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5-Iodo-A-85380, an $\alpha4\beta2$ Subtype-Selective Ligand for Nicotinic Acetylcholine Receptors[1]

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ABSTRACT

In an effort to develop selective radioligands for in vivo imaging of neuronal nicotinic acetylcholine receptors (nAChRs), we synthesized 5-iodo-3-(2(*S*)-azetidinylmethoxy)pyridine (5-iodo-A-85380) and labeled it with ^{125}I and ^{123}I. Here we present the results of experiments characterizing this radioiodinated ligand in vitro. The affinity of 5-[^{125}I]iodo-A-85380 for $\alpha4\beta2$ nAChRs in rat and human brain is defined by K_d values of 10 and 12 pM, respectively, similar to that of epibatidine (8 pM). In contrast to epibatidine, however, 5-iodo-A-85380 is more selective in binding to the $\alpha4\beta2$ subtype than to other nAChR subtypes. In rat adrenal glands, 5-iodo-A-85380 binds to nAChRs containing $\alpha3$ and $\beta4$ subunits with 1/1000th the affinity of epibatidine, and exhibits 1/60th and 1/190th the affinity of epibatidine at $\alpha7$ and muscle-type nAChRs, respectively. Moreover, unlike epibatidine and cytisine, 5-[^{125}I]iodo-A-85380 shows no binding in any brain regions in mice homozygous for a mutation in the $\beta2$ subunit of nAChRs. Binding of 5-[^{125}I]iodo-A-85380 in rat brain is reversible, and is characterized by high specificity and a slow rate of dissociation of the receptor–ligand complex ($t_{1/2}$ for dissociation ~2 h). These properties, along with other features observed previously in in vivo experiments (low toxicity, rapid penetration of the blood-brain barrier, and a high ratio of specific to nonspecific binding), suggest that this compound, labeled with ^{125}I or ^{123}I, is superior to other radioligands available for in vitro and in vivo studies of $\alpha4\beta2$ nAChRs, respectively.

Nicotinic acetylcholine receptors (nAChRs) are excitatory ligand-gated cation channels that are widely distributed in mammalian organisms, appearing in the central and peripheral nervous systems, neuromuscular junctions, and adrenal glands. The nAChR channel complex is composed of five protein subunits, which form a pore that is permeable to Na^+, K^+, and Ca^{2+} (Lindstrom, 1995; Holladay et al., 1997). To date, α, β, γ, δ, and ϵ subunits have been isolated and cloned from mammalian and avian tissues, with nine varieties of α and four varieties of β subunits identified. The $\alpha1$, $\beta1$, γ, δ, and ϵ subunits form the neuromuscular junction receptor, the very first nAChR to be characterized. The other subunits ($\alpha2$–$\alpha9$ and $\beta2$–$\beta4$) are found predominantly throughout the nervous system (Lindstrom, 1995; Holladay et al., 1997). This subunit diversity affords a large potential for a variety of nAChR subtypes, exhibiting distinct cation-conducting properties and pharmacological heterogeneity.

Based on binding properties and pharmacological sensitivity, major nAChR subtypes in mammalian brain can be categorized as α-bungarotoxin-sensitive ($\alpha7$) and α-bungarotoxin-insensitive (e.g., $\alpha4\beta2$) (Lindstrom, 1995; Holladay et al., 1997). Accordingly, ^{125}I-α-bungarotoxin has been the radioligand of choice for in vitro characterization of the $\alpha7$ subtype of nAChR, whereas tritiated agonists, such as nico-

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[1] A preliminary report of this work has been presented previously (Mukhin AG, Gündisch D, Horti AG, Koren AO, Kimes AS, Vaupel DB and London ED (1998) 5-Iodo-A-85380–a novel highly selective ligand for the $\alpha4\beta2$ subtype of nicotinic acetylcholine receptors. *Soc Neurosci Abstr* **24:**85).

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; IPH, (±)-*exo*-2-(2-iodo-5-pyridyl-7-azabicyclo[2.2.1]heptane; A-85380, 3-(2(*S*)-azetidinylmethoxy)pyridine; HSS, HEPES-salt solution; α-BTX, α-bungarotoxin; DFP, diisopropyl fluorophosphate.

tine, acetylcholine, *N*-methylcarbamylcholine, cytisine, and epibatidine, have been used to study nAChRs of the latter group in vitro (Holladay et al., 1997). Of these ligands, epibatidine has the highest known affinity for $\alpha4\beta2$ nAChRs, and outstanding in vitro binding characteristics (high specific-to-nonspecific binding ratio and slow kinetics of dissociation) (Dukat et al., 1993; Houghtling et al., 1995; Flores et al., 1996; Holladay et al., 1997; Stauderman et al., 1998; Xiao et al., 1998).

Radioligands developed for noninvasive in vivo imaging of α-bungarotoxin-insensitive nAChRs have exhibited shortcomings, such as poor subtype selectivity and high levels of nonspecific binding (Nybäck et al., 1994). The radioligands [^{123}I]IPH and [^{125}I]IPH ((±)-*exo*-2-(2-[$^{123/125}$I]iodo-5-pyridyl)-7-azabicyclo[2.2.1]heptane), recently developed iodinated analogs of epibatidine, do not distinguish well between the $\alpha4\beta2$ subtype and nAChRs containing $\alpha3$ and $\beta4$ subunits (Dávila-García et al., 1997), much like epibatidine itself (Flores et al., 1996; Xiao et al., 1998). In contrast to $\alpha4\beta2$ nAChRs, nAChRs containing $\alpha3$ and $\beta4$ subunits, possibly in combination with $\alpha5$ subunits, are distributed mostly in the peripheral nervous system and adrenal glands (Holladay et al., 1997). Therefore, high affinity for the latter receptors could contribute to the untoward cardiovascular effects of epibatidine and its analogs (Molina et al., 1997; Horti et al., 1998) and might limit the use of epibatidine-based compounds for imaging nAChRs in human subjects.

Recently, 3-(2(*S*)-azetidinylmethoxy)pyridine (A-85380, Fig. 1) has been identified as a high-affinity nAChR ligand (Abreo et al., 1996). Subsequently, a chloro analog of A-85380, ABT-594 (Fig. 1), has been developed as a promising nonopioid analgesic having affinity for $\alpha4\beta2$ nAChRs comparable to that of epibatidine, but lacking its toxicity (Bannon et al., 1998). In a search for improved radioligands suitable for noninvasive in vivo imaging of nAChRs, chemists in our group synthesized several halogenated analogs of A-85380 (Koren et al., 1998). Some of these compounds, particularly 5-iodo-A-85380 (Fig. 1), exhibited extremely high affinity for nAChRs in rat brain (Koren et al., 1998).

Initial evaluation of 5-[^{125}I]iodo- and 5-[^{123}I]iodo-A-85380 in vivo in mice (Musachio et al., 1998; Vaupel et al., 1998) and 5-[^{123}I]iodo-A-85380 in rhesus monkey (Chefer et al., 1998) and baboon (Musachio et al., 1999) demonstrated that these radioligands readily crossed the blood-brain barrier, bound to cerebral nAChRs with high specificity, and had low toxicity. Here, we present an in vitro characterization of 5-[^{125}I]iodo-A-85380, indicating that this ligand possesses excellent properties as a probe for studying the $\alpha4\beta2$ nAChR subtype.

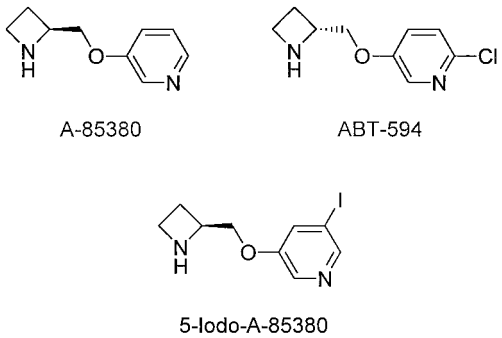

Fig. 1. Chemical structures of A-85380 and two of its analogs.

5-Iodo-A-85380 features high affinity for nAChRs, low nonspecific binding, slow dissociation from the receptor, and exceptionally high selectivity for the $\alpha4\beta2$ subtype among the major mammalian nAChR subtypes. These properties, together with the results of in vivo studies with this ligand (Chefer et al., 1998; Musachio et al., 1998, 1999; Vaupel et al., 1998), suggest that 5-[^{123}I]iodo-A-85380 may have exceptional potential as a radioligand for in vivo imaging of $\alpha4\beta2$ nAChRs with single photon emission computed tomography.

Experimental Procedures

Materials. 5-[^{123}I]iodo-A-85380 and 5-[^{125}I]iodo-A-85380 were prepared according to the literature procedures (Musachio et al., 1998; Horti et al., 1999). Specific activity of 5-[^{125}I]iodo-A-85380 was determined as described previously (Horti et al., 1999). On the day of each synthesis, the specific activities of the three batches of 5-[^{125}I]iodo-A-85380 used in these studies were 1550, 1980, and 2200 Ci/mmol, respectively. ^{125}I-α-Bungarotoxin (^{125}I-α-BTX, 100 Ci/mmol), [^{3}H]cytisine (32 Ci/mmol), and (±)-[^{3}H]epibatidine (48 Ci/mmol) were obtained from New England Nuclear Corp. (Boston, MA). 5-Iodo-A-85380 was prepared by a published method (Koren et al., 1998). (±)-*exo*-2-(2-Iodo-5-pyridyl)-7-azabicyclo[2.2.1]heptane (IPH), a gift from Dr. Kellar, was synthesized by Dr. J. L. Musachio at the Johns Hopkins University as described previously (Musachio et al., 1997). 3-(2(*S*)-Azetidinylmethoxy)pyridine dihydrochloride (A-85380), α-bungarotoxin (α-BTX), (–)- and (±)-epibatidine, (–)-cytisine, (+)- and (−)-stereoisomers of nicotine, acetylcholine, carbachol, dihydro-β-erythroidine, curare, mecamylamine, atropine, naloxone, (*R*)-(–)-apomorphine, and haloperidol were purchased from Research Biochemicals International (Natick, MA). Physostigmine, diisopropyl fluorophosphate (DFP), and all other chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO). Male Fischer-344 and Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, MA). Rats, shipped at the age of 12 weeks, were housed in a temperature- and light-controlled vivarium for at least 2 weeks before being used for this study. Mice were generated by mating parents heterozygous for a mutation in the $\beta2$ nAChR subunit (Picciotto et al., 1995). Frozen *Torpedo californica* electric organ tissue was purchased from Marinus Inc. (Long Beach, CA). Frozen samples of postmortem tissue of human cerebral cortex (four subjects, 38 to 49 years of age, death from arteriosclerotic cardiovascular disease) were obtained from the Brain and Tissue Bank for Developmental Disorders (Baltimore, MD).

All animal procedures performed at the National Institute on Drug Abuse Brain Imaging Center were approved by the National Institute on Drug Abuse Institutional Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals, as endorsed by the National Institutes of Health. All animal procedures performed at the Yale University School of Medicine were approved by the Yale Animal Care and Use Committee.

Membrane Preparation. After CO_2 euthanasia and decapitation of the rats, brains were removed and prepared as follows. Brain tissue used for binding studies was obtained by a single cut just behind the inferior colliculi to exclude the cerebellum and medulla. In some experiments, specific brain regions and the adrenal glands were isolated. Frozen samples of *Torpedo californica* electric organ and postmortem human cerebral cortical tissue were thawed at room temperature for 30 to 60 min before membrane preparation. Total membrane fractions from all tissues were isolated by homogenization of the respective tissue with a Brinkmann Polytron homogenizer in 10 to 20 volumes of a HEPES-salt solution (HSS), containing HEPES (pH 7.4, 15 mM), 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, and 1.8 mM $CaCl_2$, followed by centrifugation at 40,000*g* for 10 min. The pellets were washed twice with HSS through rehomogenization and centrifugation at the same settings. Three additional washings

were performed in the case of the total membrane fraction from rat adrenal glands and *Torpedo californica* electric organ. Crude membrane fractions (P2) were isolated as described previously (Koren et al., 1998), and were stored in aliquots at –70°C for at least 16 h but not more than 4 weeks before use. On the day of assay, pellets were thawed, homogenized in 30 volumes of HSS, and centrifuged at 40,000*g* for 10 min. The resultant pellets were resuspended in a freshly prepared HSS and used for binding assays.

Binding Assays. Assays were carried out in HSS at 22°C unless otherwise specified. Incubations were performed in polystyrene tubes except for the assays with ^{125}I-α-BTX, for which borosilicate glass tubes were used. The HSS for the studies of membranes from *T. californica* electric organ contained 0.1% of BSA. Nonspecific binding was determined in the presence of 300 μM (–)-nicotine except for the assays with ^{125}I-α-BTX, for which 1 μM α-BTX was used instead (see the figure legends for other specific conditions for particular binding assays). Incubation was terminated by filtration through Whatman GF/B glass fiber filters, presoaked in 1% polyethyleneimine, using a Brandel 48-channel cell harvester. Filters were washed three times with 3-ml aliquots of a rinse buffer (50 mM Tris · HCl, pH 7.4). In ^{125}I-α-BTX assays, the rinse buffer also contained 1% of nonfat dry milk to reduce nonspecific binding to filter material. Radioactivity was measured using a Beckman LS 3801 liquid scintillation counting system (efficiency 43%) or a LKB Wallac 1277 gamma counter (efficiency 68%).

In Vitro Autoradiography. Sagittal slices (20 μm thick) at 0.4, 0.9, 1.4, 1.9, 3.9, and 4.2 mm lateral to midline from eight frozen Fischer-344 rat brains were obtained by sectioning in a cryostat at –20°C, and were thaw-mounted onto gelatin-coated slides. Sections were refrozen and kept frozen at –80°C until the day of the assay. Slices were preincubated for 20 min in 50 mM Tris · HCl buffer (pH 7.0) containing 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, and 1 mM $MgCl_2$. Thereafter, they were incubated with 210 pM 5-[^{125}I]iodo-A-85380 (specific activity 435 Ci/mmol) in the same buffer for 2 h at 25°C, then rinsed twice in ice-cold buffer for 5 min each and once in distilled water for 1 min. The slides were dried overnight in a vacuum desiccator. Nonspecific binding was assessed in adjacent slices incubated in 210 pM 5-[^{125}I]iodo-A-85380 containing 10 μM nicotine bitartrate. Slices and appropriate ^{125}I-standards were apposed to ^{3}H-Ultrafilm for 2 days at room temperature. The resulting autoradiograms were digitized using a video camera-based system. The digitized images were analyzed using a computer program (INQUIRY, Loats Associates, Inc., Westminster, MD).

Brain slices (12 μm) from 2- to 4-month-old mice were prepared and processed using a procedure similar to that used for rat slices, but the incubation with radioligands was carried for 30 min in 50 mM Tris · HCl (pH 7.4). Details of the procedure were described previously (Perry and Kellar, 1995; Zoli et al., 1998). In each experiment, sections from three β2+/+ and three β2−/− mice were run in parallel. The brain sections were incubated with 200 pM [^{125}I]IPH (2200 Ci/mmol) or with 200 pM 5-[^{125}I]iodo-A-85380 (200 Ci/mmol). Slides were apposed to ^{3}H-Hyperfilm for 2 days ([^{125}I]IPH) or for 5 days (5-[^{125}I]iodo-A-85380) at room temperature. In addition, slides from β2−/− mice, labeled with 5-[^{125}I]iodo-A-85380, were apposed to ^{3}H-Hyperfilm for 5 weeks.

Protein Assay. Protein measurements were performed using a dye reagent kit (Bio-Rad, Richmond, CA) and BSA as a standard.

Data Analysis. Saturation binding data were subjected to Scatchard and linear regression analyses. Competition binding data were analyzed using nonlinear regression methods. Values of K_i were derived from the measured IC_{50} and K_d values for radioactive ligands using the Cheng-Prusoff equation $K_i = IC_{50}/(1+F/K_d)$, where F is the concentration of unbound radioligand. The K_d values were obtained from three to six independent experiments performed on the same membrane preparations that were used for the competition assays. Results of the kinetic experiments were analyzed using semilogarithmic plots and linear regression analysis. The values of equilibrium constant of dissociation, K_d, obtained from the kinetic studies, were calculated by the equation $K_d = k_{diss}/k_{ass}$, where k_{diss} and k_{ass} are the dissociation and association rate constants, respectively.

Results and Discussion

Kinetic and Equilibrium Binding Characteristics. The specific binding of 5-[^{125}I]iodo-A-85380, determined in rat brain membranes at 22°C and at a ligand concentration of 10 pM, reached one half the maximal (equilibrium) binding level in 67 ± 9 min (Fig. 2a). The binding was completely reversible and was characterized by a very slow dissociation ($t_{1/2}$ = 132 ± 9 min) (Fig. 2b). The rate constants of association (k_{ass}) and dissociation (k_{diss}) were (5.6 ± 1.4) · 10^{-4}/pM/min and (54 ± 4) · 10^{-4}/min, respectively. The K_d value, calculated as the ratio of k_{diss} to k_{ass}, was 9.7 ± 1.8 pM.

Based on these data, subsequent equilibrium binding studies with 5-[^{125}I]iodo-A-85380 were performed using a 4-h incubation at 22°C. At the lowest concentration of 5-[^{125}I]iodo-A-85380 (ca. 1 pM) used in saturation studies, radioligand depletion of up to 30% was observed. To account for this depletion, the concentrations of free radioligand at equilibrium were calculated by reducing the concentration of total added radioactivity by the concentration of total bound radioactivity. The specific binding of 5-[^{125}I]iodo-A-85380 in rat brain was saturable and was represented by a single population of binding sites over a radioligand concentration range of 1 to 500 pM (Fig. 3). The binding parameters (K_d and B_{max}) were 10.6 ± 0.3 pM and

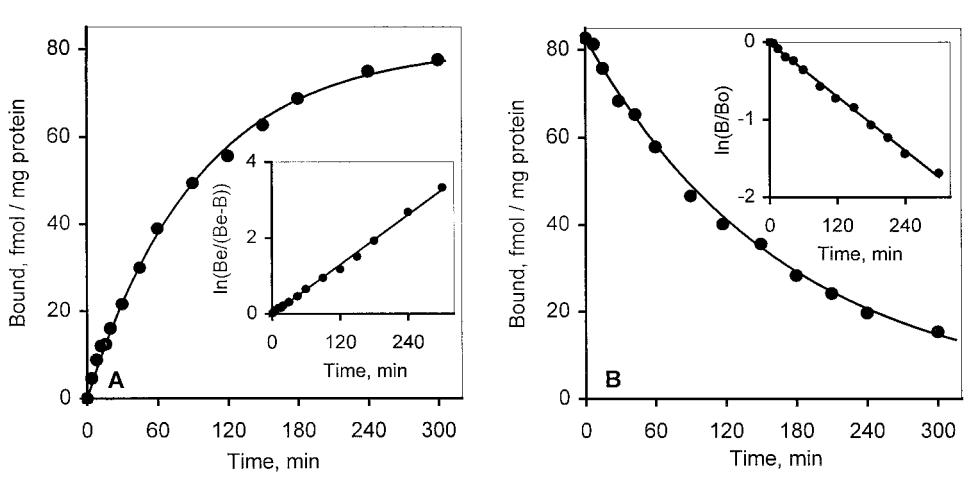

Fig. 2. Kinetics of ligand–receptor association (A) and dissociation (B) for 5-[^{125}I]iodo-A-85380 in rat brain. In association experiments, rat brain P2 membrane fractions (4–5 μg of protein) were incubated in a total volume of 0.4 ml with 10 pM 5-[^{125}I]iodo-A-85380 at 22°C. In dissociation experiments, (−)-nicotine at final concentration of 1 mM was added to rat brain P2 membrane fractions preincubated with 10 pM 5-[^{125}I]iodo-A-85380 for 4 h at 22°C. Each graph represents results of a single experiment performed in triplicate (S.E.M. < 10%). Similar results were obtained in three additional experiments for both association and dissociation studies. The mean ± S.E. values from the four corresponding experiments were: $t_{1/2ass}$, 67 ± 9 min [k_{ass}, (5.6 ± 1.4) · 10^{-4} /min/pM], and $t_{1/2diss}$, 132 ± 9 min [k_{diss}, (5.4 ± 0.4) · 10^{-3} /min].

3.8 ± 0.6 pmol/g tissue (160 ± 25 fmol/mg protein) in Fischer-344 rat forebrain (Fig. 3a), and 10.0 ± 0.2 pM and 3.9 ± 0.2 pmol/g tissue (178 ± 6 fmol/mg protein) in Sprague-Dawley rat forebrain (Fig. 3b), respectively.

The observed K_d values in saturation studies with 5-[^{125}I]iodo-A-85380 agreed with both the K_d value of 9.7 pM derived from the kinetic experiments (Fig. 2) and the K_i value of 11 pM obtained in our previous competition assays with (±)-[^{3}H]epibatidine (Koren et al., 1998). The density of 5-[^{125}I]iodo-A-85380 binding sites in rat forebrain was comparable to densities obtained using (−)-[^{3}H]cytisine and (−)-[^{3}H]nicotine (Lippiello and Fernandes, 1986; Pabreza et al., 1991; Flores et al., 1992), ligands that primarily label the $\alpha4\beta2$ nAChR subtype in rat brain. Figure 3c depicts binding of 5-[^{125}I]iodo-A-85380 in a postmortem sample of human brain cortex. As in rat brain, a single population of binding sites with a K_d value of 11.6 ± 0.7 pM was observed. The density of binding sites in the human cortex was characterized by B_{max} = 0.98 ± 0.04 pmol/g tissue, (53 ± 6 fmol/mg protein). This density was close to values obtained in studies of postmortem human cortex using (±)-[^{3}H]epibatidine, (−)-[^{3}H]nicotine, and (−)-[^{3}H]cytisine (Sihver et al., 1998).

Nonspecific binding of 5-[^{125}I]iodo-A-85380 was proportional to the concentration of the radioligand (data not shown) and, at a concentration of 100 pM (approximately 10 times the K_d value), constituted ca. 10% of total binding. Much of this value was attributable to binding of the radioligand to filter material. True nonspecific binding to tissue typically did not exceed 5% of total binding.

As seen in Table 1, the binding affinity of 5-[^{125}I]iodo-A-85380 in rat brain membranes was moderately sensitive to variations in temperature during the incubation period. Thus, increasing the incubation temperature from 4–37°C resulted in a modest increase in the K_d value (from 9.9 ± 0.8 to 20 ± 2 pM, respectively). It should be emphasized that incubation at 4°C required an extended incubation time (24 h) to reach equilibrium. The 4-h incubation time routinely used seemed to be insufficient to reach equilibrium at this temperature and resulted in an inaccurate K_d value of 15.5 ± 0.9 pM (n = 2). On the other hand, when incubating at 22°C, increasing the duration beyond 4 h (up to 18 h) did not produce significant changes in the observed K_d or B_{max} values (data not shown). This observation suggests that the 4-h incubation time was sufficient to reach equilibrium at 22°C and that neither the radioligand nor the receptor protein underwent degradation under the assay conditions used.

Competition Studies. In competition assays with 5-[^{125}I]iodo-A-85380, affinities for nAChRs in rat brain for six well-characterized nicotinic agonists and four nicotinic antagonists (Table 2) fell into an order that was consistent with that previously observed in assays using other radioligands for α-bungarotoxin-insensitive nAChRs (Pabreza et al., 1991; Decker et al., 1995; Houghtling et al., 1995). Compounds that did not effectively inhibit binding of 5-[^{125}I]iodo-A-85380 (K_i > 25 μM) included a noncompetitive nicotinic antagonist (mecamylamine), an antagonist at muscarinic acetylcholine receptors (atropine), cholinesterase inhibitors (physostigmine and DFP), an antagonist at opiate receptors (naloxone), an agonist at dopamine receptors (apomorphine), and an antagonist at D_2-like dopamine receptors (haloperidol).

A K_i value of 10 ± 1 pM, measured for the unlabeled 5-iodo-A-85380, was very close to that obtained for (–)-epibatidine (Table 2) and nearly identical with the K_d values for 5-[^{125}I]iodo-A-85380 measured in direct binding assays (Figs. 2 and 3). In addition, the K_i values for (–)-epibatidine, 5-iodo-A-85380, and A-85380 from Table 2 agreed well with those derived previously from competition assays with (±)-[^{3}H]epibatidine (Koren et al., 1998).

In our competition studies, the concentration of 5-[^{125}I]iodo-A-85380 (130 pM) was more than 10-fold higher than its K_d value. Because more than 90% of the binding sites were occupied by the radioligand at this concentration, the results obtained effectively characterized the entire population of 5-[^{125}I]iodo-A-85380 binding sites in rat forebrain. In all cases where K_i values were determined, the pseudo-Hill coefficient values were close to 1. These findings support our previous conclusion that in the rat forebrain, over the concentration range used, 5-[^{125}I]iodo-A-85380 labels a homog-

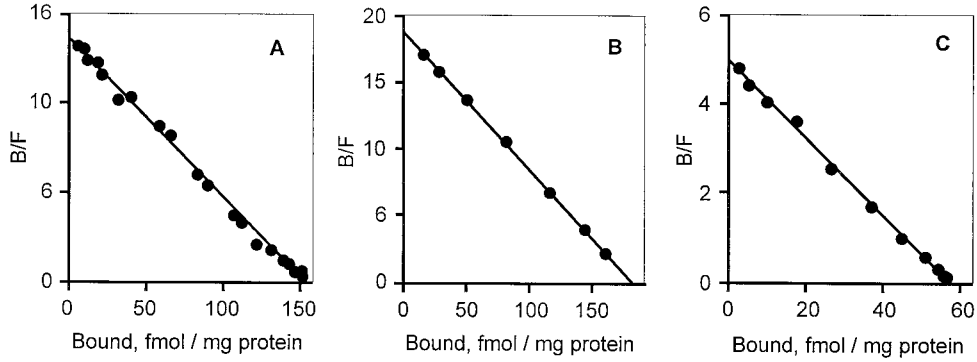

Fig. 3. Scatchard plots of 5-[^{125}I]iodo-A-85380 binding data obtained from saturation studies in P2 membrane fraction of Fischer-344 rat brain (A), in total membrane fraction of Sprague-Dawley rat brain (B), and in total membrane fraction of postmortem tissue of human cerebral cortex (C). Rat brain membranes (18–25 μg of protein) or human cortical membranes (26–30 μg of protein) were incubated in a total volume of, respectively, 1 or 0.5 ml with 1 to 500 pM 5-[^{125}I]iodo-A-85380 for 4 h at 22°C. Data were analyzed as described in *Experimental Procedures*. Each point represents the mean from three or four replicates (S.E.M. < 7%). A, pooled data from two independent experiments performed on the same membrane preparation. Four additional experiments were performed on membranes from two separate membrane preparations. The K_d and B_{max} values (mean ± S.E.) obtained from these six experiments were 10.6 ± 0.3 pM and 160 ± 25 fmol/mg protein (3.8 ± 0.6 pmol/g tissue), respectively. B, results of a single experiment. Similar binding characteristics were observed in four additional experiments performed on membranes from four independent preparations. The K_d and B_{max} values (mean ± S.E.) obtained from these five experiments were 10.0 ± 0.2 pM and 178 ± 6 fmol/mg protein (3.9 ± 0.2 pmol/g tissue), respectively. C, results of a single experiment. Similar results were obtained in three additional experiments performed on membranes of human postmortem cortical tissue obtained from different subjects. The K_d and B_{max} values (mean ± S.E.) obtained from all four experiments were 11.6 ± 0.7 pM and 53 ± 6 fmol/mg protein (0.98 ± 0.04 pmol/g tissue).

enous population of agonist binding sites associated with α-bungarotoxin-insensitive nAChRs, presumably the $\alpha4\beta2$ subtype.

Regional Distribution in Brain. To test our hypothesis that 5-[^{125}I]iodo-A-85380 labels $\alpha4\beta2$ nAChRs, we investigated distribution of the radioligand binding in the rat brain using both in vitro binding assays and autoradiography. In all brain regions studied, 5-[^{125}I]iodo-A-85380 binding was characterized by interaction with a single population of homogenous binding sites (Scatchard plots not shown) with K_d values close to 11 pM (Fig. 4). The average of K_d values from all regions studied was 11.0 ± 0.2 pM. These constants closely agreed with the K_d and K_i values observed for the whole rat forebrain (Fig. 3, Table 2). The regional distribution of binding sites (Fig. 4) closely matched that of the $\alpha4\beta2$ nAChR subtype measured previously in rat brain using (–)-[^{3}H]cytisine and (±)-[^{3}H]epibatidine (Pabreza et al., 1991; Houghtling et al., 1995); with the highest densities being observed in thalamic nuclei and superior colliculi; intermediate densities in the striatum, cortex, and hippocampus; and the lowest concentrations in the hypothalamus and cerebellum.

Results of the autoradiographic study with 5-[^{125}I]iodo-A-85380 (Fig. 5) corroborated the pattern of distribution determined in the in vitro binding experiments. The study was carried out using 210 pM 5-[^{125}I]iodo-A-85380, a concentration that theoretically should have saturated nearly 95% of the high-affinity binding sites. In the presence of 10 μM (–)-nicotine, the binding was blocked almost completely (Fig. 5a), indicating that, at the concentration used, the binding of 5-[^{125}I]iodo-A-85380 was limited to interactions with nAChRs in the rat brain.

Taken together, the regional densities of binding sites, labeled with 5-[^{125}I]iodo-A-85380, and the results of the competition studies suggest that the labeled sites correspond to agonist binding sites on the $\alpha4\beta2$ nAChR subtype. Still, these findings do not rule out the possibility that 5-iodo-A-85380 binds to other major mammalian nAChR subtypes, such as the $\alpha7$, muscle-type, or subtypes containing $\alpha3$ and $\beta4$ subunits.

Subtype Selectivity. To address the issue of binding selectivity among nAChR subtypes, we measured affinities of unlabeled 5-iodo-A-85380 in four different competition assays. To determine the affinity of 5-iodo-A-85380 for the $\alpha4\beta2$ subtype, we used previously described competition assays with (±)-[^{3}H]epibatidine or (–)-[^{3}H]cytisine and P2 membrane fractions of Fischer-344 rat forebrain. Consistent with the previous observations (Koren et al., 1998), Scatchard plots of (±)-[^{3}H]epibatidine binding to these rat brain membranes, at ligand concentrations of 1 to 800 pM, revealed a single population of binding sites (data not shown) with a K_d of 9.5 ± 0.5 pM (n = 4). Similar high-affinity binding of (±)-[^{3}H]epibatidine was observed in the rat (Houghtling et al., 1995), mouse (Marks et al., 1998), and human brain (Houghtling et al., 1995; Sihver et al., 1998), as well as in transfected cells stably expressing the $\alpha4\beta2$ nAChR subtype (Houghtling et al., 1995; Whiteaker et al., 1998). In addition, the affinity of 5-iodo-A-85380 for the $\alpha4\beta2$ nAChR subtype was measured in competition assays with (–)-[^{3}H]cytisine, which is the most widely used ligand for characterization of this subtype. In our saturation experiments, (–)-[^{3}H]cytisine revealed binding sites with a K_d value of 300 ± 50 pM (n = 3) in the rat brain membranes, consistent with published data (Pabreza et al., 1991).

To characterize binding of 5-iodo-A-85380 to the $\alpha7$ and muscular nAChR subtypes, we modified (see *Experimental*

TABLE 1

Effect of temperature on binding parameters of 5-[^{125}I]iodo-A-85380

Rat brain P2 membrane fractions (5 μg of protein) were incubated in a total volume of 0.3 ml with 1 to 500 pM 5-[^{125}I]iodo-A-85380 for 4 h at 22°C or 37°C, or for 24 h at 4°C. Results were analyzed as described in *Experimental Procedures*. Data represent mean ± S.E. obtained from three to four experiments per incubation temperature. Experiments were performed in quadruplicate.

Binding Parameter	Incubation Temperature		
	4°C	22°C	37°C
K_d, pM	9.9 ± 0.8	10.6 ± 0.3	20 ± 2
B_{max}, fmol/mg protein	162 ± 5	167 ± 5	166 ± 6

TABLE 2

Inhibition of 5-[^{125}I]iodo-A-85380 binding by nAChR and non-nAChR ligands

Rat brain P2 membrane fractions (10–11 μg of protein) were incubated in a total volume of 0.2 ml with 130 pM 5-[^{125}I]iodo-A-85380 and 9 to 11 concentrations of competitors for 4 h at 22°C and analyzed as described in *Experimental Procedures*. The inhibition constants (K_i values) were calculated by the Cheng-Prusoff equation from measured IC_{50} values using a K_d value of 10 pM for 5-[^{125}I]iodo-A-85380 binding. In all assays, the pseudo-Hill coefficients (n_H) did not differ significantly from 1. Data represent mean ± S.E. obtained from four to six experiments per compound. Experiments were performed in duplicate.

nAChR Ligands	K_i, nM	Non-nAChR Ligands	K_i, nM
Agonists		Atropine	>25,000
(–)-Epibatidine	0.009 ± 0.001	Physostigmine	>25,000
5-Iodo-A-85380	0.010 ± 0.001	DFP	>1,000,000
A-85380	0.015 ± 0.002	Naloxone	>25,000
(–)-Cytisine	0.16 ± 0.02	Apomorphine	>25,000
(–)-Nicotine	0.96 ± 0.06	Haloperidol	>25,000
Acetylcholine	4.0 ± 0.1		
(+)-Nicotine	10 ± 1		
Carbachol	73 ± 6		
Antagonists			
Dihydro-β-erythroidine	7.4 ± 0.8		
Curare	730 ± 70		
α-Bungarotoxin	>2,500		
Mecamylamine	>25,000		

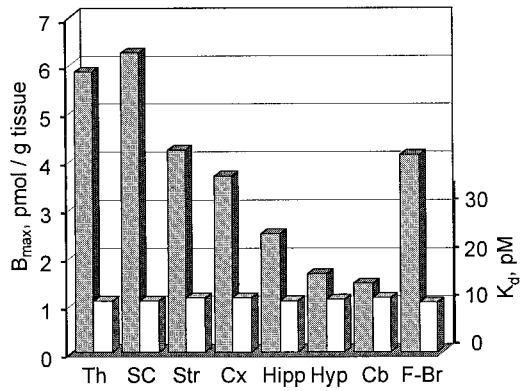

Fig. 4. Distribution of 5-[^{125}I]iodo-A-85380 in Fischer-344 rat brain. The individual brain structures obtained from ten animals were pooled and total membrane fractions from each region were isolated as described in *Experimental Procedures*. Membrane samples from each brain region (correspond to 0.1–0.5 mg of wet tissue) were incubated in a total volume of 0.25 ml with 1 to 500 pM 5-[^{125}I]iodo-A-85380 for 4 h at 22°C. For all brain regions, Scatchard analyses produced data consistent with homogeneous populations of binding sites with similar K_d values. Gray (B_{max} values) and open (K_d values) columns represent means obtained from three to four saturation assays performed on membranes from two separate preparations. For all regions studied, S.E.M. values were less than 10% except for the hypothalamus, where S.E.M. = 25%. Cb, cerebellum; Cx, frontal cortex; F-Br, forebrain; Hipp, hippocampus; Hyp, hypothalamus; SC, superior colliculus; Str, striatum; Th, thalamus.

Procedures) previously described techniques (Bougis et al., 1986; Arneric et al., 1994). These techniques use ^{125}I-α-bungarotoxin and membrane fractions isolated either from the rat brain (for the $\alpha7$ subtype) or from *T. californica* electroplax (for muscle-type nAChRs). In our experiments, ^{125}I-α-bungarotoxin bound to a single population of binding sites in each of the two membrane fractions, exhibiting K_d values of 1.5 ± 0.2 nM (n = 3) in rat brain and 2.3 ± 0.3 nM (n = 3) in electroplax, consistent with published data (Zeghloul et al., 1988; Quik et al., 1996).

To complete the study on nAChR subtype selectivity, we developed an assay using (±)-[^{3}H]epibatidine and a membrane fraction from rat adrenal glands to estimate the affinity of 5-iodo-A-85380 for nAChRs containing $\alpha3$ and $\beta4$ subunits. This assay was based on a previous study, which showed that (±)-[^{3}H]epibatidine, in addition to its high affinity for $\alpha4\beta2$ nAChRs in rat brain (Houghtling et al., 1995), bound to cells stably expressing receptors of the $\alpha3\beta4$ subtype (Stauderman et al., 1998; Xiao et al., 1998) and to membranes from rat adrenal glands (Houghtling et al., 1995; Flores et al., 1997). Results of studies with bovine adrenals (Criado et al., 1997; Wenger et al., 1997) and cultured rat pheochromocytoma cells (PC12) (Rogers et al., 1992; Henderson et al., 1994) suggested that the adrenal glands were rich in nAChR subtype(s) containing $\alpha3$ and $\beta4$

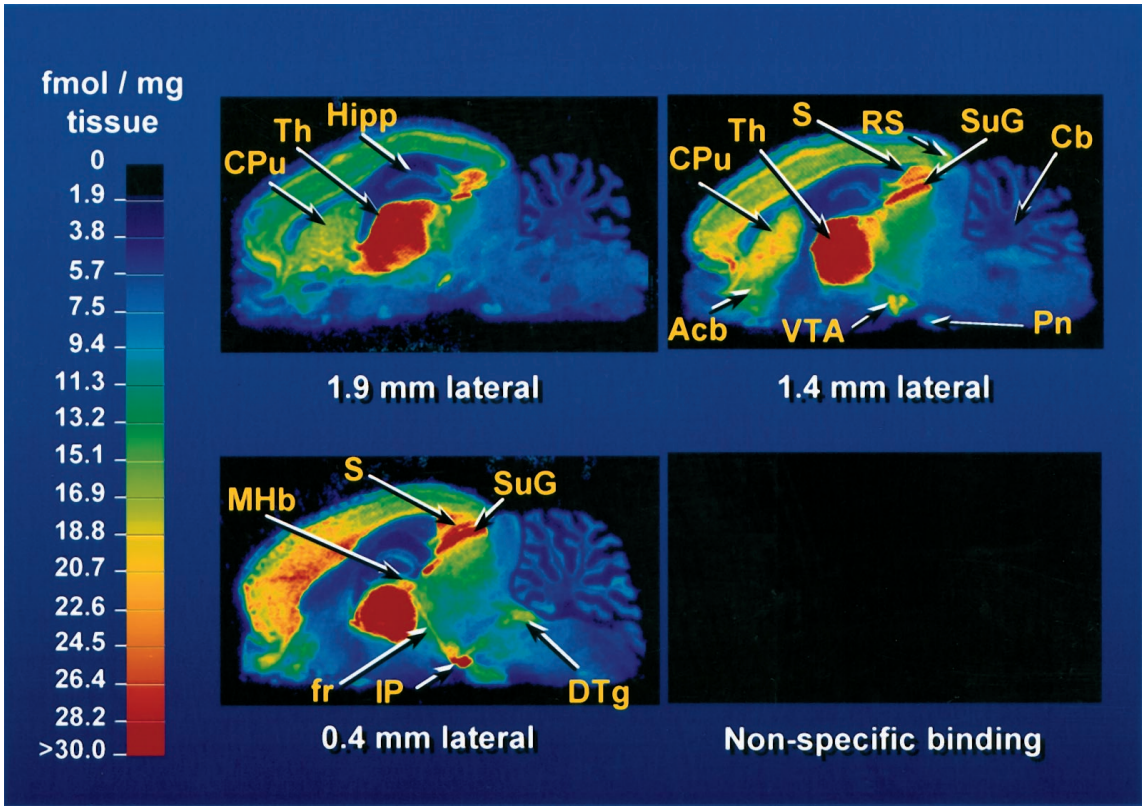

Fig. 5. In vitro autoradiography of rat brain with 5-[^{125}I]iodo-A-85380. Slices (20-μm thick; taken approximately 0.4, 1.4, and 1.9 mm lateral to the midline) were incubated with 210 pM 5-[^{125}I]iodo-A-85380 for 2 h. Nonspecific binding was assessed in the presence of 10 μM (–)-nicotine. Pseudocolor-transformed autoradiograms were expressed in femtomoles per milligram of tissue. Acb, nucleus accumbens; Cb, cerebellum; CPu, caudate putamen; DTg, dorsal tegmental area; fr, fasciculus retroflexus; Hipp, hippocampus; IP, interpeduncular nucleus; MHb, medial habenula; Pn, pontine nucleus; RS, retrosplenial cortex; S, subiculum; SuG, superior colliculus, superficial gray; Th, thalamus; VTA, ventral tegmental area.

TABLE 3

Binding selectivity of 5-iodo-A-85380 and other nAChR ligands at four major mammalian nAChR subtypes

In assays of the $\alpha4\beta2$ subtype, rat brain P2 membrane fractions (200 μg of protein) were incubated in a total volume of 0.5 ml with 0.5 nM (±)-[^{3}H]epibatidine and 9 to 11 concentrations of competitors for 1.5 h at 22°C. In assays of the nAChRs containing $\alpha3$ and $\beta4$ subunits ($\alpha3\beta4$x), total membrane fractions of rat adrenal glands (250 μg of protein) were incubated in a total volume of 1 ml with 0.4 nM (±)-[^{3}H]epibatidine and five to nine concentrations of competitors for 1.5 h at 22°C. In assays of the $\alpha7$ subtype, rat brain P2 membrane fractions (100 μg of protein) were incubated in a total volume of 0.1 ml with 2 nM ^{125}I-α-bungarotoxin and 9 to 11 concentrations of competitors for 3 h at 22°C. In assays of the muscle subtype, total membrane fractions from *T. californica* electric organ (0.1 μg of protein) were incubated in a total volume of 0.1 ml with 2 nM ^{125}I-α-bungarotoxin and 9 to 11 concentrations of competitors for 1.5 h at 22°C. The inhibition constants (K_i values) were calculated by the Cheng-Prusoff equation from measured IC_{50} values using the following K_d values: 10 and 55 pM for (±)-[^{3}H]epibatidine binding in rat forebrain ($\alpha4\beta2$) and in rat adrenal glands ($\alpha3\beta4$x), respectively; and 1.5 and 2.3 nM for ^{125}I-α-bungarotoxin binding in rat forebrain ($\alpha7$) and in *T. californica* electric organ ($\alpha1\beta1\delta\varepsilon$), respectively. These K_d values were obtained from three to six separate saturation assays per constant. Data represent means ± S.E. obtained from three to seven competition assays per constant.

Ligand	K_i at nAChR Subtype, nM (Ratio to K_i at $\alpha4\beta2$)			
	$\alpha4\beta2$	$\alpha3\beta4$x	$\alpha7$	Muscle
(±)-IPH	0.027 ± 0.004 (1)	0.11 ± 0.04 (4)	30 ± 3 (1,100)	6.5 ± 0.1 (240)
(±)-Epibatidine	0.008 ± 0.001 (1)	0.049 ± 0.02 (6)	4.0 ± 0.5 (500)	7.5 ± 0.5 (900)
(−)-Nicotine	0.84 ± 0.13 (1)	100 ± 20 (120)	130 ± 10 (150)	1000 ± 100 (1,200)
(−)-Cytisine	0.18 ± 0.02 (1)	54 ± 9 (300)	260 ± 20 (1,400)	190 ± 20 (1,100)
A-85380	0.017 ± 0.002 (1)	14 ± 2 (800)	17 ± 2 (1,000)	320 ± 20 (19,000)
5-Iodo-A-85380	0.010 ± 0.001 (1)	51 ± 5 (5,000)	250 ± 20 (25,000)	1400 ± 200 (140,000)

subunits as well as $\alpha7$ subtype, but expressed few, if any, receptors of the $\alpha4\beta2$ subtype. Binding assays with (±)-[^{3}H]epibatidine using rat adrenal gland membranes demonstrated a single population of binding sites (data not shown) with a K_d value of 55 ± 5 pM (n = 3). (±)-[^{3}H]Epibatidine binding to the rat adrenal gland membranes at a radioligand concentration of 0.5 nM was not blocked (data not shown) by α-bungarotoxin at concentrations as high as 10,000 times its affinity (K_i = 1 nM) at $\alpha7$ nAChRs (Quik et al., 1996). This observation suggests that, at conditions used for the competition assays, the binding of (±)-[^{3}H]epibatidine in rat adrenal glands does not reflect interactions with the $\alpha7$ subtype. In light of the above-cited reports, the present data are consistent with the view that [^{3}H]epibatidine binds to nAChRs containing $\alpha3$ and $\beta4$ subunits. Nonetheless, we cannot exclude the possibility that some portion of binding could reflect interactions with nAChRs including some other subunits (e.g., $\alpha5$).

Results of the competition assays for different nAChR ligands/subtypes are summarized in Table 3. It is notable that the affinity of 5-iodo-A-85380 for the $\alpha4\beta2$ receptor exceeded its affinities for other major mammalian nAChR subtypes by three to five orders of magnitude. In this regard, 5-iodo-A-85380 is vastly superior to all $\alpha4\beta2$-specific nAChR ligands known to date, including (–)-cytisine, which has long been the ligand of choice for characterizing the $\alpha4\beta2$ subtype. The high affinity of 5-iodo-A-85380 for the $\alpha4\beta2$ nAChR subtype measured in competition assays with (±)-[^{3}H]epibatidine was confirmed in additional assays with (−)-[^{3}H]cytisine, which yielded a nearly identical K_i value of 10.5 ± 0.7 pM (n = 3), and was consistent with results from binding assays with radiolabeled 5-iodo-A-85380 (Figs. 2 and 3), which provided K_d values of 9.7 to 10.6 pM. It should be noted that the exceptionally high $\alpha4\beta2$-subtype selectivity of 5-iodo-A-85380 is consistent with previous studies on interactions of the structurally related compounds, A-85380 (Sullivan et al., 1996) and ABT-594 (Bannon et al., 1998), with the $\alpha4\beta2$, $\alpha7$, and muscle-type nAChRs.

The high selectivity of 5-iodo-A-85380 for the $\alpha4\beta2$ nAChR subtype was additionally confirmed by in vitro autoradiographic studies of the $\beta2$-knockout mouse brain. In brain from the wild-type mouse, distribution of 5-[^{125}I]iodo-A-85380 binding resembled that of [^{125}I]IPH (Fig. 6), and the known pattern of distribution of $\alpha4\beta2$ nAChRs. Unlike the case of the wild-type mouse, 5-[^{125}I]iodo-A-85380 did not exhibit binding in any brain region of mice homozygous for a mutation in the $\beta2$ subunit of nAChRs. Unlabeled were the medial habenula and interpeduncular nucleus (Fig. 6), which were labeled with [^{125}I]IPH (Fig. 6), and which were labeled previously with [^{3}H]epibatidine and [^{3}H]cytisine in mice that had a mutation in the $\beta2$ subunit (Zoli et al., 1998). As shown previously (Perry and Kellar, 1995), the medial habenula and interpeduncular nucleus contain substantially higher densities of binding sites for [^{3}H]epibatidine than for [^{3}H]cytisine. Taken together, the results of studies of nAChRs in the medial habenula and interpeduncular nucleus suggest the presence of at least two distinct types of nAChRs in these regions. Of these types, only one, namely, that containing $\beta2$ subunit (presumably, the $\alpha4\beta2$ nAChR subtype), can be labeled with 5-[^{123}I]iodo-A-85380. Thus, 5-[^{125}I]iodo-A-85380 appears to be more selective than either epibatidine or, more importantly, cytisine, which has been accepted heretofore as the most selective high-affinity ligand for the $\alpha4\beta2$ nAChR subtype.

Available data do not rule out the possibility that 5-iodo-A-85380 has high affinity for subtypes other than $\alpha4\beta2$, which are far less abundant in mammalian brain. The investigation of such a possibility is a subject for future studies. Nonetheless, taking into consideration recent unpublished findings (K. J. Kellar, personal communication) that the parent compound, A-85380, has picomolar affinities toward several $\beta2$-containing nAChR subtypes (including the $\alpha3\beta2$ subtype) but only nanomolar affinities toward $\beta4$-containing nAChRs (including $\alpha4\beta4$), it is reasonable to assume that 5-iodo-A-85380 would follow the same pattern. Additionally, taken together with the above-cited results from Dr. Kellar's laboratory, the present observation of low affinity of A-85380 toward nAChRs in rat adrenal glands suggests that these receptors do not contain $\beta2$ subunits and are represented by $\alpha3\beta4x$ nAChRs, where x may or may not represent another subunit, e.g., $\alpha5$. In this regard, it is noteworthy that the ratios of affinity for the $\alpha4\beta2$ subtype to affinity for $\alpha3\beta4$x nAChRs, derived in the present work for (±)-epibatidine, cytisine, (−)-nicotine, and A-85380 (Table 3), closely matched recently published data obtained for the same compounds using rat brain membranes ($\alpha4\beta2$ nAChRs) and a cell line stably expressing $\alpha3\beta4$ nAChRs (Xiao et al., 1998).

In summary, the present results demonstrate that 5-iodo-A-85380 is an excellent ligand for studying nAChRs. It features extremely high affinity, slow dissociation from the receptor-ligand complex, high specific-to-nonspecific binding

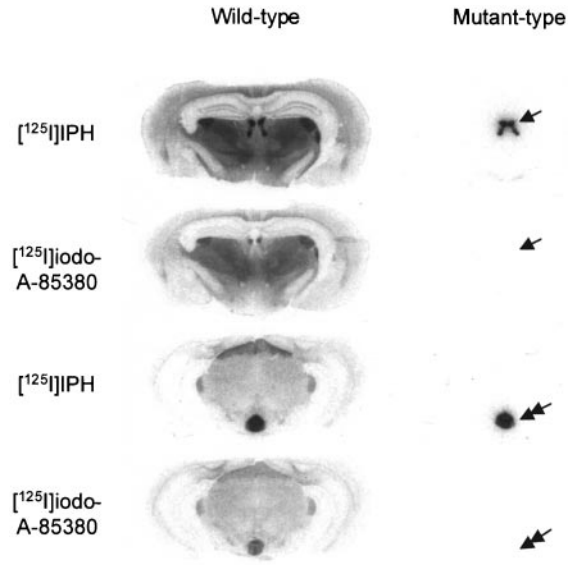

Fig. 6. In vitro autoradiography of wild-type mouse brain and of brains from mice homozygous for a mutation in the $\beta2$ subunit of nAChRs performed with [^{125}I]IPH and with 5-[^{125}I]iodo-A-85380. Slices (12-μm thick) at bregma –1.5 mm (four top panels) and at bregma –3.4 mm (four bottom panels) were incubated with 200 pM [^{125}I]IPH (2200 Ci/mmol) and with 200 pM 5-[^{125}I]iodo-A-85380 (420 Ci/mmol) for 0.5 h. Processed slides were exposed to Hyperfilm for 2 days ([^{125}I]IPH) or 5 days (5-[^{125}I]iodo-A-85380). Autoradiograms from mice homozygous for a mutation in the $\beta2$ subunit showed an absence of 5-[^{125}I]iodo-A-85380 binding. Similar results were observed in all slices analyzed. Arrow and double arrow indicate, respectively, the medial habenula and interpeduncular nucleus, where binding of [^{125}I]IPH, but not of 5-[^{125}I]iodo-A-85380, was seen in brains of $\beta2$-subunit knockout mice. Exposure for 5 weeks of 5-[^{125}I]iodo-A-85380-labeled slices from mice homozygous for a mutation in the $\beta2$ subunit of nAChRs to Hyperfilm did not result in the detection of any additional specific signal. Results shown were reproduced in additional experiments with two control and two mutant animals as well as in experiments with 5-[^{123}I]iodo-A-85380 in three control and three mutant animals.

ratio, and exceptionally high selectivity for the $\alpha4\beta2$ nAChR subtype. Furthermore, the ability to produce 5-[^{125}I]iodo-A-85380 with a specific activity of up to 2200 Ci/mmol makes it possible to detect nAChRs in the femtomolar range.

Recent in vivo studies with 5-[$^{123/125}$I]iodo-A-85380 in the mouse (Musachio et al., 1998; Vaupel et al., 1998) and rhesus monkey (Chefer et al., 1998) and baboon (Musachio et al., 1999) demonstrated that this radioligand readily crosses the blood-brain barrier, specifically accumulates in the brain regions enriched with the $\alpha4\beta2$ nAChRs, and exhibits low toxicity. These data, together with the results of the present in vitro characterization of 5-iodo-A-85380, suggest that radiolabeled with ^{123}I, this compound would be particularly promising for noninvasive imaging of nAChRs with single photon emission computed tomography in both animals and humans.

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