In Vitro Competitive Binding Experiments

To investigate binding selectivity, the affinity of ^{127}I-iododexetimide was examined across mAchR subtypes M_1–M_5. Chinese hamster ovary cell membranes overexpressing human mAchR M_1–M_5 subtypes were incubated with a concentration of ^{3}H-NMS that was close to the calculated K_d for each receptor (M_1 200 pM, K_d = 196 pM; M_2 700 pM, K_d = 769 pM; M_3 700 pM, K_d = 642 pM; M_4 200 pM, K_d 142 pM; M_5 400 pM, K_d = 410 pM), in the presence or absence of 11 different concentrations of ^{127}I-iododexetimide.

All experiments were performed in assay buffer (buffer composition: 20 mM HEPES, 100 mM NaCl and 10 mM $MgCl_2$, pH 7.5) and used 10 μg of protein/well in a total assay volume of 1 mL using deep well blocks. All assay incubations were initiated by the addition of membrane suspensions, and deep well blocks were shaken for 5 min to ensure complete mixing. Incubation was then carried out for 2 h at 21°C. Binding reactions were terminated by rapid filtration through GF/A filters (Perkin Elmer) presoaked with 0.5% w/v poly-ethylene-imine (PEI) for 1 h. Filters were then washed 3 times with ice-cold assay buffer. Dried filters were counted with Meltilex A scintillant using a Trilux 1450 scintillation counter (Perkin Elmer).

The specific bound counts (dpm) were expressed as a percentage of the maximal binding observed in the absence of test compound (total) and nonspecific binding determined in the presence of 10 μM atropine.

To assess functional antagonism of ^{127}I-iododexetimide, further in vitro profiling was performed using a GTPγ^{35}S binding methodology. Membranes acquired from Chinese hamster ovary cells overexpressing human muscarinic M_1R–M_5R subtypes were used at 20 mg/well. MAchR expression levels (B_{max} values) were as follows: hM_1 (3.3 pmol/mg), hM_2 (11 pmol/mg), hM_3 (6.1 pmol/mg), hM_4 (3.3 pmol/mg), and hM_5 (2.4 pmol/mg). An EC_{80} acetylcholine concentration (20 μM) was used to assess antagonism. Rabbit polyclonal antibodies $G\alpha_q$ and Ga_{i-3} were added with a 1:400 or 1:1,200 dilution.

LC-MS/MS Ex Vivo Characterization Experiments

^{127}I-iododexetimide concentrations were measured with a Shimadzu SIL-20AC/HT HPLC autosampler (Shimadzu Corp.) linked to a Triple Quad 5500 mass spectrometer (AB SCIEX). An Agilent XDB-C18 column (2.1 × 50 mm) was used for the HPLC. The precursor-to-production transition monitored was Q1 = 489.0, Q3 = 217.1. A gradient method lasting 5 min consisted of a mobile phase of various ratios of water (A) and acetonitrile (ACN) (B) with 0.1% formic acid. The initial conditions were 95% A and 5% B, and final conditions were 5% A and 95% B. Standards were prepared by adding known quantities of analyte to samples of brain tissues and plasma from nontreated animals and processed as described above. The proportion of the injected ligand dose reaching brain region tissue, normalized for animal weight, was expressed as standardized uptake value (%SUV), which was calculated as follows: %SUV = (ligand amount measured in tissue [ng/g] / injected ligand dose [mg/kg]) × 100. Levels of ^{127}I-iododexetimide measured in frontal cortex and striatum (rich in M_1R)

were taken to represent total ligand binding in a tissue (*1,2*). Cerebellar levels were used to represent nonspecific binding, because the expression of mAchR is low in this brain area (*3*). The difference between the total ligand concentration measured in the frontal cortex and striatum, and that measured in the cerebellum was used to assess specific binding to mAchR. The binding potential was calculated by taking the ratio of total binding measured in M_1R-rich brain areas (frontal cortex, striatum) divided by nonspecific binding (cerebellum) – 1.

To examine the brain distribution of ^{127}I-iododexetimide, the compound was formulated in 25% β-cyclodextran. On the basis of previous experience, β-cyclodextran is a suitable intravenous vehicle that created hydrophilic sugar groups surrounding dexetimide, increasing the solubility and bioavailability of ^{127}I-iododexetimide. Initial in vivo tracer evaluation and screening can be expedited by using ^{127}I- instead of ^{123}I-labelled compounds. Various time points were selected to assess rat brain kinetics of ^{127}I-iododexetimide. The 40-min time point after tracer administration was selected to conduct the dose occupancy study. It afforded a reasonable signal-to-noise ratio without compromising detection of the tracer. In both cases, tissues were dissected out, weighed, and placed in conical centrifuge tubes on ice. Four volumes (w/v) of acetonitrile containing 0.1% formic acid were added to each tube. These samples were then homogenized using an ultrasonic probe and centrifuged at 3.75*g* for 20 min. Supernatant liquid (100 μL) was diluted by adding sterile water, as previously published (*4*).

Tracer Dose

For tracer kinetic studies, occupancy of receptors by the radiotracers should be less than 3% (*5*). In the present study, we showed that acute treatment with olanzapine (2.5 mg/kg) was able to block at least 50% of striatal ^{123}I-iododexetimide binding (Fig. 6). This dose of olanzapine represents approximately 10 μmol/kg (molecular weight of olanzapine = approximately 312). So, approximately 10 μmol/kg olanzapine is needed to block approximately 50% of striatal ^{123}I-iododexetimide binding in rat brain. In other words, the ED_{50} is around 10 μmol/kg. In the present study, a typical dose of ^{123}I-iododexetimide is around 0.2 nmol/animal (SA, 185 MBq/nmol; dose was 37 MBq), which corresponds to 1 nmol/kg (weight of rat, 200–250 g). This is nearly 10,000 times less than required to occupy 50% of M_1 receptors in vivo, indicating an occupancy that is well below the pharmacologic dose.

References

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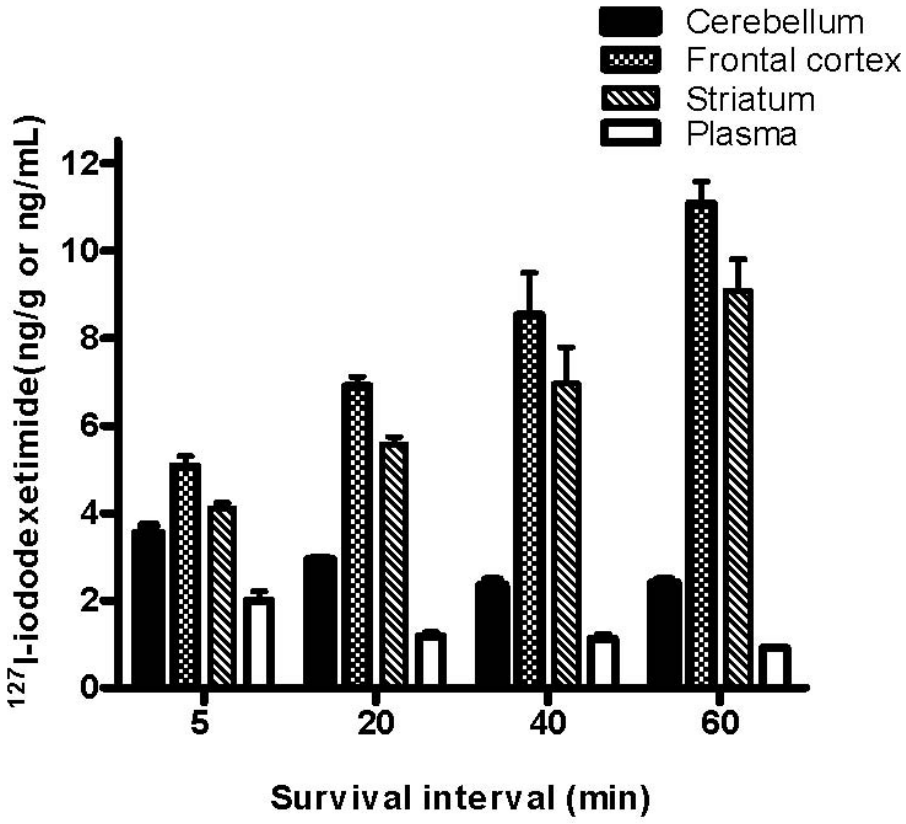
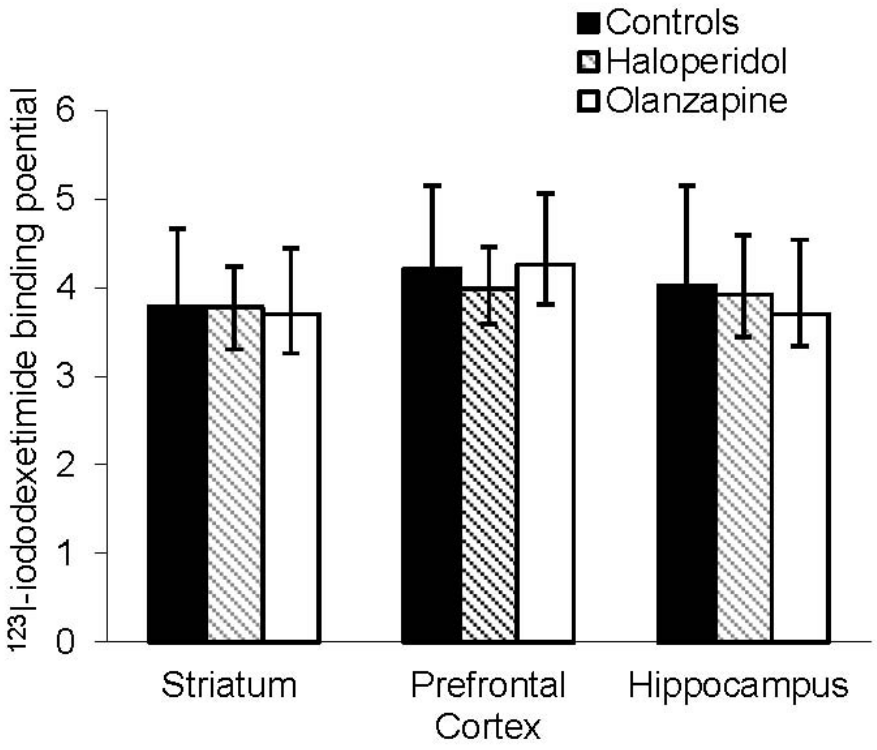


Supplemental Figure 1. Rat time course and distribution of nonlabeled ^{127}I-iododexetimide over 1 h in cerebellum, frontal cortex, striatum, and plasma (n = 4). Data points represent mean specific binding ± SEM.



Supplemental Figure 2. Binding potential ^{123}I-iododexetimide binding in the ROI (striatum, prefrontal cortex, and hippocampus). Binding potential was calculated as specific binding (total binding minus nonspecific binding) in the ROI divided by nonspecific binding (measured in the cerebellum). ^{123}I-iododexetimide binding was measured 2 h after intravenous injection of ^{123}I-iododexetimide. Rats (*n* = 8/group) were pretreated twice daily for 2 wk with saline, haloperidol (1 mg/kg), or olanzapine (2.5 mg/kg), up to 24 h before injection with the radiotracer.