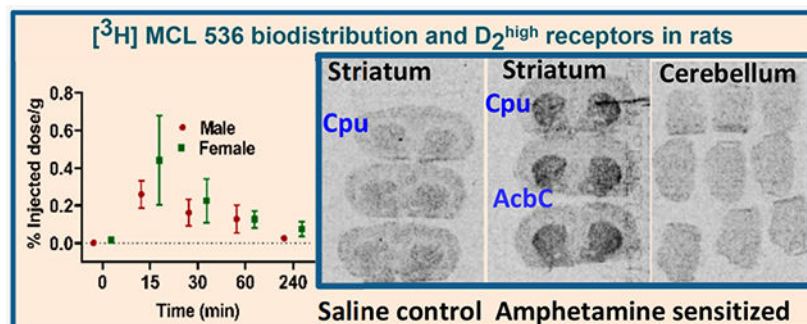
Author Contributions

S.S. carried out biodistribution studies, amphetamine sensitization, autoradiography, and data analysis and wrote the manuscript; AWS carried out synthesis of precursors and cold ligands; S.S. and A.W.S. carried out metabolism studies in microsomes; all authors discussed the results and contributed to the final manuscript.

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organs, uptake peaked at 15 min but declined to baseline at 60 min, indicating good clearance from the body. *In vitro* autoradiography on transaxial and coronal brain sections showed specific binding of [³H]MCL-536, which was abolished by preincubation with D₂/D₃ ligands sulpiride, NPA, and raclopride and in the presence of the stable GTP analogue guanylylimidodiphosphate. In amphetamine-sensitized animals, striatal binding was higher than in controls, indicating specificity for the D₂^{high} receptor state. [³H]MCL-536's unique properties make it a valuable tool for research on neurological disorders involving the dopaminergic system like Parkinson's disease or schizophrenia.

Graphical Abstract



Keywords

dopamine D₂^{high} receptor; Parkinson's disease; schizophrenia; aporphine; tritiated radioligand

INTRODUCTION

The neurotransmitter dopamine is a key mediator of signaling in the brain and disturbance of the dopaminergic system, especially if it involves the D₂ dopamine receptor, which has been closely associated with a variety of neurological disorders including Parkinson's disease (PD), schizophrenia, restless leg syndrome, and attention deficit disorder, to name just a few.¹⁻⁶ Some of these neurological and psychiatric diseases become symptomatic long after the first significant changes in brain physiology occur. An earlier diagnosis is desirable in these cases, since this would allow for earlier and more targeted intervention. Ultimately, broadening the arsenal of treatment options would greatly benefit patients with dopaminergic dysfunction.

Dopamine receptors are G-protein coupled receptors (GPCRs), divided into two subfamilies based on their signal transduction characteristics. The subfamily of D₂-like receptors (D₂, D₃, and D₄) inhibit cAMP production by adenylate cyclase.⁷⁻⁹ The D₂ receptor subtype is a major target in the pathophysiology and treatment of schizophrenia and PD. Typical for GPCRs, it exhibits interconvertible high- and low-affinity states for agonists *in vitro*.¹⁰⁻¹² In the high-affinity state, which is considered to be the active form of the receptor, D₂ is coupled to the G-protein.¹³ In the low-affinity state, in contrast, the receptor is uncoupled from the G-protein and is inactive. A plethora of evidence indicates that alterations

in the density of D₂ receptors in the high-affinity state may be more relevant to the pathophysiology of neuropsychiatric disorders than alterations in the total receptor density.¹²

Currently, there are many high affinity antagonist D₂/D₃ receptor ligands, which are widely used, for example, in PET imaging. However, in light of the evidence above, focus has recently been redirected to the development of agonist D₂ receptor ligands, which have great promise with regard to their potential to differentiate the high- and low-affinity states of the D₂ receptor; notable examples include [¹¹C]MNPA,¹⁴⁻¹⁹ [¹¹C]NPA,²⁰ and [¹¹C]-(+)-PHNO.^{21,22}

We have synthesized a highly promising high affinity dopamine D₂ receptor ligand, *R*-(-)-2-(3-fluoropropanoxy-11-hydroxy-*N*-*n*-propyl(1,2-[³H])noraporphine (MCL-536), as a potential tritiated radioligand for applications in receptor binding assays and autoradiography studies *in vitro*.²³ MCL-536 is an aporphine agonist that binds with high affinity to dopamine D₂ receptors but not to other receptors of the D₂ receptor family.^{24,25} MCL-536 also exhibits little to no affinity for other receptors found in the brain.²⁵ MCL-536 has a high affinity for the D₂ receptor in its active state, with a *K*_d value of 0.8 nM and *K*_i values of 0.16 nM for (*R*)-(-)-*N*-*n*-propylnorapomorphine (NPA) and 0.9 nM for raclopride, respectively, in competition binding assays.²⁵

The ability to label a radiotracer with ¹⁸F instead of ¹¹C greatly increases its utility and value to the research and medical communities, since ¹⁸F has a much longer half-life than ¹¹C (110 min versus 20 min) and does not limit the use of the radiotracer to facilities with an on-site cyclotron.

Previous attempts to develop ¹⁸F-labeled D₂/D₃ ligands from nonfluorinated analogues were not successful, underscoring the fact that incorporation of ¹⁸F into a tracer and preserving its biological activity is not a trivial task.²⁶⁻²⁸ In contrast, MCL-536 possesses a fluoropropanoxy side chain rather than *N*-fluoroalkyl moiety, and it lacks the unstable catechol moiety found in [¹¹C]MNPA and [¹¹C]NPA.

Here, we further characterize MCL-536 with regard to its metabolic stability using a human liver microsome assay, biodistribution in rats *in vivo*, and binding characteristics in rat brain tissue, evaluated by *in vitro* autoradiography in the presence or absence of competing ligands, and in amphetamine sensitized rats *ex vivo*, which have increased numbers of D₂ receptors in the high affinity state.

RESULTS AND DISCUSSION

Microsomal Stability.

Investigation of metabolic stability of MCL-536 (Figure 1A) in human liver microsomes resulted in a high recovery at 1 h of about 75% of the ligand, indicating good metabolic stability (Figure 1B).

Biodistribution of MCL-536 in the Brain and Peripheral Organs.

The highest brain uptake of [³H]MCL-536 was observed after 15 min in the striatum, with an uptake of 0.28% injected dose per gram wet tissue (%ID/g) in males and 0.42 %ID/g in females (Figure 2A,B). The difference in % ID/g between males and females was not significant. The striatum is known to be rich in D₂ receptors.²⁹ In contrast, the cerebellum, a region that contains almost no D₂ receptors, showed a baseline binding between 0.1 and zero (Figure 2A,B). The washout rate of [³H]MCL-536 was comparatively fast in both sexes, showing a decrease in concentrations of about 50–70% by 60 min. Values reached almost baseline 4 h after injection of the ligand (Figure 2B).

Specific uptake ratios, defined as [%ID/g striatum]/[%ID/g cerebellum] reached a maximum of about 4 for both males and females. In males, the ratio was increased during the first 60 min after injection. In females, however, it reached the maximum at 15 min, followed by a slow decline to about 3 at 60 min (Figure 2C). For all other brain regions, namely, the cortex, thalamus, hypothalamus, and medulla, the specific uptake ratios were between about 0.5 and 2, confirming that in regions with low D₂ receptor expression, binding of [³H]MCL-536 is low.

In peripheral organs and tissues, uptake of [³H]MCL-536 was comparatively low in heart, spleen, and muscle in both sexes, but higher in fat and lungs (Figure 3A,B). Highest %ID/g was observed in liver and kidneys. In all peripheral tissues the maximum %ID/g was at 15 min, followed by a washout (Figure 3A,B).

The biodistribution studies showed that the uptake of [³H]MCL-536 was highest in the striatum and in the kidneys, with comparable maxima. However, %ID/g in the striatum was stable in females and declined only by about 25% in males at 60 min postinjection, while in the kidneys, the decline was about 60% by 60 min in both sexes. The data suggest an efficient passage of the radioligand through the blood–brain barrier as well as an efficient excretion from the body.

In Vitro Autoradiography.

In vitro autoradiography on transaxial brain sections showed specific binding of [³H]MCL-536 (Figure 4D) in the striatum, which could be abolished by preincubation with receptor ligand sulpiride (10 nM) and by blocking with cold ligand MCL-536 (100 nM), as well as in the presence of guanylylimidodiphosphate (Gpp(NH)p, 200 μM) and raclopride (100 nM). Without preincubation with buffer, the presence of endogenous ligand at the receptors inhibited binding of [³H]MCL-536.

³H microscales were exposed simultaneously as a reference for total radioactivity quantitative analysis (Figure 4C), and the gray values were converted into relative optical densities (ROD, Figure 4B). Quantitative analysis of D₂ receptors (fmol/mg) and the receptor density ratios between D₂-rich caudate putamen versus D₂ poor cerebellum as well as D₂ poor cortex versus D₂ poor cerebellum are shown in Figure 4E,F, respectively. From the data, it is clear that [³H]MCL-536 is able to detect the elevated number of D₂ receptors in the striatum as compared to cerebellum or cortex.

Figure 5A shows that MCL-536 specifically detects a dense population of D₂ receptors in the caudate putamen and nucleus accumbens. Again, this binding could be abolished by co-incubation with D₂ receptor antagonists sulpiride and raclopride, as well as by presence of cold MCL-536 and (Gpp(NH)p.

Ex Vivo Autoradiography in D₂^{high} Rat Model.

Since amphetamine sensitization markedly up-regulates D₂ receptors in the high affinity state *in vitro*,¹² this model was used to determine whether [³H]MCL-536 detects such an elevation in D₂^{high}. As shown in Figure 5B, [³H]MCL-536 detected more D₂ receptors in the striatum of amphetamine sensitized animals compared to controls. In the cerebellum, a D₂ poor region, as expected, no increase in the binding of [³H]MCL-536 was detected.

Results confirm that [³H]MCL-536 binds preferentially at D₂^{high} receptors in D₂ rich regions like the striatum, especially the area of the caudate putamen and nucleus accumbens, confirming selectivity of the ligand in brain tissue.

Most of the commonly used antipsychotic drugs act at dopamine D₂ receptors. Binding and blocking of these receptors is important in the efficacy of these drugs toward psychiatric illness, for example, against the positive symptoms of schizophrenia. Since drugs with very different pharmacological and therapeutic profiles act at D₂ receptors, these receptors are still subjected to intense research both *in vitro* and *in vivo*. Tools that enable a comprehensive study of D₂ receptors are therefore in demand. An agonist ligand is preferable in this context because it is able to distinguish between the active D₂^{high} state of the receptor and its inactive D_{2low} state, while antagonist ligands fail to do that.

There are many examples of radioligands for D₂/D₃ receptors, including the antagonists [³H]spiperone and its more lipophilic derivative [³H]*N*-methylspiperone (NMSP),³⁰ [³H]domperidone,³¹ [³H]raclopride,³² [³H]nemonapride,³³ and [³H]remoxipride.³⁴ Among the agonists are [³H]-apomorphine³⁵ [³H]-*N*-propyl-norapomorphine ([³H]-NPA),³⁶ and [³H]quinpirole.³⁷ However, the usefulness of these substances is limited, either because of their antagonist nature, which excludes distinction between high and low affinity D₂ receptors, or because of their cross-reactivity with other members of the dopamine receptor family or even other receptor families, for example, adrenergic or serotonergic receptors, which compromises specificity.

The D₂ receptor agonist MCL-536 has shown highly favorable *in vitro* properties toward D₂ receptors, with a subnanomolar binding affinity (K_d 0.8 nM) for human cloned D₂ long receptors expressed in CHO cells but not for the other receptors of the D₂ subfamily. The biphasic nature of its curves shows that MCL-536 clearly differentiates between the active high affinity and the inactive low affinity state of the receptor.²⁵ In *in vitro* competition experiments, [³H]MCL-536 had a K_i of 0.16 nM against the D₂/D₃ agonist (*R*-(-)-*N*-*n*-propylnor-apomorphine, NPA) and a K_i of 0.9 nM against the D₂/D₃ antagonist raclopride. Co-incubation with guanylimidodiphosphate abolished binding to D₂^{high}. MCL-536 exhibited little to no affinity for other receptors tested.^{25,38} These properties make MCL-536 superior to other agonist ligands, which are less specific and usually have high binding affinities to more than one receptor.

One of the conditions for the suitability of MCL-536 as a D_2^{high} receptor radioligand for application in research, diagnosis, and treatment is metabolic stability. When tested in an assay with human liver microsomes, MCL-536 showed good metabolic stability with about 75% of the ligand still intact after 60 min of incubation. The rationale of this strategy is that the *in vitro* metabolic stability in the preparations from humans should reasonably well predict *in vivo* clearance in humans.

Another prerequisite for the usefulness of a radioligand is a good pharmacological profile, especially for *in vivo* applications, and good penetration of the blood–brain barrier, since neurological and psychiatric symptoms originate in D_2 rich areas in the brain. Figure 2 shows the results of the *ex vivo* biodistribution studies in rodent brain. [^3H]MCL-536 exhibited a rapid (15 min after injection into the tail vein) and highly specific binding in the striatum, a region enriched in D_2 receptors, which persisted for at least 60 min at a high level in both male and female rats. If compared to the binding in the cerebellum, a region expressing a low number of D_2 receptors, the ratio striatum/cerebellum was much higher than the ratios for other brain regions such as the cortex, thalamus, hypothalamus, and medulla, all regions expressing low numbers of D_2 receptors.²⁹ This not only suggests good penetration through the blood–brain barrier, which is a crucial property of a potential radioligand for receptors located in the brain but also confirms the preference of the ligand for D_2 receptors in tissue.

A good pharmacological profile, as mentioned above, especially for applications that involve humans, also requires that the ligand be excreted rapidly from the body. Figure 3 shows that after an initial peak, [^3H]MCL-536 is excreted rapidly from the body in both male and female rats. Highest concentrations were found in the body organs that mediate excretion, namely, the liver and kidney. In all other organs examined, heart, lungs, spleen, and muscle, initial uptake was much lower and excretion was rapid. A bit of an exception is the fatty tissue, where initial peaks were a bit higher; however, at 60 min, [^3H]MCL-536 levels were reduced by about 2/3 in this compartment as well. From all these data, it can be concluded that [^3H]MCL-536 has a very good ADME (absorption, distribution, metabolism and excretion) profile, suggesting its superiority to other D_2 receptor ligands in reaching the target tissue while limiting or minimizing the dose to other organs.

Examination of the binding of [^3H]MCL-536 to D_2 rich versus D_2 poor tissues in the brain *in situ* in the presence or absence of D_2 receptor antagonists provided valuable information concerning the specific displaceable binding to the dopamine D_2 receptor. Using autoradiography on transaxial and transversal brain slices, we could demonstrate enhanced binding in the D_2 receptor rich striatum, especially the caudate putamen and nucleus accumbens. In contrast, binding at other dopamine innervated regions including the cortex, thalamus, hypothalamus, and medulla was much lower.

Co-incubation with an excess of D_2/D_3 receptor antagonists sulpiride or raclopride completely abolished binding of [^3H]MCL-536. Raclopride, similar to other antagonists, has a high affinity to D_2 receptors but does not have the capability to distinguish between D_2^{high} and $D_{2\text{low}}$ in whole, unfrozen rat anterior pituitary cells *ex vivo*.^{4,5,32}

Likewise, competition with nonradioactive MCL-536 prevented binding of the ligand. It has been shown before that the percentage of D₂ sites in the high-affinity agonist state is high in tissue sections;³⁹ with [³H]spiperone (competing with dopamine), 90% of the dopamine receptors were shown to be in the high-affinity state on rat striatal slices. Addition of the GTP analogue GmP-PnP resulted in a total shift to the low-affinity state.³⁹ In more recent studies on both fresh and frozen and thawed rat brain sections, 13% and 22% of the receptors, respectively, were determined to be in the high-affinity state in striatal slices.⁵ This contrasts with homogenate binding where the percentage is usually much lower, varying from 28% to 56% when using dopamine as a competitor.⁴⁰⁻⁴² The first formal demonstration of a greater displaceability of an agonist ligand compared to an antagonist in the amphetamine challenge model was performed in mouse striatum using *N*-[³H]-*n*-propylnorapomorphine and [¹¹C]-raclopride.⁴³ It appears that higher proportions of D₂^{high} receptors are found for *in vivo* experiments⁴⁴ or in brain sections,^{39,45} where the tissue structure is more intact, than in disrupted and homogenized tissue. The abundance of agonist binding sites in striatum seems to be lower than that of antagonist binding sites: agonist B_{max} was found to be about 75–80% of antagonist B_{max} in two independent studies.^{44,46} However, this may be due to experimental differences in the molar activity determination of various radioligands or an experimental artifact caused by depletion of G-protein in brain sections, which may cause the D₂^{high} receptor to revert to D₂^{low} in the presence of high agonist concentrations. A review of available data did not strongly argue for a substantial difference between agonist and antagonist B_{max}.⁴⁵

The dopamine D₂ receptor is a G protein-coupled receptor. Its G protein alpha subunit binds GTP in the activated state, and the receptor becomes inactivated when GTP is metabolized to GDP and Pi. In our previous binding studies, the specific binding of [³H]MCL-536 was found to separate into two sites in the presence of GppNHp. The binding component removed by the guanine nucleotide represented the dopamine D₂^{High} receptors, confirming that [³H]MCL-536 is a D₂^{high} agonist.²⁵ In our present study, co-incubation with GppNHp abolished binding of [³H]MCL-536; this was especially clear in the caudate putamen and nucleus accumbens, which depicted an intense labeling that was reduced to background levels by GDP. This is strong evidence for the specific binding of MCL-536 to D₂^{high} receptors.

Quantification of the receptor binding in the caudate putamen yielded a mean specific binding of [³H]MCL-536 of about 145 fmol/mg protein. Seeman and Tellerico⁴⁷ found three sets of D₂ receptors in homogenates of rat striatum: D₂ low-affinity sites (9.8 pmol/g), D₂ high-affinity sites that were unmasked in the presence of D₁ receptor blockade by SCH-23390 (4.2 pmol/g), and D₂ high-affinity sites that were not unmasked by SCH-23390 but were converted to low-affinity sites in the presence of guanilylimidodiphosphate (3 pmol/g). In an *in vivo* study in contrast, the D₂ receptor density for the caudate putamen was determined to be 784 ± 60 fmol/mg protein.⁴⁸

Further evidence for the specificity of [³H]MCL-536 for D₂^{high} receptors could be demonstrated in our experiments with amphetamine-sensitized rats, which display elevated D₂ receptors in the high affinity state.¹² Amphetamine typically induces about a 2.36-fold increase in D₂^{high}, measured in the striatum *in vivo*, which corresponds nicely to a 2.44-fold

elevation of D_2^{high} *in vitro* in the same tissue.⁴⁹ As expected, in the present experiment, binding of [³H]MCL-536 in the striatum of amphetamine sensitized rats was much higher than that in control animals. This suggests that in its tritiated form, MCL-536 can be used experimentally to investigate D_2 receptor activity and dynamics more effectively as a function of neurological conditions. A recent study compared healthy volunteers who received a mildly sensitizing regimen of repeated oral amphetamine to unmedicated schizophrenia patients with a first-episode psychosis.⁵⁰ Amphetamine sensitization of healthy volunteers significantly increased dopamine release in the prefrontal cortex as evaluated by PET using the D_2/D_3 receptor agonist ligand [¹¹C]-(+)-PHNO. The dopamine release in amphetamine-sensitized volunteers was similar to that seen in the patient group. This study not only confirms the hypothesis of “endogenous sensitization” but at the same time also seconds the concept of impairment of prefrontal dopaminergic system in schizophrenia.

In summary as discussed in detail above, agonist ligands are superior, because their preferential binding to the receptors in their high affinity state makes them an ideal tool for studying the role of the receptor state in the pathogenesis of disorders associated with the dopaminergic system. MCL-536 with its high affinity to D_2^{high} receptors *in vitro* and *in vivo* with its good ADME profile is an excellent tool in this regard. Thus, MCL-536 will be enabling more detailed studies and enhancing the knowledge of dopamine transmission and D_2 receptor dynamics in these disorders.

METHODS

Microsomal Stability.

The NADPH-dependent metabolism of MCL-536 was studied using human liver microsomes (pooled) according to the supplier's instructions (Life Technologies; HMMCPL). Briefly, MCL-536 (2 μL of 100 \times stock) was preincubated with pooled human liver microsomes (5 μL of 20 mg/mL) in 183 μL of 100 mM phosphate buffer (pH 7.4) for 5 min at 37 $^\circ\text{C}$; then the reaction was initiated with 10 μL of NADPH (20 mM; final concentration 1 mM). After 30 or 60 min incubation at 37 $^\circ\text{C}$ (water bath), the reaction was stopped by addition of 200 μL of cold acetonitrile (MeCN). The samples were vortexed and then centrifuged at 3000 rpm (4 $^\circ\text{C}$) for 5 min to precipitate proteins. The supernatant (about 180 μL) was transferred to a vial and filtered through a 0.2 μm syringe filter prior to injection; then 100 μL of each filtrate was analyzed with a Varian Prostar HPLC system on Agilent Microsorb-MV 100 C18 columns 150 mm \times 4.6 mm, 5 μm particle size, fitted with a Microsorb 100–5 C18 MetaGuard column, 100 mm \times 4.6 mm. Samples were analyzed using a 1 mL flow rate and gradient method beginning at 20% B going to 90% B for 4 min, going to 20% in 2 min, and holding for 4 min (20 min total time). The mobile phases used were phase A, 100 mM phosphate buffer, pH 2.9, and mobile phase B, acetonitrile, with detector set at 265 nm to determine remaining MCL-536. Dextromethorphan (DXM) was used as positive control (detector set at 278 nm). Negative controls included DXM incubated with heat-inactivated microsomes (HDXM) and one incubation mix without NADPH. The metabolic stability was calculated based on the 0 min sample. Each experiment was performed in triplicate.

Ligand Studies.

[³H]MCL-536 was custom synthesized (maximum specific activity 60 Ci/mmol; American Radiolabeled Chemicals, Inc. St. Louis, MO 63146 USA).

Animals.

For biodistribution studies, a total of 60 adult (30 male and 30 female) Sprague–Dawley rats (Charles River Laboratories) were used for evaluation of the tritiated ligand. Animals were housed four rats per cage and allowed access to food and water *ad libitum*. The animal room was maintained on a reverse 12:12 light/dark cycle with lights off from 8:00 AM to 8:00 PM. Ten animals (5 male, 5 female) were used as controls; 50 animals (25 male, 25 female) were used for studies with [³H]MCL-536. All procedures involving animals were approved by the McLean Hospital IACUC Committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Biodistribution Studies.

[³H]MCL-536 in saline (6 μ Ci/300 g body weight, 200 μ L total volume, $n = 3$ –5 per group) was injected into the tail vein of male or female SD rats. Saline was used as a control. At 15, 30, 60, and 240 min after injection, selected organs (heart, liver, kidneys, lungs, spleen, fat, muscle, and brain) were removed. The striatum, cortex, thalamus, hypothalamus, medulla, and cerebellum were dissected. The organs and brain regions were weighed and dissolved in 1 mL of 0.8 N NaOH. After addition of 5 mL of scintillant, radioactivity was measured in a β -counter. The percent injected dose per gram (%ID/g) was calculated by normalizing the total counts of the organ by the weight of the organ as a percentage of the total injected radioactivity. Uptake in brain regions was calculated as the ratio of %ID/g of the region versus 5 the cerebellum, where D₂ receptors are scarce.

Autoradiography *In Vitro*.

Brains were sectioned at -12 °C into transaxial and transversal 20 μ m sections using a cryostat (HM560 Microm), and the sections were mounted onto Superfrost plus Gold adhesive slides (Fisher Scientific). Slides were organized in pairs, and for each pair, new sections were mounted alternatively on the first or second slide of the pair so that sections on the corresponding positions on the two slides forming a pair would represent adjacent tissue layers. The sections were allowed to dry and were stored at -80 °C with silica gel desiccant until used.

On the day of experiment, the slides were equilibrated 20 min at -20 °C and then allowed to dry at room temperature for 5–10 min. For binding studies, slides were equilibrated for 30 min with buffer (50 mM Tris-HCl, 1 mM EDTA, 5 mM KCl, 1.5 mM CaCl₂, 4 mM MgCl₂, 120 mM NaCl, 0.001% ascorbic acid and protease inhibitor cocktail tablet (Roche) pH = 7.4) at room temperature (RT).

Following preincubation, sections were incubated with 2 nM of [³H]MCL-536 alone or in combination with other ligands: nonspecific binding was determined in adjacent sections by addition of 10 μ M sulpiride (D₂ receptor antagonist), cold ligand MCL-536, or raclopride (D₂ receptor antagonist, 100 nM).

In order to examine the effects of guanine nucleotides on [³H]MCL-536 binding, a separate set of slices was incubated with 2 nM [³H]MCL-536, as described above, in the presence of 200 μM guanylimidodiphosphate (Gpp[NH]p), Millipore-Sigma, St. Louis, MO).

In a final set of slides, the preincubation step was omitted in the [³H]MCL-536 assay in order to verify the effects of endogenous ligands (data not shown).

After 2 h incubation at room temperature, sections were washed in 5 the appropriate buffer (2 × 5 min at 4 °C followed by a quick dip in ice cold distilled water) and left to dry at room temperature for 1 h. For visualization and optical density analysis, the sections were opposed to BioMax MR Film (Carestream) for 2–3 weeks alongside microscale-calibrated ³H standards ART0123C (0.03–4.24 nCi/mg, tissue equivalents) and ART0123B (0.7–27.3 nCi/mg, tissue equivalents) (American Radiolabeled Chemicals, St. Louis, MO).

Quantitative Analysis.

Analysis of optical density was carried out using the public NIH ImageJ program. The binding to the D₂ receptor was quantified by determining the gray levels of the pixels (relative optical density (ROD)). The binding density was quantified by setting the optical densities of the tissue autoradiograms in relation to those of the radioactive microscale standards, which were the same thickness as the tissue sections and for which the amounts of ⁷ radioactivity are known. A ROD measured in a region was interpolated to a value expressed as nCi/mg protein using the calibration curve. Specific binding was determined by the following relationship: fmol/mg protein = (*X* nCi/mg protein) × (fmol/V nCi), where *X* = tissue equivalent value over the region being studied and *V* = specific activity of the radiolabeled ligand. The left and right side of the brain was quantified separately on three consecutive sections, and the background level on each section was measured and subtracted. For each brain area, three consecutive sections were processed to determine the total binding, and one was processed to evaluate the nonspecific binding. Both total and nonspecific binding were calculated in the same way, and nonspecific binding was subtracted from total binding to give specific binding. The number of animals was *n* = 5 for each experimental group.

Amphetamine Sensitization and *Ex Vivo* Autoradiography.

Adult male Sprague–Dawley rats, weighing 120 g at the start of the experiment, were used. They were housed one per cage with free access to food and water at a constant temperature of 20 °C on a 12:12 reverse light/dark cycle. Lights were off at 8:00 AM.

Each rat received an intraperitoneal injection of 1.5 mg/kg d-amphetamine sulfate (Sigma, St. Louis, MO) or 0.9% saline (1 mL/kg), daily for 10 days, followed by an additional 10 days without any injections.^{47,51}

Rats were briefly anesthetized with 1.5–2% isoflurane, and tails were prewarmed for 5 min with a disposable heating pad to increase vasodilation. A catheter was placed into the lateral caudal tail vein. Subsequently, [³H]MCL-536 (6 μCi/300 g body weight in 200 μL of saline) was injected into the catheter using a 25-gauge needle, followed by 300 μL of saline to ensure that no ligand remained in the catheter. Immediately after injection, the catheter was

removed from the tail vein, and the puncture site was sealed with a wound closure strip. At the end of the experiment (60 min), rats were sacrificed, and the brains were removed, cut into transverse sections, and exposed to BioMax MR Film (Carestream) as described above.

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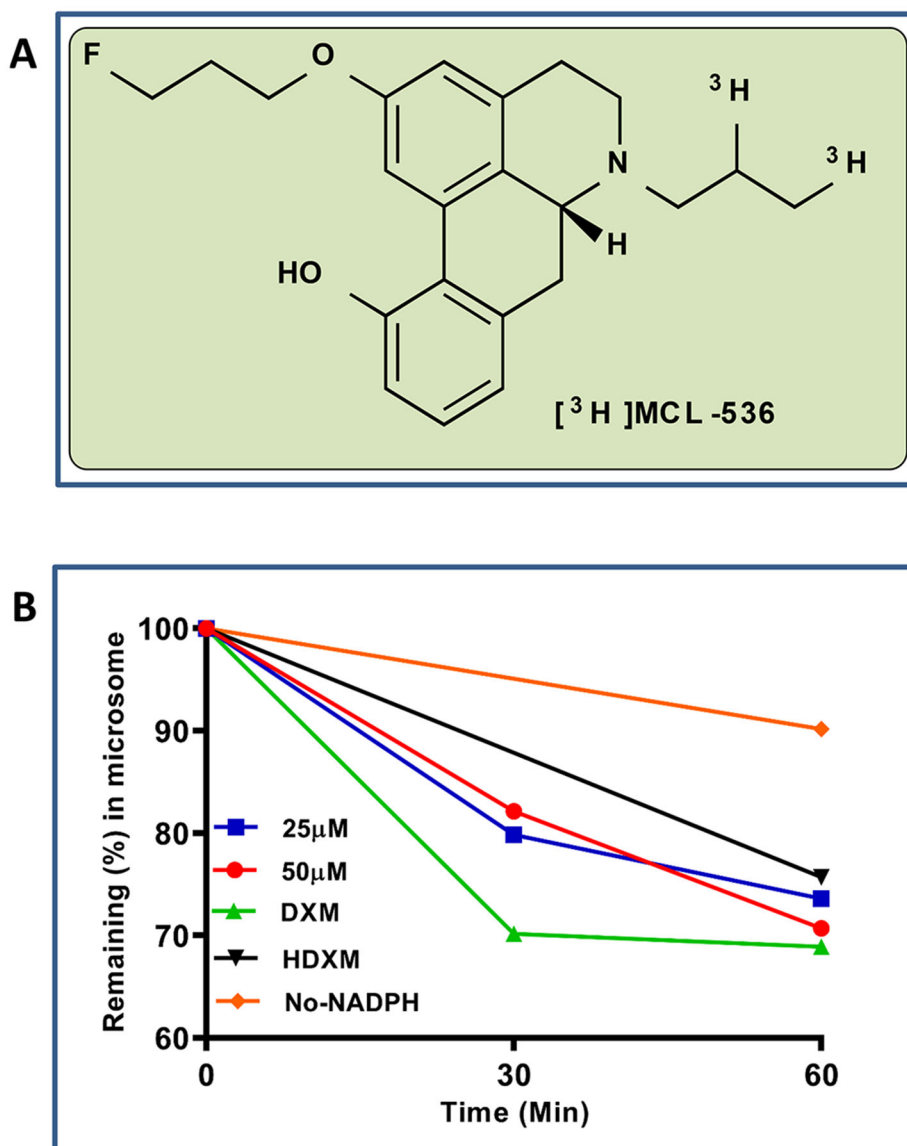


Figure 1. Human liver microsome stability assay of MCL-536. Briefly, MCL-536 was preincubated with pooled human microsomes in 100 mM phosphate buffer; then NADPH (final concentration 1 mM) was added. After 30 or 60 min incubation, the reaction was stopped, and samples were analyzed with a Varian Prostar HPLC system on Agilent Microsorb-MV 100 C18 columns fitted with a Microsorb 100-5 C18 MetaGuard column and a detector set at 265 nm. Dextromethorphan (DXM) was used as positive control (detection at 278 nm); negative controls included DXM incubated with heat-inactivated microsomes (HDXM) and one incubation mix without NADPH. The experiment was performed in triplicate. (A) Structure of MCL 536. (B) Microsome stability assay.

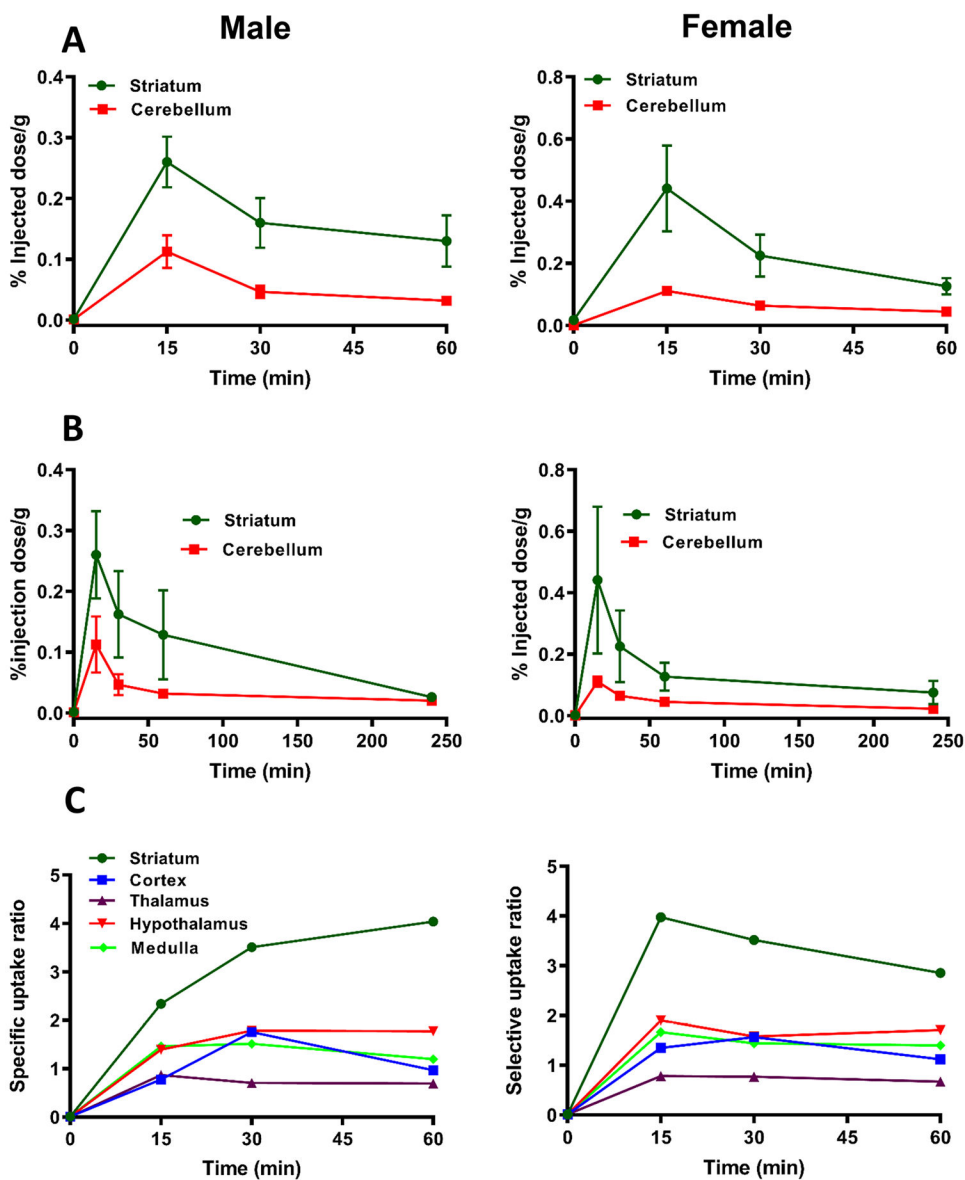


Figure 2. Biodistribution of [^3H]MCL-536 in the rat brain. A total amount of $6\ \mu\text{Ci}$ of [^3H]MCL-536 was injected into the tail vein of male or female Sprague–Dawley rats, and 15, 30, 60, and 240 min after injection, brain regions were dissected, and the tissue was weighed and dissolved in 1 mL of 0.8 N NaOH. After addition of 5 mL of scintillant, samples were counted in a β -counter and the %ID/g wet tissue was calculated. Data are presented as mean \pm SEM of 4 animals per time point and ligand. (A, B) Biodistribution of [^3H]MCL-536 in the striatum and cerebellum of female and male rats. (C) Biodistribution of [^3H]MCL-536 presented as the brain region/cerebellum ratio of female and male rats. Uptake in brain regions was calculated as the ratio of percent dose per gram of the region versus the cerebellum, where D_2 receptors are scarce.

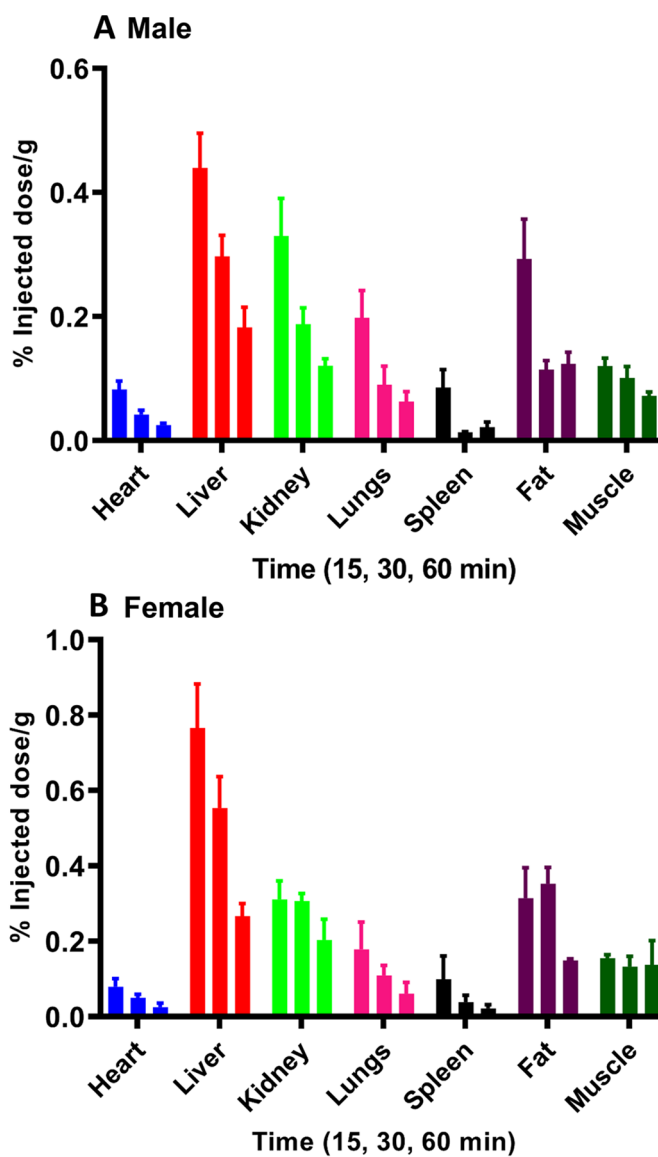


Figure 3. Biodistribution of [^3H]MCL-536 in peripheral organs in male and female rats. [^3H]MCL-536 ($6 \mu\text{Ci}/300 \text{ g}$ body weight) was injected into the tail vein; 15, 30, and 60 min after injection, peripheral organs were dissected, and the tissue weighed and dissolved in 1 mL of 0.8 N NaOH. After addition of 5 mL of scintillant, samples were counted in a β -counter and the %ID/g wet tissue was calculated. Data are presented as mean \pm SEM of 4 animals per time point and ligand. (A) Male rats. (B) Female rats.

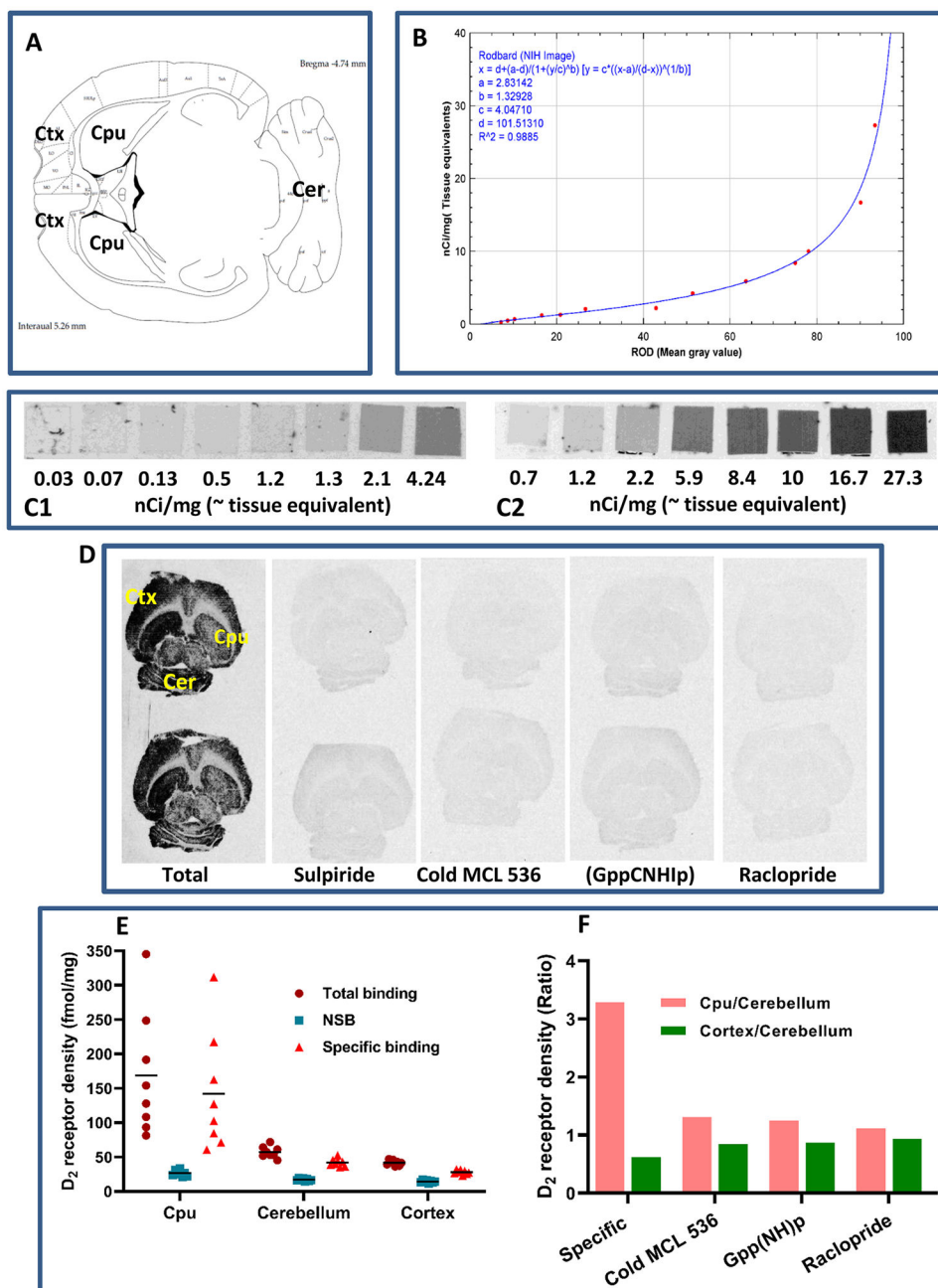


Figure 4. *In vitro* autoradiography showing the binding of [³H]MCL-536 in the rat brain. Transaxial brain sections were preincubated with buffer to eliminate nonspecific binding. Afterward, sections were incubated with [³H]MCL-536 alone or in combination with D₂ receptor antagonists or cold MCL-536 to show specificity. Dried sections were exposed to autoradiography film for visualization. (A) Schematic of a transaxial brain section; CPU, caudate putamen; Ctx, cortex; Cer, cerebellum. (B) Conversion of gray scale values to relative optical densities (ROD). (C) ³H microscale were coexposed on the same films as the experimental samples to allow quantification. (D) *In vitro* binding of radioligand to

10 μm thick rat brain transaxial slices in the presence or absence of 10 M sulpiride, 100 nM cold ligand, 200 μM Gpp(NH)p, or 100 nM raclopride. (E) Quantification of receptor density for D_2 (fmol/mg). (F) D_2 receptor density ratio in striatum versus cerebellum and cortex versus cerebellum in the presence or absence of other radioligands or cold MCL 536.

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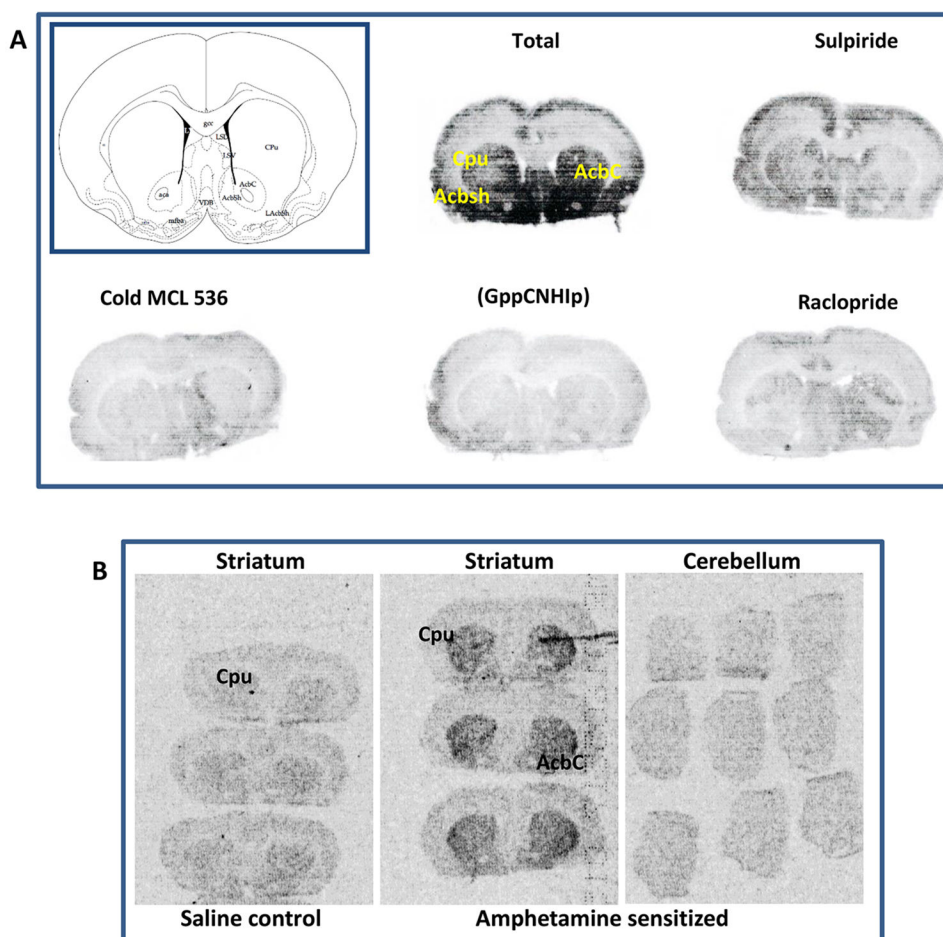


Figure 5. *In vitro* and *ex vivo* autoradiography of [³H]MCL-536 binding on transverse brain sections of untreated and amphetamine-sensitized rats. Transverse brain sections of control or amphetamine-sensitized rats were preincubated with buffer to eliminate nonspecific binding. Afterward, sections were incubated with [³H]MCL-536 alone or in combination with D₂ receptor antagonists sulpiride and raclopride, cold ligand, or Gpp(NH)p. (A) Schematic of a transverse brain section at the level of the anterior commissure (CPU, caudate putamen; AcbC, nucleus accumbens, core; Acbsh, nucleus accumbens, shell) and *in vitro* binding of [³H]MCL-536 to transverse rat brain slices at the level of the nucleus accumbens in the absence or presence of D₂ receptor antagonists sulpiride and raclopride, cold ligand, or Gpp(NH)p. (B) *Ex vivo* binding of [³H]MCL-536 to transverse rat brain sections of amphetamine-sensitized rats in comparison with control rats. Note that panels A and B are separate experiments and that exposure times of the autoradiographs on the film differ between panels A and B for better visualization.