0026-895X/91/010009-04$03.00/0

MOLECULAR PHARMACOLOGY, 39:9-12

ACCELERATED COMMUNICATION

[^{3}H]Cytisine Binding to Nicotinic Cholinergic Receptors in Brain

LAURIE A. PABREZA, SUBHASH DHAWAN, and KENNETH J. KELLAR
Department of Pharmacology, Georgetown University School of Medicine, Washington, DC 20007
Received August 14, 1990; Accepted October 22, 1990

SUMMARY

Cytisine, a ganglionic agonist, competes with high affinity for brain nicotinic cholinergic receptors labeled by any of several nicotinic ^{3}H-agonist ligands. Here we have examined the binding of [^{3}H]cytisine in rat brain homogenates. [^{3}H]Cytisine binds with high affinity (K_d < 1 nM), and specific binding represented 60–90% of total binding at all concentrations examined up to 15 nM. The nicotinic cholinergic agonists nicotine, acetylcholine, and carbachol compete with high affinity for [^{3}H]cytisine binding sites, whereas among nicotinic receptor antagonists only dihydro-β-erythroidine competes with high affinity (in the nanomolar range). Comparison of binding in several brain regions showed that [^{3}H]cytisine binding is higher in the thalamus, striatum, and cortex than in the hippocampus, cerebellum, or hypothalamus. The pharmacology and brain regional distribution of [^{3}H]cytisine binding sites are those predicted for neuronal nicotinic receptor agonist recognition sites. The high affinity and low nonspecific binding of [^{3}H]cytisine should make it a very useful ligand for studying neuronal nicotinic receptors.

Nicotinic cholinergic binding sites in brain can be labeled by several agonist ligands, including [^{3}H]nicotine (1–3), [^{3}H]acetylcholine (4), and *N*-[^{3}H]methylcarbamylcholine (5, 6). All of these ligands appear to label the same agonist recognition site of neuronal nicotinic receptors with affinities of 3–12 nM, and each has been useful for characterizing the pharmacological properties, anatomical distribution, and regulation of these receptors (1–11).

Cytisine (Fig. 1), an alkaloid found in the seed of *Laburnum anagyroides*, is an agonist at autonomic ganglia; in binding competition studies it has the highest affinity of all drugs examined for brain nicotinic cholinergic binding sites (1, 4, 12, 13). In this paper, we report the characteristics of [^{3}H]cytisine binding to rat brain membrane homogenates. We found that [^{3}H]cytisine binds with a K_d of less than 1 nM to nicotinic cholinergic recognition sites in brain. Its high affinity, low rate of dissociation from its binding site, low nonspecific binding, and high stability should make [^{3}H]cytisine very useful for further studies of these receptors.

Materials and Methods

[^{3}H]Cytisine (12.4 Ci/mmol) was synthesized by Dr. Ernest Do of Dupont New England Nuclear Products. The purity was determined to be greater than 98% by high performance liquid chromatography. Drugs and chemicals were purchased from Sigma Chemical Co., except α-bungarotoxin, which was purchased from Biotoxins, Inc. (St. Cloud, FL), and Dβ E and mecamylamine, which were kind gifts from Merck Sharp & Dohme Research Laboratories (West Point, PA).

Rat brain tissues were homogenized in 50 mM Tris·HCl buffer (pH 7.0 at room temperature) containing 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2.5 mM $CaCl_2$. The homogenate was centrifuged at 40,000 × *g* for 10 min, the pellet was resuspended in fresh buffer and centrifuged a second time, and the final pellet was resuspended in fresh buffer. Aliquots of homogenate equivalent to 10 or 15 mg of tissue (600 or 900 μg of protein) were added to test tubes containing buffer and the indicated concentrations of [^{3}H]cytisine and were incubated in a final volume of 250 μl for 75 min at 2°, unless otherwise indicated. Nonspecific binding was determined in tissues incubated in parallel in the presence of 10 μM (−)-nicotine bitartrate, which was added before [^{3}H]cytisine. In competition studies, drugs were dissolved in buffer and added to the tubes at the indicated concentrations before the [^{3}H]cytisine. When acetylcholine was used in competition studies, 200 μM DFP, a cholinesterase inhibitor, was added to the tissue homogenate approximately 30 min before initiation of the assay. The incubations were terminated by rapid vacuum filtration through Whatman GF/C filter paper mounted on a Brandel cell harvester. The filters were washed rapidly three times with 4-ml aliquots of cold buffer and then counted in a scintillation counter.

Data were analyzed by nonlinear regression analyses (Accufit Saturation-Two Site and Accufit Competition programs; Beckman Instruments Inc, Fullerton, CA). The data were fit to both a one-site and a two-site binding model. The simpler model was chosen unless the two-site model gave a statistically better fit of the data ($p < 0.05$ by *F* test).

Results

Specific binding of [^{3}H]cytisine in membranes of rat cerebral cortex is saturable and represents 60–90% of total binding at

This work was supported by National Institute on Drug Abuse Grant DA06486.

ABBREVIATIONS: Dβ E, dihydro-β-erythroidine; DFP, diisopropyl-fluorophosphate.

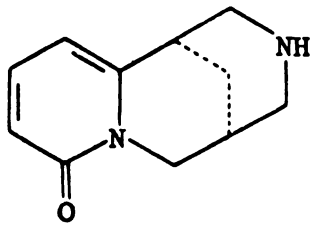

Fig. 1. Structure of cytisine.

all concentrations of [^{3}H]cytisine examined between 0.2 and 15 nM (Fig. 2A). The data for specific binding were fit best to a model for a single class of binding sites. Scatchard plots (Fig. 2B) indicate that the equilibrium dissociation constant (K_d) for [^{3}H]cytisine binding is approximately 0.9 nM and that the density (B_{max}) of binding sites in the cortex is 4 fmol/mg of tissue (66 fmol/mg of protein). The Hill coefficient (n_H) of [^{3}H]cytisine saturation binding is close to 1 (Fig. 2B, *inset*). The density of [^{3}H]cytisine binding sites in rat cortex is similar to the density of sites labeled by other nicotinic cholinergic agonist ligands (2–6), but the affinity of [^{3}H]cytisine is 3–15 times higher than that of other ligands for this site. At 37°, [^{3}H]cytisine labeled the same number of sites but its affinity was decreased, resulting in a K_d of 2.5 nM (data not shown). We have detected no decrease in the binding affinity of [^{3}H]cytisine over a period of 8 months when it is stored at −20°; thus, it appears to be very stable.

Under standard assay conditions (2°), the half-time ($t_{1/2}$) for [^{3}H]cytisine association to its binding site is approximately 11 min (Fig. 3A) and its $t_{1/2}$ for dissociation is approximately 13 min (Fig. 3B). The rate constants for association (k_1) and dissociation (k_{-1}) were derived from first-order kinetic plots (Fig. 3, *insets*), and the K_d calculated from the ratio k_{-1}/k_1 was 0.6 ± 0.3 nM, in reasonable agreement with the K_d value determined in saturation experiments.

In preliminary studies, the affinity of [^{3}H]cytisine binding in whole rat brain, as well as in several subcortical regions of brain, did not vary significantly from the affinity found in the cerebral cortex (data not shown). Therefore, the relative density of [^{3}H]cytisine binding sites in several grossly dissected areas of rat brain was determined by measurement of binding of [^{3}H]cytisine at a concentration of 4 nM, which should occupy 80–85% of the sites in each brain area. These studies indicated that the relative density of [^{3}H]cytisine binding sites in rat brain is higher in the thalamus, striatum, and cortex than in the hippocampus, hypothalamus, or cerebellum (Fig. 4).

In competition studies, unlabeled cytisine competed for [^{3}H]cytisine binding sites with an IC_{50} of less than 1 nM and with a Hill slope consistent with a single binding site (Fig. 5 and Table 1). Acetylcholine (in the presence of DFP to inhibit cholinesterases) and nicotine also have high affinity for [^{3}H]cytisine binding sites, whereas the affinity of carbachol is about 20-fold lower than that of acetylcholine (Fig. 5 and Table 1). In all cases, the competition curves for these nicotinic agonists had Hill slopes close to 1 and the data were fit best by a model for a single binding site.

The nicotinic antagonist DβE has high affinity for [^{3}H]cytisine binding sites, competing with an IC_{50} of about 57 nM (Fig. 5 and Table 1), whereas *d*-tubocurarine (curare) competes with an IC_{50} of about 30 μM and mecamylamine with an IC_{50} of greater than 100 μM (Table 1). α-Bungarotoxin, a potent antagonist at nicotinic receptors in skeletal muscle and electroplax tissues, did not compete for brain [^{3}H]cytisine binding sites at concentrations up to 2 μM (Table 1). Neither the muscarinic antagonist atropine nor the cholinesterase inhibitors DFP and physostigmine competed effectively for [^{3}H]cytisine binding sites (Table 1).

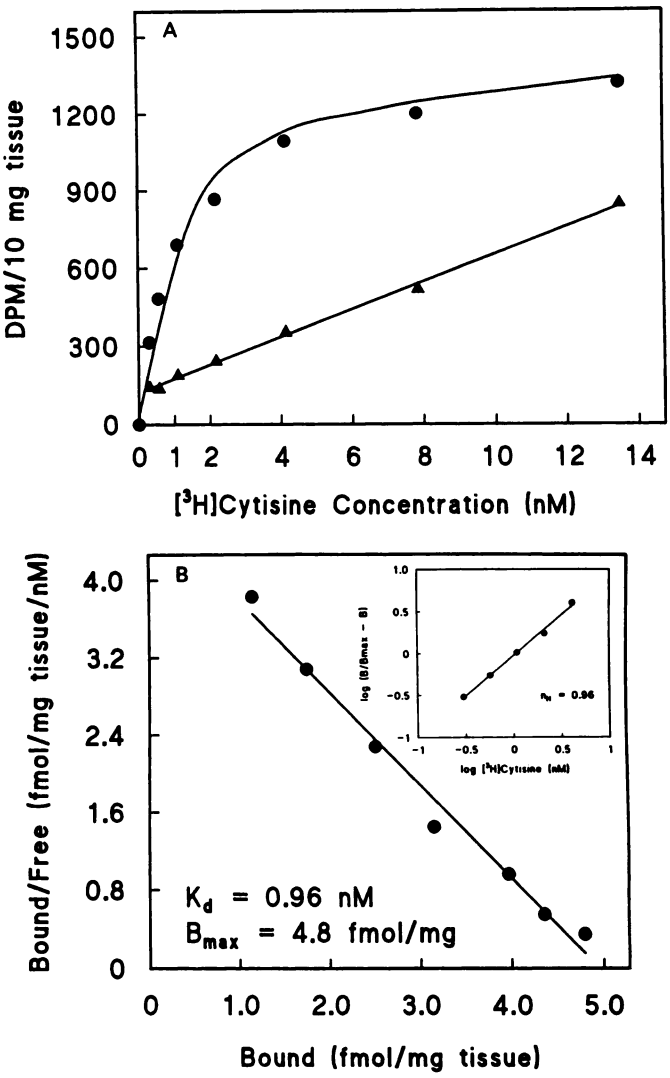

Fig. 2. [^{3}H]Cytisine binding in rat cerebral cortex. A, Saturation binding of [^{3}H]cytisine (0.2–14 nM). Washed cortical membranes were incubated for 75 min at 2° with the concentrations of [^{3}H]cytisine shown. Nonspecific binding (▲) was determined in the presence of 10 μM nicotine. Specific binding (●) was defined as the difference between total and nonspecific binding. B, Scatchard plot of specific binding data shown in A. The means ± standard errors of K_d and B_{max} values from seven such analyses were 0.9 ± 0.1 nM and 4.0 ± 0.2 fmol/mg of tissue, respectively. *Inset*, Hill plot of specific binding data shown in A. The mean ± standard error of the Hill coefficients (n_H) from seven such analyses was 0.96 ± 0.1.

Discussion

[^{3}H]Cytisine binds with high affinity to sites in brain that have the characteristics of nicotinic cholinergic receptor agonist recognition sites. The pharmacology and the relative distribution of the binding site in grossly dissected brain areas are nearly identical to those that have been found using [^{3}H]acetylcholine (4, 12, 13), [^{3}H]nicotine (1, 2, 12, 13), and *N*-[^{3}H]methylcarbamylcholine (6, 7). What distinguishes [^{3}H]cytisine

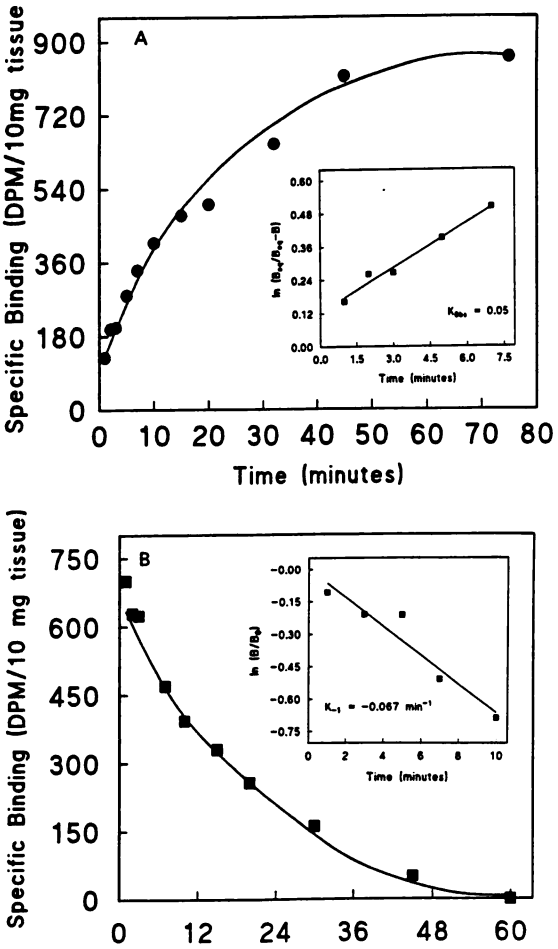

Fig. 3. Association and dissociation of [³H]cytisine binding in rat cerebral cortex. A, The association rate was determined by incubation of tissues with 2 nM [³H]cytisine at 2° for the times shown, before filtration. *Inset*, psuedo-first-order kinetic plot of [³H]cytisine binding. The association rate constant, k_1, was calculated from the equation $k_1 = (k_{obs} - k_{-1})/[^3H]$ cytisine concentration. This experiment was replicated three times. B, The dissociation rate was determined by incubation of tissues with 2 nM [³H]cytisine at 2° for 75 min and then addition of 10 μM nicotine. The reaction was then stopped by filtration at the times shown. *Inset*, first-order kinetic plot of dissociation of [³H]cytisine from its binding site. The rate constant for dissociation, k_{-1}, is given by the slope of the line. This experiment was replicated three times.

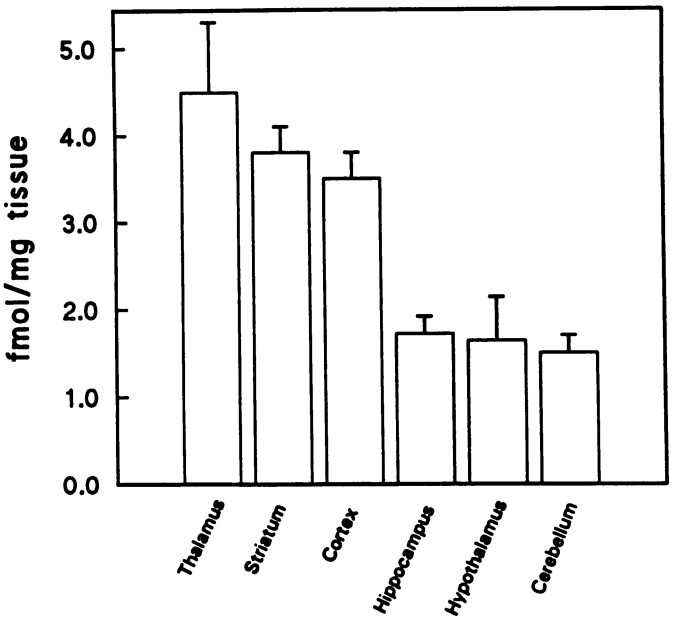

Fig. 4. Relative distribution of [³H]cytisine binding in several areas of rat brain. Binding of [³H]cytisine (4 nM) in the brain areas shown was measured as described in the text. Values are the mean and standard error of five independent measurements.

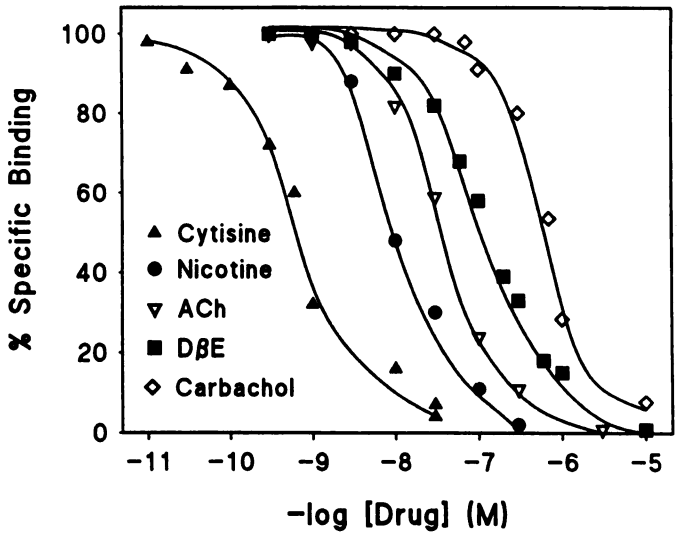

Fig. 5. Competition for [³H]cytisine binding sites by nicotinic cholinergic drugs. Cerebral cortical homogenates were incubated with 8–10 concentrations of drugs before addition of approximately 1.5 nM [³H]cytisine. Curves are representative of three to five such determinations for each drug. When competition with acetylcholine (*ACh*) was examined, the tissues were preincubated for 30 min with the cholinesterase inhibitor DFP. See Table 1 for IC_{50} values.

as a ligand for neuronal nicotinic receptors is its higher affinity, very low nonspecific binding, and slow rate of dissociation from the receptor. These characteristics, as well as its stability, should make [³H]cytisine very useful as a ligand for neuronal nicotinic receptors.

In contrast to nicotinic receptors in vertebrate muscle and fish electric organs, which are assembled from four different but homologous protein subunits arranged as the pentamer $\alpha_2,\beta,\gamma,\delta$ (14, 15), available evidence suggests that neuronal nicotinic receptors are composed of only α and β subunits (16). As in the muscle receptor, the agonist recognition site of neuronal nicotinic receptors is located on the α subunit, but the total number of subunits of the neuronal receptor and the subunit stoichiometry is at present uncertain.

Although most radioligand binding studies indicate that ³H-agonists bind to a homogeneous population of brain nicotinic cholinergic receptors, mammalian brain expresses mRNA coding for at least four different α subunits (17–21) and three different β subunits (21–24). In *Xenopus* oocytes injected with synthetic mRNA encoding different subunits of neuronal nicotinic receptors, the sensitivity to activation by agonists, the sensitivity to blockade by neurotoxins, and the channel properties of the nicotinic receptors expressed appear to depend on both the particular α subunit and β subunit that the receptor contains (24–26). If different combinations of these subunits form functional receptors in brain, it could provide a basis for significant diversity with respect to receptor function and regulation (for reviews, see Refs. 27 and 28).

The binding characteristics of [³H]cytisine should make it

TABLE 1

Competition by drugs for [^{3}H]cytisine binding sites in rat cerebral cortex

Homogenates of cortex were incubated with 8–10 concentrations of each drug and approximately 1.5 nM [^{3}H]cytisine. Values are the mean ± standard error from at least three determinations.

Drug	IC_{50}	n_H
	nM	
Cytisine	1.2 ± 0.4	0.9 ± 0.1
Nicotine	8.9 ± 2.6	0.9 ± 0.1
Acetylcholine	32.7 ± 10	0.9 ± 0.2
Carbachol	600 ± 89	0.9 ± 0.1
DβE	57.4 ± 20	0.8 ± 0.1
Curare	28,000 ± 2,000	
Mecamylamine	>100,000	
α-Bungarotoxin	>2,000	
DFP	>1,000,000	
Physostigmine	>1,000,000	
Atropine	>1,000,000	

particularly useful for further studies of neuronal nicotinic receptors in tissues with a relatively low density of receptors, such as ganglia and neuronal cells in culture, for further characterization of the receptors in brain, and possibly for labeling of specific subtypes of these receptors. Although the [^{3}H]cytisine used in the present studies had a specific activity of only 12.4 Ci/mmol, [^{3}H]cytisine with a specific activity of 42 Ci/mmol has been synthesized recently. We tested this batch of [^{3}H]cytisine and found that it bound in rat cortex with an affinity of 0.4 nM.

Acknowledgments

We thank Dr. Ernest Do and Dupont NEN for making [^{3}H]cytisine available.

References

1. Romano, C., and A. Goldstein. Stereospecific nicotine receptors on rat brain membranes. *Science (Washington D. C.)* **210**:647–649 (1980).
2. Marks, M. J., and A. C. Collins. Characterization of nicotine binding in mouse brain and comparison with the binding of *alpha*-bungarotoxin and quinuclidinyl benzilate. *Mol. Pharmacol.* **22**:554–562 (1982).
3. Lippiello, P. M., and K. G. Fernandes. The binding of 1-[^{3}H]nicotine to a single class of high affinity sites in rat brain. *Mol. Pharmacol.* **29**:448–454 (1986).
4. Schwartz, R. D., R. McGee, and K. J. Kellar. Nicotinic cholinergic receptors labeled by [^{3}H]acetylcholine in rat brain. *Mol. Pharmacol.* **22**:56–62 (1982).
5. Abood, L. G., and S. Grassi. [^{3}H]Methylcarbamylcholine, a new radioligand for studying brain nicotinic receptors. *Biochem. Pharmacol.* **35**:4199–4202 (1986).
6. Boska, P., and R. Quiron. [^{3}H]*N*-Methylcarbamylcholine, a new radioligand specific for nicotinic acetylcholine receptors in rat brain. *Eur. J. Pharmacol.* **139**:323–333 (1987).
7. Clarke, P. B. S., R. D. Schwartz, S. M. Paul, C. B. Pert, and A. Pert. Nicotinic binding in rat brain: autoradiographic comparison of [^{3}H]acetylcholine, [^{3}H]nicotine, and [^{125}I]α-bungarotoxin. *J. Neurosci.* **5**:1307–1315 (1985).
8. Schwartz, R. D., and K. J. Kellar. Nicotinic cholinergic receptor binding sites in brain: regulation *in vivo*. *Science (Washington D. C.)* **220**:214–216 (1983).
9. Schwartz, R. D., and K. J. Kellar. *In vivo* regulation of [^{3}H]acetylcholine recognition sites in brain by nicotinic cholinergic drugs. *J. Neurochem.* **45**:427–433 (1985).
10. Marks, M. J., J. B. Burch, and A. C. Collins. Effects of chronic nicotine infusion on tolerance development and nicotinic receptors. *J. Pharmacol. Exp. Ther.* **226**:817–825 (1983).
11. Marks, M. J., J. A. Stitzel, and A. C. Collins. Time course study of the effects of chronic nicotine infusion on drug response and brain receptors. *J. Pharmacol. Exp. Ther.* **235**:619–628 (1985).
12. Marks, M. J., J. A. Stitzel, E. Romm, J. M. Weher, and A. C. Collins. Nicotinic binding sites in rat and mouse brain: comparison of acetylcholine, nicotine and α-bungarotoxin. *Mol. Pharmacol.* **30**:427–436 (1986).
13. Martino-Barrows, A. M., and K. J. Kellar. [^{3}H]Acetylcholine and [^{3}H](−)nicotine label the same recognition site in rat brain. *Mol. Pharmacol.* **31**:169–174 (1987).
14. Reynolds, J. S., and A. Karlin. Molecular weight in detergent solution of acetylcholine receptor from *Torpedo californica*. *Biochemistry* **17**:2035–2038 (1978).
15. Kubalek, E., S. Ralston, J. Lindstrom, and N. Unwin. Location of subunits within the acetylcholine receptor: analysis of tubular crystals from *Torpedo marmorata*. *J. Cell Biol.* **105**:9–18 (1987).
16. Whiting, P., and J. Lindstrom. Purification and characterization of a nicotinic acetylcholine receptor from rat brain. *Proc. Natl. Acad. Sci. USA* **84**:595–599 (1987).
17. Boulter, J., K. Evans, D. Goldman, G. Martin, D. Treco, S. Heinemann, and J. Patrick. Isolation of cDNA clone coding for a possible neural nicotinic acetylcholine receptor *alpha* subunit. *Nature (Lond.)* **319**:368–374 (1986).
18. Boulter, J., J. Connolly, E. Deneris, D. Goldman, S. Heinemann, and J. Patrick. Functional expression of two neural nicotinic acetylcholine receptors from cDNA clones identifies a gene family. *Proc. Natl. Acad. Sci USA* **84**:7763–7767 (1987).
19. Goldman, D., E. Deneris, W. Luyten, A. Kochlar, J. Patrick, and S. Heinemann. Members of a nicotinic acetylcholine receptor gene family are expressed in different regions of the mammalian central nervous system. *Cell* **48**:965–973 (1987).
20. Wada, K., M. Ballivet, J. Boulter, J. Connolly, E. Wada, E. Deneris, L. Swanson, S. Heinemann, and J. Patrick. Functional expression of a new pharmacological subtype of brain nicotinic acetylcholine receptor. *Science (Washington D. C.)* **240**:330–334 (1988).
21. Nef, P., C. Oneyser, C. Alliod, S. Couturier, and M. Ballivet. Genes expressed in the brain define three distinct neuronal acetylcholine receptors. *EMBO J.* **7**:595–601 (1988).
22. Deneris, E. S., J. Connolly, J. Boulter, E. Wada, K. Wada, L. W. Swanson, J. Patrick, and S. Heinemann. Primary structure and expression of β2: a novel subunit of neuronal nicotinic acetylcholine receptors. *Neuron* **1**:45–54 (1988).
23. Deneris, E. S., J. Boulter, L. W. Swanson, J. Patrick, and S. Heinemann. β3: a new member of nicotinic acetylcholine receptor gene family is expressed in brain. *J. Biol. Chem.* **264**:6268–6272 (1989).
24. Duvoisin, R. M., E. S. Deneris, J. Patrick, and S. Heinemann. The functional diversity of the neuronal nicotinic acetylcholine receptors is increased by a novel subunit: β4. *Neuron* **3**:487–496 (1989).
25. Papke, R., J. Boulter, J. Patrick, and S. Heinemann. Single channel current of rat neuronal nicotinic acetylcholine receptors expresed in *Xenopus laevis* oocytes. *Neuron* **3**:589–596 (1989).
26. Luetje, C. W., K. Wada, S. Rogers, S. N. Abramson, K. Tsuji, S. Heinemann, and J. Patrick. Neurotoxins distinguish between different neuronal nicotinic acetylcholine receptor subunit combinations. *J. Neurochem.* **55**:632–640 (1990).
27. Steinbach, J. H., and C. Ifune. How many kinds of nicotinic acetylcholine receptors are there? *Trends Neurosci.* **12**:3–6 (1989).
28. Luetje, C. W., J. Patrick, and P. Seguela. Nicotine receptors in mammalian brain. *FASEB J.* **4**:2753–2760 (1990).

Send reprint requests to: Kenneth J. Kellar, Department of Pharmacology, Georgetown University School of Medicine, Washington, DC 20007.