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The Nicotinic Receptor in the Rat Pineal Gland is an α3β4 Subtype

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Running Title: Rat Pineal Gland α3β4 nAChRs

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[^{3}H]EB, [^{3}H](±)-epibatidine; [^{125}H]EB, [^{125}H](±)-epibatidine; [^{125}H]α-BTX, [^{125}H]alpha-bungarotoxin; α-BTX, alpha-bungarotoxin; EB, epibatidine; DHβE, dihydro-β-erythroidine; DMPP, 1,1-dimethly-4-phenylpiperazinium; nAChR, nicotinic acetylcholine receptor.

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Abstract

The rat pineal gland contains a high density of neuronal nicotinic receptors (nAChRs). We characterized the pharmacology of the binding sites and function of these receptors, measured the nAChR subunit mRNA and used subunit-specific antibodies to establish the receptor subtype as defined by subunit composition. In ligand binding studies, [^{3}H]epibatidine binds with an affinity of ~ 100 pM to nAChRs in the pineal, and the density of these sites is ~ 5-times that in rat cerebral cortex. The affinities of nicotinic drugs for binding sites in the pineal gland are similar to those at α3β4 nAChRs heterologously expressed in HEK293 cells. In functional studies, the potencies and efficacies of nicotinic drugs to activate or block whole cell currents in dissociated pinealocytes match closely their potencies and efficacies to activate or block $^{86}Rb^+$ efflux in the cells expressing heterologous α3β4 nAChRs. Measurements of mRNA indicated the presence of transcripts for α3, β2 and β4 nAChR subunits but not those for α2, α4, α5, α6, α7, or β3 subunits. Immunoprecipitation with subunit-specific antibodies showed that virtually all [^{3}H]EB-labeled nAChRs contained α3 and β4 subunits associated in one complex. The β2 subunit was not associated with this complex. Taken together, these results indicate that virtually all of the nAChRs in the rat pineal gland are the α3β4 nAChR subtype, and that the pineal gland can therefore serve as an excellent and convenient model in which to study the pharmacology and function of these receptors in a native tissue.

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Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated cation channels composed of α and β subunits. Nine α (α2 - α10) and three β (β2 - β4) subunits have been identified in vertebrates, and different subunit combinations define specific receptor subtypes. All of these subtypes pass Na^+, K^+ and Ca^{2+}, but they exhibit distinct biophysical and pharmacological properties. Studies of nAChRs in *Xenopus* oocytes and transfected mammalian cells have provided valuable information on the biophysical properties, pharmacology and possible regulation of several different well-defined nAChR subtypes that might play important physiological roles. However, the precise subunit compositions of the subtypes of nAChRs that actually exist in most native tissues are not well defined. Therefore, identifying the subunit composition of native nAChRs is a crucial step in establishing the physiological roles played by the different receptor subtypes that exist *in vivo*.

Considerable progress has been made in determining the subunit composition of the predominant receptor subtypes in the rat forebrain, namely the α4β2 subtype (Whiting and Lindstrom, 1987; Flores et al., 1992), which has high affinity for most agonists, and the α7 subtype, which has high affinity for α-bungarotoxin (α-BTX) (Couturier et al., 1990; Schoepfer et al., 1990; Orr-Urtreger et al., 1997). However, other nAChR subtypes are found in various amounts throughout many regions of the CNS (Marks et al., 1998; Perry et al., 2002), and some of these less prevalent receptors may play critical roles because of their strategic location--e.g., α6-containing receptors on dopamine axons (Quik et al., 2002; Champtiaux et al., 2003; Salminen et al., 2004). Moreover, under some conditions in vivo, such as when α4β2 and/or α7 receptors are desensitized or inactivated by exposure to nicotine or during certain disease states that may involve loss of specific nAChR subtypes, the less prevalent receptors may take on critical roles in mediating cholinergic signals. In addition, α3β2 and α3β4 nAChR subtypes are the predominant nAChRs in

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autonomic ganglia and thus are critical to the homeostatic functioning of virtually all organ systems in the body.

The pineal gland is part of the photoneuroendocrine system of vertebrates and functions in response to signals from photoreceptor cells in the retina and endogenous oscillators within the suprachiasmatic nucleus (SCN) to translate light stimuli into neuroendocrine responses. The main role of the pineal appears to be to produce and secrete the hormone melatonin, which influences circadian and seasonal biological rhythms in animals. Melatonin production is stimulated when norepinephrine released by sympathetic axons from the superior cervical ganglia activate β-adrenergic receptors in the pineal (Axelrod, 1974). More recent studies have demonstrated a cholinergic innervation of the pineal from the parasympathetic nervous system (Korf et al., 1996; Larsen et al., 1998; Schafer et al., 1998) and possibly from the medial habenula within the brain (Schafer et al., 1998). This cholinergic innervation appears to play an important role in pineal gland physiology by inhibiting melatonin synthesis via the activation of nAChRs (Stankov et al., 1993; Yamada et al., 1998b).

The pineal expresses mRNA encoding the α3, β2, and β4 nAChR subunits (Wada et al., 1989; Zoli et al., 1995), and nAChR binding sites have been found in mouse and rat pineal gland (Marks et al., 1998; Hernandez et al., 1999; Perry et al., 2002; Dávila-García et al., 2003). Here we studied the pharmacology of the rat pineal nAChR binding sites and functional responses and used subunit-selective antibodies to determine the subunit composition of the nAChR subtype expressed in the rat pineal gland. Our data indicate that the pineal gland expresses a single subtype of nAChR; it thus provides a simple and convenient model system in which to study a native nAChR of defined subtype.

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Materials and Methods

Materials. [^{3}H]Epibatidine ([^{3}H]EB) was purchased from Perkin Elmer Life Sciences (Boston, MA). [^{125}I]Epibatidine ([^{125}I]EB) used for autoradiography was a kind gift from Dr. John Musachio (NIMH, Bethesda, MD). [^{125}I]alpha-bungarotoxin ([^{125}I]α-BTX) and [α–^{32}P]ATP were purchased from Amersham Pharmacia (Piscataway, NJ). Tissue culture medium, fetal bovine serum, and antibiotics were purchased from Invitrogen Corp. (Carlsbad, CA). DNase 1 was purchased from Boehringer Mannheim Corporation (Indianapolis, IN). Bacterial cell walls containing protein-A (Pansorbin) and protein G (Omnisorb) were purchased from Calbiochem (San Diego, CA). Whatman GF/C filters were obtained from Brandell (Gaithersberg, MD). Other drugs and chemicals were purchased from Sigma Chemical Co (St. Louis, MO) or Fischer Scientific Co. (Fairlawn, NJ). The α3 nAChR subunit specific polyclonal antibody was raised in rabbits and has been described previously (Yeh et al., 2001). The β4 nAChR subunit specific polyclonal antibody was a generous gift from Dr. Scott Rogers (University of Utah, Salt Lake City, UT). The β2 nAChR subunit specific monoclonal antibody mAb 270 was produced from hybridoma stocks purchased from ATCC (Manassas, VA). This mAb was originally developed and characterized by Whiting and Lindstrom (1987). It is an excellent antibody for immunoprecipitation but it does not detect rat nAChR β2 subunits in western blots. Therefore, a nAChR β2 subunit-specific polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and used for western blot analysis.

Tissues. Frozen pineal glands and cerebral cortex from male Sprague-Dawley rats weighing 225 - 250 g were purchased from Zivic-Miller Laboratories (Portersville, PA) and stored at –80°C until assayed.

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Primary Cell Culture. Male Sprague-Dawley rats purchased from Taconic (Germantown, NY) were group housed with food and water ad libitum in a temperature- and light-controlled room (24°C, lights on 7:00 am to 7:00 pm). At 30 – 35 days old (150 – 200 g), the rats were anesthetized with methoxyflurane inhalation and decapitated. Fresh pineal glands were dissected and then used for primary tissue culture. The isolated pineal glands were washed two times in phosphate buffered saline (PBS) and cut into small pieces under a dissecting microscope. The tissue pieces were transferred to a sterile tube containing 5 ml of 0.02% trypsin and 1% DNase in PBS and incubated with gentle shaking for 10 min at 37°C. The tissues were triturated with a fire polished pasture pipette and incubated for another 10 min at 37°C. The tissues were then triturated a second time, and 0.5 ml of fetal bovine serum was added to stop the enzymatic reaction. The dissociated cells were washed four times by centrifugation at 800 x g in culture media containing basal Eagles medium supplemented with 10% fetal bovine serum, 25 mM glutamine, and 50 μg/ml gentamycin. The dissociated pineal cells were resuspended in fresh culture media and plated onto poly-D-lysine coated cover slips in a 30 mm dish. Cultures were maintained at 37°C with 5% CO_2 in a humidified incubator for 1 –3 days. HEK 293 cells stably expressing the α3β4 nAChR (KXα3β4 cells) and α3β2 nAChR (KXα3β2 cells) were grown and maintained as described previously (Xiao et al., 1998).

Radioligand Binding Assays. nAChR receptor binding sites in membrane homogenates from rat pineal gland and HEK cells expressing α3β4 and α3β2 nAChRs were measured with [^{3}H]EB. Tissues were homogenized with a Polytron homogenizer in 50 mM Tris-HCl (pH 7.4) and the homogenates were centrifuged at 35,000 x g for 10 min. Membrane pellets were washed twice and then resuspended in fresh buffer. Membrane aliquots (~ 35 μg of protein) were incubated with [^{3}H]EB (~5 - 3000 pM) for 4 hours at 24°C in a volume of 1 ml of Tris-HCl buffer. In competition

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binding experiments a series of concentrations of each drug was incubated with ~500 pM [^{3}H]EB. Bound and free radioligand were separated by vacuum filtration through Whatman GF/C filters pretreated with 0.5% polyethylenimine. The radioactivity bound to the filter was measured by liquid scintillation counting. Nonspecific binding was determined in the presence of 300 μM (-)-nicotine, and specific binding was defined as the difference between total and non-specific binding. In saturation binding experiments, B_{max} and K_d values were determined by non-linear regression analysis (GraphPad Prism software, San Diego, CA). In competition binding experiments, inhibition curves and the IC_{50} values were determined by non-linear regression analysis (GraphPad Prism). The affinities of drugs (K_i values) at nAChRs were calculated from the IC_{50} values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

Autoradiography. Autoradiography of [^{125}I]EB and [^{125}I]-α-BTX binding sites was carried out in 20 μm cryostat-cut sections of the rat brain through the superior colliculus with the pineal gland in place, as described by Perry et al. (2002). Adjacent brain sections were mounted onto slides and incubated with ~ 500 pM [^{125}I]EB or ~ 5 nM [^{125}I]α-BTX and then rinsed, air dried, and apposed to autoradiographic film for 1 to 6 days. Nonspecific labeling was determined in the presence of 300 μM nicotine.

Immunoprecipitation. Rat pineal glands were prepared as for a radioligand binding assay, and the membrane pellet was resuspended in fresh buffer and incubated with ~ 3 nM [^{3}H]EB for 2 hr at room temperature. The tissue was then solubilized by the addition of Triton X-100 at a final concentration of 2% with gentle mixing for 2 h at room temperature. Following solubilization, the mixture was centrifuged at 35,000 x g for 30 min. Aliquots of clear supernatant equivalent to 4 mg of original tissue weight were added to tubes containing either crude antiserum directed at the β4 subunit, or affinity purified antibody directed at the α3 subunit or a monoclonal antibody (mAb)

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directed at the β2 subunit. The β4 antiserum was tested at dilutions from 1:50 to 1:6 to determine the optimal concentration. The stock α3 and β2 antibodies each contained 1 μg/μl and were tested at dilutions from 2:100 to 15:100 to determine the optimal concentration. Tubes containing normal rabbit serum (NRS) or an irrelevant monoclonal antibody served as controls. The mixtures were incubated for 4 h to 16 h at 4°C with gentle rotation. After this incubation period, 50 μl aliquots of stripped Protein A (Pansorbin) or Protein G (Omnisorb) (Wall et al., 1991) were added to each assay tube, and incubated for an additional hour at 4°C with gentle rotation. The [^{3}H]EB-labeled receptor-antibody complex was precipitated by centrifugation at 14,000 x g for 30 sec. The pellets were washed two times with 1 ml of Tris-EDTA buffer, dissolved in 400 μl of 0.1 NaOH, 3% deoxycholate and counted by liquid scintillation spectroscopy. For the sequential immunoprecipitation assays, the individual clear supernatant from the first immunoprecipitation was collected and immediately added to tubes containing antisera or antibody directed at another nAChR subunit and allowed to incubate for an additional 4 hr. The immunoprecipitation procedure with the second antibody was carried out as described above.

Western Blots. Western blot analyses to measure subunit proteins were carried out as previously described (Yeh et al., 2001), using the polyclonal antibodies directed at each of the subunits.

RNA isolation and RNase protection assay. Expressions of mRNAs encoding nAChR subunits were determined as described previously (Xiao et al., 1998) with modifications. Briefly, total cellular RNA was isolated using RNA-STAT-60 (Tel Test B, Friendswood, TX). Antisense riboprobes for the α2, α3, α4, α5, α6, α7, β2, β3 and β4 nAChR subunits were generated from DNA templates using T7 RNA polymerase and [α-^{32}P]CTP. The RNase protection assays were carried out using the RPA II kit (Ambion, Austin, TX). Total RNA (20 μg) from the tissue samples

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was hybridized overnight at 42ºC with the subunit riboprobes and a riboprobe for rat GAPDH, which was used as an internal and loading control. Specific activities of [α-^{32}P]CTP used for synthesizing the probes of rat nAChR subunit genes and the probe of GAPDH were 800 and 40 Ci/mmol, respectively. Following hybridization, non-protected fragments were digested with a combination of RNase A and RNase T1 for 30 min at 37ºC. The numbers of bases of the full length probes and the protected fragments of the probes were as follows: α2, 416 and 332; α3, 306 and 230; α4, 496 and 408; α5, 411 and 380; α6, 462 and 396; α7, 450 and 376; β2, 322 and 263; β3, 430 and 394; β4, 252 and 170 and GAPDH, 204 and 135. To avoid overlap of signal bands, three reactions were carried out for each RNA sample separately using three groups of probes: (group 1 contained α2, α3, α4 and GAPDH; group 2 contained α5, α6, β4 and GAPDH; and group 3 contained α7, β2, β3 and GAPDH). The protected probe fragments were separated by electrophoresis on a 6% denaturing polyacrylamide gel, and the fragments were visualized using X-ray film or by phosphor-imaging.

Electrophysiology. Functional responses of nAChRs in rat pineal primary cell culture were measured using the whole-cell configuration of the voltage patch clamp technique. Dissociated rat pinealocytes were plated onto glass cover slips and positioned into a recording chamber (1 ml volume) mounted on the stage of an upright microscope (Axioskop; Carl Zeiss, Jena, Germany) used to visually identify the cells under study. The cells were bathed with an extracellular solution containing 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 5 mM HEPES, and adjusted to 325 mOsM with sucrose (pH adjusted to 7.4 with NaOH). The recording chamber was continuously perfused with the extracellular solution at a rate of 1ml/min. Ionic currents were monitored with an Axopatch 1-D patch amplifier (Axon Instruments, Foster City, CA). Recording patch electrodes were pulled from borosilicate glass capillaries to a resistance of 5 to 7 MΩ when

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filled with internal electrode solution containing 145 mM potassium-gluconate, 5 mM EGTA, 5 mM $MgCl_2$, 10 mM HEPES, 5 mM ATP 0.5 mM GTP and 10 mM BAPTA (pH adjusted to 7.42 with CsOH). Pinealocytes were distinguished from glial cells visually and selected for whole cell recording. The size and capacitance were very similar between pinealocytes, and recordings were made from single isolated cells. No differences in responses between cells kept in culture for 1 day or 3 days were noted. Drugs were applied using a gravity-fed Y-shaped tubing system positioned within 500 μm of the cell under investigation. Drugs were applied and exchanged rapidly with an onset of <100 ms. For experiments requiring α-BTX incubation, the extracellular solution was exchanged with the same solution with the addition of toxin and allowed to incubate for 2.5 min. Generation of voltage-clamp protocols and acquisition of data were carried out using the pCLAMP software. All experiments were performed at room temperature.

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Results

Expressions of nAChR subunit mRNAs in rat pineal glands. Expressions of mRNAs encoding nine nAChR subunits (α2, α3, α4, α5, α6, α7, β2, β3 and β4) in rat pineal glands were examined with a multiplex RNAse protection assay. The mRNA expression pattern detected in the rat pineal gland is shown in figure 1. The pineal gland expressed nAChR mRNAs encoding the α3, (lane 1), β4 (lane 2), and β2 (lane 3) subunits only, and the intensity of the bands indicated that the expression levels of the α3 and β4 subunit mRNAs were somewhat higher than that of the β2 subunit mRNA. Although no signals for the mRNA encoding any other nAChR subunits were detected in the pineal gland, strong signals for these other subunits were detected in tissues used as positive controls for the assay (data not shown).

nAChR binding sites in the rat pineal. Autoradiographic studies of nAChR binding sites in coronal brain sections at the level of the superior colliculus revealed dense [^{125}I]EB binding sites in the pineal gland (Fig. 2A); while in contrast, autoradiography of [^{125}I]α-BTX binding in the pineal did not exceed the background level (Fig. 2B). These autoradiographic results combined with the mRNA analysis indicate that the pineal gland expresses one or more heteromeric nAChRs but not α7 receptors or other subtypes that bind [^{125}I]α-BTX. As shown in figure 3, saturation binding measurements of [^{3}H]EB binding sites in membrane homogenates demonstrate that the pineal expresses a high density of nAChRs (~300 fmol/mg protein), which is ~ 5 times the density found in the rat forebrain (~ 60 fmol/mg protein). The [^{3}H]EB binding curves in the pineal fit a model for a single site with a K_d of ~ 100 pM.

Based on the nAChR subunit mRNA it expresses, the pineal could contain either an α3β2 or an α3β4 subtype, both subtypes, or a receptor comprised of α3 subunits in association with both β2

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and β4 subunits (i.e., an α3β2β4 subtype). To begin to determine the characteristics of the nAChR subtype(s) in the pineal, the affinities of drugs for the [^{3}H]EB-labeled sites were assessed in binding competition assays and compared to the affinities at defined α3β4 and α3β2 receptors heterologously expressed in HEK293 cells. The agonists A-85380, cytisine and nicotine all competed for [^{3}H]EB binding sites in the pineal gland with affinities between ~ 24 nM and 200 nM, while the antagonist dihydro-β-erythroidine (DHβE) displayed an affinity of ~ 65 μM (Fig. 4A and Table 1). These affinities are much closer to those found at the defined α3β4 receptor than at the α3β2 receptor, especially for A85380 and DHβE, two drugs that are good discriminators between these subtypes (Fig. 4B, 4C and Table 1). In fact, the correlation line between the K_i values for the pineal and the α3β4 subtype was very close to the line of identity (Fig. 4D), suggesting that, as is seen in Table 1, the pharmacology of the pineal nAChR binding sites is very similar to, and probably indistinguishable from, that of the α3β4 subtype.

Functional responses of nAChRs in the pineal gland. The functional responses of the nAChRs in the pineal were examined in whole-cell patch clamp studies of dissociated pineal cells maintained in culture for ~ 3 days. All nicotinic agonists examined activated inward currents in a concentration-dependent manner, with epibatidine being at least 100-times more potent than any other agonist tested and ~ 1,000-times more potent than acetylcholine (Fig. 5A, B and Table 2). Most of the agonists examined here, including cytisine, functioned as full agonists compared to acetylcholine, eliciting peak currents of 250 - 300 pA; the exception was DMPP, which appeared to be a partial agonist with about half the efficacy of acetylcholine (Fig. 5C). The receptors appeared to desensitize in a manner that was also concentration-dependent, but they recovered their sensitivity within about 2 min after removal of agonists (data not shown). At agonist concentrations ≥ 500 μM, there was usually an obvious loss of receptor function, which probably reflects channel

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blockade by most of the agonists (data not shown). That could account for the apparently slightly higher efficacy seen with epibatidine, which because of its high potency can fully activate the receptors at concentrations that do not significantly block the channel.

As shown in Table 2, the EC_{50} values for these six agonists to activate whole cell currents via nAChRs in the pineal gland are very similar to their EC_{50} values to stimulate $^{86}Rb^{+}$ efflux via defined α3β4 receptors heterologously expressed in HEK293 cells. In fact, as shown in figure 6, comparison of the EC_{50} values for these two very different functional assays carried out in different cell types, one expressing its native receptor and the other expressing a heterologous receptor, reveals that the values are highly correlated ($r = 0.99$); and, more important, the best fit line for the correlation is again close to the line of identity, indicating that the absolute EC_{50} values are very similar, as would be expected if both assays were measuring the same receptor.

The potencies of antagonists to block nicotine-activated currents were evaluated by first measuring activation by 30 μM nicotine alone followed at 2 min intervals by measurements after simultaneous application of 30 μM nicotine and decreasing concentrations of mecamylamine, curare or DHβE in the same cell. Each of these antagonists blocked nicotine-activated currents (Fig. 7A), and the block was concentration-dependent (Fig. 7B). In contrast, α-BTX, which is a highly selective α7 nAChR antagonist, failed to block the nicotine-induced currents in the pineal cells even when the cells were perfused with the toxin during and for 2.5 minutes before application of nicotine (Fig. 7A).

As shown in Table 3, the IC_{50} values for the 3 antagonists to block nicotine-activated whole cell currents in the pineal are similar to their IC_{50} values to block $^{86}Rb^{+}$efflux via defined α3β4 receptors heterologously expressed in HEK293 cells. Moreover, for DHβE, the only effective competitive antagonist examined here, its K_i value calculated from its IC_{50} to inhibit whole cell

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currents in pineal cells was indistinguishable from its K_i value calculated from its IC_{50} to inhibit $^{86}Rb^+$efflux in the heterologously expressed cells (20 μM vs 22 μM).

Subunit composition of nAChRs in pineal gland. The pharmacological characteristics of the nAChRs in the pineal are consistent with an α3β4 receptor subtype; but pharmacology can not prove subtype, which is defined by subunit composition. Therefore, we carried out immunoprecipitation assays to determine the subunits that comprise the pineal nAChRs. To do this, we used a polyclonal antibody selective for the α3 subunit (Yeh et al., 2001) and another directed at the β4 subunit (Flores et al., 1996), and the monoclonal antibody mAb 270, which is selective for the β2 subunit (Whiting and Lindstrom, 1987). As shown in figure 8, the polyclonal antibodies directed at the α3 and β4 subunits each immunoprecipitated [^{3}H]EB-labeled nAChRs from detergent-solubilized extracts of pineal tissue in a concentration-dependent manner. In contrast, the β2 subunit-selective mAb270 did not immunoprecipitate any [^{3}H]EB-labeled receptors from the pineal extracts (Fig. 8). To confirm that mAb270 was, in fact, capable of immunoprecipitating nAChRs that contained β2 subunits, it was tested in similarly prepared extracts from rat cerebral cortex, where it effectively immunoprecipitated the α4β2 receptor (Fig. 8, inset), which predominates in that tissue (Whiting and Lindstrom, 1987; Flores et al., 1992).

The immunoprecipitation of nAChRs in the pineal extracts only with antibodies directed at α3 and β4 subunits indicates that these two subunits probably comprise the nAChR in the pineal gland. But to further test this, sequential immunoprecipitation assays were carried out to determine if the α3 and β4 subunits are physically associated with each other in the pineal gland**.** In these studies, [^{3}H]EB-labeled nAChRs in pineal membranes were solubilized and immunoprecipitated with one subunit-specific antibody and then, after centrifugation to collect the immunoprecipitated receptors in the pellet, the remaining (“cleared”) supernatant was subjected to a second

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immunoprecipitation with a second subunit-specific antibody. The degree to which the first antibody decreases immunoprecipitation by the second antibody indicates the degree of association between the subunits (Flores et al., 1992).

The results from these sequential immunoprecipitation studies are shown in Figure 9. When the pineal extracts were first immunoprecipitated with the α3 subunit-specific antibody, subsequent immunoprecipitations of the supernatant with the antibodies directed at the α3, β4, or β2 subunits yielded essentially no additional immunoprecipitation (Fig. 9A). Nearly identical results were found when the extracts were first immunoprecipitated with the β4 subunit-specific antisera (Fig. 9B). In contrast, the β2 subunit-specific mAb 270 antibody did not immunoprecipitate significant amounts of [^{3}H]EB labeled receptors from the pineal extracts; thus, after a first exposure to mAb 270, subsequent exposure to the antibodies directed at either the α3 or β4 antisera subunits immunoprecipitated virtually all of the receptors available in the supernatant (Fig. 9C). Western blot analyses of pineal extracts clearly demonstrated the presence of the α3 and β4 subunits, but no signal was detected for the β2 subunit (data not shown).

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Discussion

nAChRs mediate the actions of acetylcholine, nicotine and other nicotinic agonists throughout the central and peripheral nervous systems. Multiple subtypes of these receptors may form from the 8 α and 3 β subunits known to be expressed in mammalian tissues, but since the rules of assembly are not known the number of theoretically possible heteromeric nAChR subtypes is not yet established. More important, there are very few tissues where the subunit composition of native nAChRs is known with some certainty. The α7 subtype is identifiable in brain by its rapid kinetics and especially by its sensitivity to α-BTX. In contrast the many different potential subtypes of heteromeric receptors are not so readily distinguished. The predominant heteromeric nAChR in mammalian brain is the α4β2 subtype (Whiting and Lindstrom, 1987; Flores et al., 1992); however, other subtypes also are found in many areas of brain and spinal cord (Marks et al., 1998; Perry et al., 2002), usually making it difficult to know whether a nicotinic response is mediated by a single nAChR subtype or is a composite response reflecting more than one receptor. Peripheral ganglia appear to contain both α3β2 and α3β4 receptors, possibly incorporating an α5 subunit in some cases (Conroy and Berg, 1995; Flores et al., 1996; Xu et al., 1999); so again, even with their more limited number of nAChR subunits, it is usually not readily apparent which subtype mediates a particular response.

The main finding of this study is that the rat pineal gland expresses the α3β4 subtype of nAChR apparently exclusively. The evidence for this is based on the pharmacology of the receptor and on direct studies of its subunit composition. The pharmacology of both the receptor's binding site and its function correspond quantitatively (K_i and EC_{50} values) to the defined α3β4 receptor heterologously expressed in HEK293 cells. Moreover, definitive evidence for the subunit composition of the receptor was derived from the immunoprecipitation studies with subunit-specific

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antibodies, which indicate clearly that the α3 and β4 subunits in the pineal are associated exclusively with each other in the same [^{3}H]EB binding complex. This conclusion is consistent with data from autoradiography studies using [^{125}I]EB in conjunction with drugs that mask certain receptor subtypes (Perry et al., 2002) . The pineal thus serves as one of the few tissues available for studies of a defined subtype of native heteromeric nAChR.

We measured strong signals for α3 and β4 subunit mRNA, as well as a somewhat weaker mRNA signal for the β2 subunit in the rat pineal. These results are consistent with previous mRNA analyses of nicotinic receptor subunits in the rat pineal gland (Wada et al., 1989; Zoli et al., 1995). However, we found no evidence that rat pineal gland nAChRs incorporate β2 subunits; and in fact, we found no evidence for the presence of the β2 protein via western blots. Based on the sensitivity of our binding and immunoprecipitation assays, we estimate we would have detected β2-containing receptors if they constituted ~ 5 % or more of the pineal receptors. The presence of the β2 mRNA might reflect a transient nAChR present during an earlier developmental stage or even a role in another protein expressed at low levels.

The main function of the pineal gland is the coupling of central circadian timing systems to effectors by the rhythmic production and release of melatonin. Accordingly, pineal function and melatonin have been implicated in reproductive cycles, gonad size (in some species) and sleep-wake cycles (including jet lag). The pineal appears to be innervated by both limbs of the autonomic nervous system. Sympathetic axons from the superior cervical ganglia synapse on pinealocytes where they release norepinephrine at β-adrenergic receptors, which leads to an increase in cyclic AMP and activation of melatonin synthesis (Axelrod, 1974). Parasympathetic cholinergic axons to the pineal probably originate in the sphenopalantine ganglia (Korf et al., 1996; Larsen et al., 1998). In addition, there is a central cholinergic innervation of the pineal from the medial habenula in some

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species (Schafer et al., 1998). Previous studies have found [^{3}H]EB binding sites in the pineal, indicating the presence of nAChRs (Marks et al., 1998; Hernandez et al., 1999; Perry et al., 2002; Dávila-García et al., 2003). Moreover, application of acetylcholine to isolated pinealocytes stimulates membrane depolarization that is coupled to activation of L-type calcium channels, and this action is mimicked by nicotine and blocked by d-tubocurarine (Letz et al., 1997).

Previous studies have found that nAChRs mediate inhibition of melatonin synthesis in rat pineal explants (Stankov et al., 1993; Yamada et al., 1998b). In an elegant series of studies, Yamada et al. (Yamada et al., 1996a; Yamada et al., 1996b; Yamada et al., 1998a; Yamada et al., 1998b) established a link between pineal nAChRs, L-type calcium channels, glutamate release, metabotropic glutamate receptors and inhibition of cyclic AMP in a signaling pathway leading to inhibition of melatonin synthesis. The signaling pathway begins with activation of the nAChRs, which by depolarizing the pinealocyte membrane activate L-type calcium channels. The increased intracellular Ca^{2+} levels trigger exocytotic release of glutamate, which activates mGluR3 metabotropic glutamate receptors and leads to decreased cyclic AMP. This results in decreased transcriptional activation of serotonin N-acetyltransferase, the rate-limiting enzyme in melatonin synthesis. Our studies identify the nAChR in pinealocytes that begins this signaling cascade as an $\alpha 3\beta 4$ subtype. This conclusion is consistent with observations from previous reports. For example, our EC_{50} values for activation of whole cell currents by nicotine and ACh in dissociated pinealocytes are similar to the values reported for release of glutamate and inhibition of melatonin synthesis (Yamada et al., 1998b). Moreover, both nicotine-activated whole cell currents (present data) and acetylcholine-induced release of glutamate (Yamada et al., 1998b) in pinealocytes are blocked by d-tubocurarine but not by α-BTX.

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A second major finding in this paper is the remarkably close correspondence between the function of α3β4 nAChRs measured by whole cell patch clamp analysis in pinealocytes and $^{86}Rb^{+}$ efflux in transfected HEK293 cells that heterologously express these receptors. Thus, the EC_{50} values for the activation of whole cell currents in pinealocytes by six nicotinic agonists were nearly indistinguishable from their EC_{50} values for the activation of $^{86}Rb^{+}$efflux measured in HEK293 cells expressing the α3β4 nAChR subtype. Moreover, cytisine, which is a full agonist at β4-containing receptors but a weak partial agonist at β2-containing receptors (Luetje and Patrick, 1991; Papke and Heinemann, 1994) is a full agonist at the pineal receptor as well as at the heterologously expressed α3β4 receptor (Meyer et al., 2001). Similarly, DMPP appears to be a partial agonist at the pineal receptors, as it is at the heterologously expressed α3β4 receptors (Meyer et al., 2001). In addition to the evidence from the activation of the receptors by agonists, the K_i values for the competitive antagonist DHβE derived from functional studies in pineal cells and the heterologously expressed α3β4 receptors are nearly identical.

Nevertheless, although the pharmacological evidence that the pineal nAChR is an α3β4 subtype is very strong, there are at present no drugs that can conclusively establish the identity of a heteromeric nAChR subtype. Furthermore, very little is known about the pharmacological properties of a possible α3β2β4 receptor (Colquhoun and Patrick, 1997). Therefore, the immunoprecipitation studies with subunit specific antibodies provide the most definitive evidence for the identity of the pineal receptor subtype. Only the antibodies that recognize the α3 and β4 subunits were effective in immunoprecipitating the nAChR in the pineal gland; moreover, immunoprecipitation with either antibody removed virtually all of the receptors from the remaining supernatant, indicating that the α3 and β4 subunits are physically associated. In contrast, the β2-directed antibody did not immunoprecipitate nAChRs from the pineal extracts, although it

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efficiently immunoprecipitated receptors from the cerebral cortex, which is known to contain nAChRs with the β2 subunit, namely the α4β2 subtype (Whiting and Lindstrom, 1987; Flores et al., 1992).

In summary, these studies have shown that the rat pineal gland expresses a high density of nAChRs and that this receptor is virtually exclusively an α3β4 subtype. The characteristics of this receptor are consistent with the one that mediates the cholinergic signals that lead to decreased melatonin production and thereby plays an important role in pineal physiology. Thus, the rat pineal gland provides a readily obtainable native tissue with a high concentration of an identified subtype of nAChR. This should allow detailed studies of, for example, the channel properties, regulation and turnover rate of this receptor in its native cell, as opposed to receptors expressed by transfection into heterologous cell systems. In addition, studies to determine proteins associated with the α3β4 nAChR require a native tissue that expresses a high level of receptor. The pineal is such a tissue and should be useful for this purpose.

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This article has not been copyedited and formatted. The final version may differ from this version.

References

Axelrod J (1974) The pineal gland: a neurochemical transducer. *Science* **184**:1341-8.

Champtiaux N, Gotti C, Cordero-Erausquin M, David DJ, Przybylski C, Lena C, Clementi F, Moretti M, Rossi FM, Le Novere N, McIntosh JM, Gardier AM and Changeux JP (2003) Subunit composition of functional nicotinic receptors in dopaminergic neurons investigated with knock-out mice. *J Neurosci* **23**:7820-9.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099-108.

Colquhoun LM and Patrick JW (1997) Alpha3, beta2, and beta4 form heterotrimeric neuronal nicotinic acetylcholine receptors in Xenopus oocytes. *J Neurochem* **69**:2355-62.

Conroy WG and Berg DK (1995) Neurons can maintain multiple classes of nicotinic acetylcholine receptors distinguished by different subunit compositions. *J Biol Chem* **270**:4424-31.

Couturier S, Bertrand D, Matter JM, Hernandez MC, Bertrand S, Millar N, Valera S, Barkas T and Ballivet M (1990) A neuronal nicotinic acetylcholine receptor subunit (alpha 7) is developmentally regulated and forms a homo-oligomeric channel blocked by alpha-BTX. *Neuron* **5**:847-56.

Dávila-García MI, Musachio JL and Kellar KJ (2003) Chronic nicotine administration does not increase nicotinic receptors labeled by [125I]epibatidine in adrenal gland, superior cervical ganglia, pineal or retina. *J Neurochem* **85**:1237-46.

Flores CM, DeCamp RM, Kilo S, Rogers SW and Hargreaves KM (1996) Neuronal nicotinic receptor expression in sensory neurons of the rat trigeminal ganglion: demonstration of alpha3beta4, a novel subtype in the mammalian nervous system. *J Neurosci* **16**:7892-901.

Flores CM, Rogers SW, Pabreza LA, Wolfe BB and Kellar KJ (1992) A subtype of nicotinic cholinergic receptor in rat brain is composed of alpha 4 and beta 2 subunits and is up-regulated by chronic nicotine treatment. *Mol Pharmacol* **41**:31-7.

Hernandez SC, Musachio JL, Wang Y, Dávila-García MI, Ebert S, Wolfe BB and Kellar KJ (1999) The rat pineal gland expresses a nicotinic receptor with the characteristics of the alpha3beta4 subtype. *Soc. Neurosci. Abstracts* **25**:980.

Korf HW, Schomerus C, Maronde E and Stehle JH (1996) Signal transduction molecules in the rat pineal organ: Ca2+, pCREB, and ICER. *Naturwissenschaften* **83**:535-43.

Larsen PJ, Enquist LW and Card JP (1998) Characterization of the multisynaptic neuronal control of the rat pineal gland using viral transneuronal tracing. *Eur J Neurosci* **10**:128-45.

Letz B, Schomerus C, Maronde E, Korf HW and Korbmacher C (1997) Stimulation of a nicotinic ACh receptor causes depolarization and activation of L-type Ca2+ channels in rat pinealocytes. *J Physiol* **499**:329-40.

Luetje CW and Patrick J (1991) Both alpha- and beta-subunits contribute to the agonist sensitivity of neuronal nicotinic acetylcholine receptors. *J Neurosci* **11**:837-45.

Marks MJ, Smith KW and Collins AC (1998) Differential agonist inhibition identifies multiple epibatidine binding sites in mouse brain. *J Pharmacol Exp Ther* **285**:377-86.

Meyer EL, Xiao Y and Kellar KJ (2001) Agonist regulation of rat alpha 3 beta 4 nicotinic acetylcholine receptors stably expressed in human embryonic kidney 293 cells. *Mol Pharmacol* **60**:568-76.

Orr-Urtreger A, Goldner FM, Saeki M, Lorenzo I, Goldberg L, De Biasi M, Dani JA, Patrick JW and Beaudet AL (1997) Mice deficient in the alpha7 neuronal nicotinic acetylcholine

receptor lack alpha-bungarotoxin binding sites and hippocampal fast nicotinic currents. *J Neurosci* **17**:9165-71.

Papke RL and Heinemann SF (1994) Partial agonist properties of cytisine on neuronal nicotinic receptors containing the beta 2 subunit. *Mol Pharmacol* **45**:142-9.

Perry DC, Xiao Y, Nguyen HN, Musachio JL, Dávila-García MI and Kellar KJ (2002) Measuring nicotinic receptors with characteristics of alpha4beta2, alpha3beta2 and alpha3beta4 subtypes in rat tissues by autoradiography. *J Neurochem* **82**:468-81.

Quik M, Polonskaya Y, McIntosh JM and Kulak JM (2002) Differential nicotinic receptor expression in monkey basal ganglia: effects of nigrostriatal damage. *Neuroscience* **112**:619-30.

Salminen O, Murphy KL, McIntosh JM, Drago J, Marks MJ, Collins AC and Grady SR (2004) Subunit composition and pharmacology of two classes of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. *Mol Pharmacol* **65**:1526-35.

Schafer MK, Eiden LE and Weihe E (1998) Cholinergic neurons and terminal fields revealed by immunohistochemistry for the vesicular acetylcholine transporter. I. Central nervous system. *Neuroscience* **84**:331-59.

Schoepfer R, Conroy WG, Whiting P, Gore M and Lindstrom J (1990) Brain alpha-bungarotoxin binding protein cDNAs and MAbs reveal subtypes of this branch of the ligand-gated ion channel gene superfamily. *Neuron* **5**:35-48.

Stankov B, Cimino M, Marini P, Lucini V, Fraschini F and Clementi F (1993) Identification and functional significance of nicotinic cholinergic receptors in the rat pineal gland. *Neurosci Lett* **156**:131-4.

Wada E, Wada K, Boulter J, Deneris E, Heinemann S, Patrick J and Swanson LW (1989) Distribution of alpha 2, alpha 3, alpha 4, and beta 2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. *J Comp Neurol* **284**:314-35.

Wall SJ, Yasuda RP, Hory F, Flagg S, Martin BM, Ginns EI and Wolfe BB (1991) Production of antisera selective for m1 muscarinic receptors using fusion proteins: distribution of m1 receptors in rat brain. *Mol Pharmacol* **39**:643-9.

Whiting P and Lindstrom J (1987) Purification and characterization of a nicotinic acetylcholine receptor from rat brain. *Proc Natl Acad Sci U S A* **84**:595-9.

Xiao Y and Kellar KJ (2004) The comparative pharmacology and up-regulation of rat neuronal nicotinic receptor subtype binding sites stably expressed in transfected mammalian cells. *J Pharmacol Exp Ther* **310**:98-107.

Xiao Y, Meyer EL, Thompson JM, Surin A, Wroblewski J and Kellar KJ (1998) Rat alpha3/beta4 subtype of neuronal nicotinic acetylcholine receptor stably expressed in a transfected cell line: pharmacology of ligand binding and function. *Mol Pharmacol* **54**:322-33.

Xu W, Gelber S, Orr-Urtreger A, Armstrong D, Lewis RA, Ou CN, Patrick J, Role L, De Biasi M and Beaudet AL (1999) Megacystis, mydriasis, and ion channel defect in mice lacking the alpha3 neuronal nicotinic acetylcholine receptor. *Proc Natl Acad Sci U S A* **96**:5746-51.

Yamada H, Ogura A, Koizumi S, Yamaguchi A and Moriyama Y (1998b) Acetylcholine triggers L-glutamate exocytosis via nicotinic receptors and inhibits melatonin synthesis in rat pinealocytes. *J Neurosci* **18**:4946-52.

Yamada H, Yamamoto A, Takahashi M, Michibata H, Kumon H and Moriyama Y (1996b) The L-type Ca2+ channel is involved in microvesicle-mediated glutamate exocytosis from rat pinealocytes. *J Pineal Res* **21**:165-74.

Yamada H, Yamamoto A, Yodozawa S, Kozaki S, Takahashi M, Morita M, Michibata H, Furuichi T, Mikoshiba K and Moriyama Y (1996a) Microvesicle-mediated exocytosis of glutamate is a novel paracrine-like chemical transduction mechanism and inhibits melatonin secretion in rat pinealocytes. *J Pineal Res* **21**:175-91.

Yamada H, Yatsushiro S, Ishio S, Hayashi M, Nushi T, Yamamoto A, Futai M, Yamaguchi A and Moriyama Y (1998a) Metabotropic Glutamate Receptors Negatively Regulate Melatonin Synthesis in Rat Pinealocytes. *J Neurosci* **18**:2056-2062.

Yeh JJ, Yasuda RP, Davila-Garcia MI, Xiao Y, Ebert S, Gupta T, Kellar KJ and Wolfe BB (2001) Neuronal nicotinic acetylcholine receptor alpha3 subunit protein in rat brain and sympathetic ganglion measured using a subunit-specific antibody: regional and ontogenic expression. *J Neurochem* **77**:336-46.

Zoli M, Le Novere N, Hill JA and Changeux JP (1995) Developmental regulation of nicotinic ACh receptor subunit mRNAs in the rat central and peripheral nervous systems. *J Neurosci* **15**:1912-39.

FOOTNOTES

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Hernandez, SC, Vicini S, Kellar KJ (2000) The functional characteristics of neuronal nicotinic acetylcholine receptors in the rat pineal gland. *Soc. Neurosci. Abstracts* **26**:105.

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FIGURE LEGENDS

Figure 1. Expression of nAChR subunit mRNA in the rat pineal gland. RNAse protection assays were carried out as described in Materials and Methods. Total RNA from rat pineal gland was hybridized with a combination of [^{32}P] labeled anti-sense probes corresponding to the nine rat nAChR subunit genes α2, α3, α4 (lane 1); α5, α6, β4 (lane 2); and α7, β2 and β3 (lane 3). The probe corresponding to GAPDH gene was used as an internal and loading control. The gel shown is representative of 3 to 6 independent experiments for the different subunits.

Figure 2. Autoradiographic images of [^{125}I]EB and [^{125}I]α-BTX binding to rat pineal gland. Autoradiography was carried out as described in *Materials and Methods*. Coronal sections (20 μm) of brain at the level of the superior colliculus with the pineal gland intact were mounted onto slides and incubated with (A) 500 pM [^{125}I]EB or (B) 5 nM [^{125}I]α-BTX to measure total binding (TB) to the sections. Non-specific binding (NSB) was measured in parallel by incubating adjacent brain sections with the radioligands in the presence of 300 μM nicotine. Slides were washed, dried and apposed to autoradiographic X-ray film in cassettes for 2-6 days. Abbreviations for the brain regions indicated are as follows: CTX, cerebral cortex; SC, superior colliculus; and Pin, pineal.

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Figure 3. Saturation Binding of [^{3}H]EB to membrane homogenates from rat pineal gland. Binding curves represent specific binding (■) and nonspecific binding (□) of [^{3}H]EB to membrane homogenates form rat pineal gland. The inset graph is a comparison of the B_{max} value for [^{3}H]EB specific binding to membrane homogenates form rat pineal gland vs. the rat cerebral cortex to demonstrate the relative densities of nAChRs expressed in the two tissues. Saturation data were analyzed by a nonlinear regression program (Graph Pad). Data shown are representative of six pineal gland and three cortex independent saturation experiments. The K_d and B_{max} values for the pineal gland were 103 ± 15 pM and 321 ± 37 fmol/mg protein, respectively.

Figure 4. Comparison between ligand binding competition profiles of nAChRs in membrane homogenates from rat pineal gland and HEK293 cells expressing α3β4 or α3β2 nAChRs. Competition of nicotinic drugs for [^{3}H]EB binding sites in (A) rat pineal gland, (B) α3β4 cells and (C) α3β2 cells. The membrane homogenates were incubated with 500 pM [^{3}H]EB, and the competing ligands were added at the concentrations indicated. All curves fit a one-site binding model, and the K_i values are provided in Table 1. Data shown are representative of 3 to 6 independent experiments. (D) Correlation between K_i values for nAChRs in the pineal and the α3β4 and α3β2 nAChRs in transfected cells. The logs of the K_i values in Table 1 were fit to a linear regression equation, and the Pearson correlation coefficient, r, was determined for each regression line. The dashed line represents the line of identity for the data and indicates that the pineal gland K_i values corresponded most closely to the α3β4 transfected cells. Data shown are the mean values of 3 to 6 independent experiments.

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Figure 5. Concentration response of different nicotinic agonists at nAChRs in dissociated rat pinealocytes. (A) Sample traces of currents activated by EB, A85380, nicotine (Nic), cytisine (Cyt) acetylcholine (ACh) and DMPP. Currents were recorded at a constant holding potential of –60 mV. Traces shown are representative of independent recordings from six different pinealocytes at the indicated drug concentration. (B) Concentration response curves for currents activated by increasing concentrations of EB (O), A85380 (■), NIC (♦), Cyt (▲), ACh (◊) and DMPP (*). Increasing concentrations of each agonist were applied at two minute intervals, with washout between each application. Currents were normalized to the maximal response induced by each individual agonist and data were fit to a sigmoidal concentration-response relationship using GraphPad. (C) The peak currents activated by each drug. Data are expressed in pA units and are the mean ± SEM for five to seven independent experiments.

Figure 6. Correlation between EC_{50} values for agonist-stimulated whole-cell currents in pineal cells and agonist stimulated $^{86}Rb^{+}$ efflux in transfected cells expressing $\alpha 3\beta 4$ receptors. The logs of the K_i values in Table 2 were fit to a linear regression equation, and the Pearson correlation coefficient, r, was determined. The dashed line represents the line of identity and indicates a close correspondence between the EC_{50} values derived from the two different types of assays.

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Figure 7. Concentration-dependent inhibition of nicotine-activated whole-cell currents by nicotinic antagonists in dissociated rat pinealocytes. (A) Sample traces of currents activated by 30 μM nicotine in rat pinealocytes in the absence and presence of mecamylamine (Mec), curare, DHβE and α-BTX. The cells first received an initial control application of 30 μM nicotine, followed by simultaneous application of 30 μM nicotine plus the indicated concentration of antagonist, except for α-BTX, which was added 2.5 min prior to and during the second nicotine application. (B) Concentration-response inhibition curves for Mec (○), curare (□) and DHβE (▼). Before curve fitting, the peak currents were normalized to the maximal current induced by 30 μM nicotine and expressed as percent of control. Data were fit to a sigmoidal concentration-response relationship using GraphPad. Data shown are mean ± SEM of three to five independent experiments.

Figure 8. Concentration dependence of antibodies for immunoprecipitation of solubilized nAChRs from the rat pineal gland. Pineal gland membrane homogenates were solubilized and [^{3}H]EB-labeled nAChRs equivalent to 4 mg of rat pineal gland tissue were incubated with the indicated dilutions of anti-β4 (■) antiserum, anti-α3 (▲) antibody or anti-β2 (○) monoclonal antibody (mAb 270), and specific immunoprecipitation was measured as described in *Materials and Methods*. As shown, only the anti-α3 antibody and anti-β4 antiserum immunoprecipitated [^{3}H]EB-labeled nAChRs from the pineal. Inset: In rat cerebral cortex, in contrast to the pineal, the anti-β2 monoclonal antibody immunoprecipitated [^{3}H]EB-labeled nAChRs. Data shown are mean ± SEM of three independent experiments.

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Figure 9. Association of α3 and β4 subunits in the rat pineal gland nAChR. Sequential immunoprecipitations were performed as described in *Materials and Methods*. Aliquots of solubilized, [^{3}H]EB-labeled nAChRs from rat pineal gland were incubated sequentially with anti-α3 antibody, anti-β4 antiserum or anti-β2 monoclonal antibody (mAb 270). Initial immunoprecipitation with α3 antibody (A), β4 antiserum (B), or β2 monoclonal antibody (C) was followed in each case by a second immunoprecipitation with α3 antibody, β4 antiserum or the β2 monoclonal antibody. Results are expressed as % of specific immunoprecipitation of total solubilized receptors. Data are the mean ± SEM from three independent experiments.

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TABLE 1.

Comparison of K_i values of nAChR ligand binding in membrane homogenates from rat pineal gland, α3β4 cells and α3β2 cells. Competition binding assays were carried out as described in *Materials and Methods* using ~ 500pM [^{3}H]EB. The K_i values were calculated using the Cheng-Prusoff equation. Values shown are the mean ± SEM of three to six independent experiments.

	K_i ± SEM (nM)		
Drug	Rat Pineal Gland	α3β4	α3β2[a]
A85380	24 ±2.8	54 ±4.8	0.21 ±0.04
Cytisine	63 ±11	128 ±15	37 ±9.0
Nicotine	202 ±26	318 ±42	47 ±11
DHβE	64,833 ±22,190	98,700 ±22,124	3800 ±1300

[a]Data for transfected cells expressing α3β2 receptors were taken from Xiao and Kellar (2004).

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TABLE 2.

Comparison of EC_{50} values of nicotinic agonists for activation of currents through nAChRs in rat pineal cells and stimulation of $^{86}Rb^+$ efflux from α3β4 transfected cells. Dissociated pineal cells were prepared from ~30-day-old male Sprague Dawley rats as described in *Materials and Methods*. See Figure 5 for description of data analyses and curve fittings of pineal whole cell recordings. Values shown are the mean ± SEM from five to seven different cell recordings and at least three $^{86}Rb^+$ efflux assays.

	EC_{50} (μM) ± SEM	
Agonist	Rat Pineal Gland (whole cell patch clamp)	α3β4 HEK Cells[a] ($^{86}Rb^+$ Efflux)
Epibatidine	0.03 ± 0.02	0.06 ± 0.01
A85380	6.0 ± 1.4	5.7 ± 0.3
DMPP	14 ± 12	28 ± 3.9
Nicotine	21 ± 16	31 ± 1.5
Cytisine	28 ± 16	24 ± 7.4
ACh	60 ± 16	110 ± 11

[a]Data for transfected cells expressing α3β4 receptors were taken form Meyer et al. (2001)

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TABLE 3.

Comparison of IC_{50} values of nicotinic antagonists for inhibition of nicotine (30 μM) activated currents through nAChRs in rat pineal cells and inhibition of nicotine (100 μM) stimulated $^{86}Rb^+$ efflux from α3β4 cells. Dissociated pineal cells were prepared from ~30-day-old male Sprague Dawley rats as described in *Materials and Methods*. See Figure 5 for description of data analyses and curve fittings of pineal whole cell recordings. Values shown are the mean ± SEM from four to five whole-cell recordings and at least three $^{86}Rb^+$ efflux assays.

	IC_{50} (μM) ± SEM	
Antagonist	Rat Pineal Gland (whole cell patch clamp)	α3β4 HEK Cells[a] ($^{86}Rb^+$ Efflux)
Mecamylamine	0.9 ± 0.2	1.2 ± 0.4
Curare	16 ± 14	9.6 ± 1.4
DHβE	50 ± 10 (K_i = 20 μM ± 4.0)	100 ± 6 (K_i = 22 μM ± 1.3)

[a]Data for transfected cells expressing α3β4 receptors were taken form Xiao et al. (1998).

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Figure 1

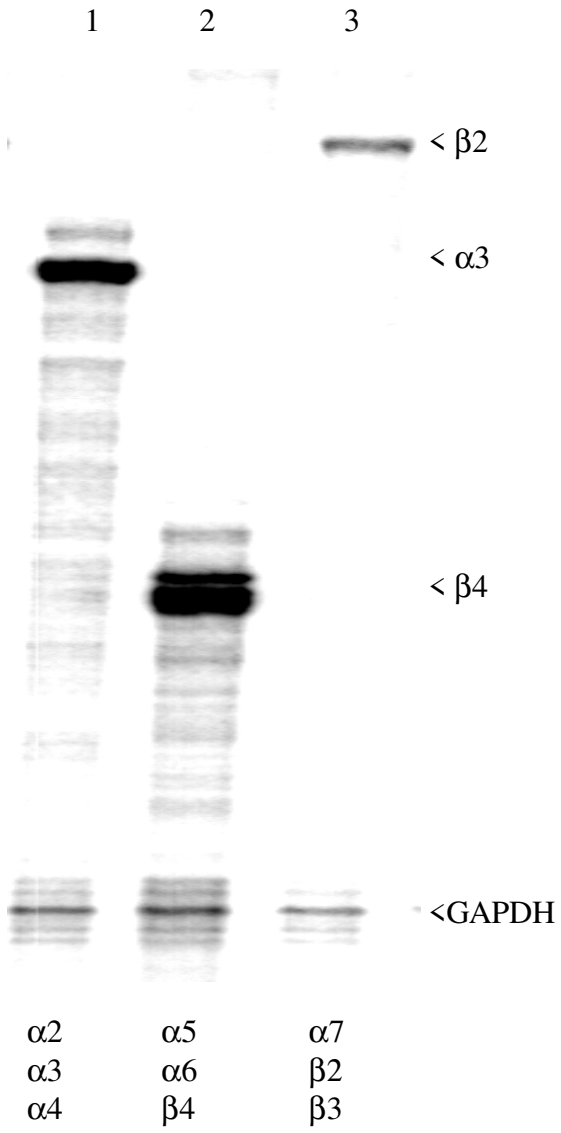

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Figure 2

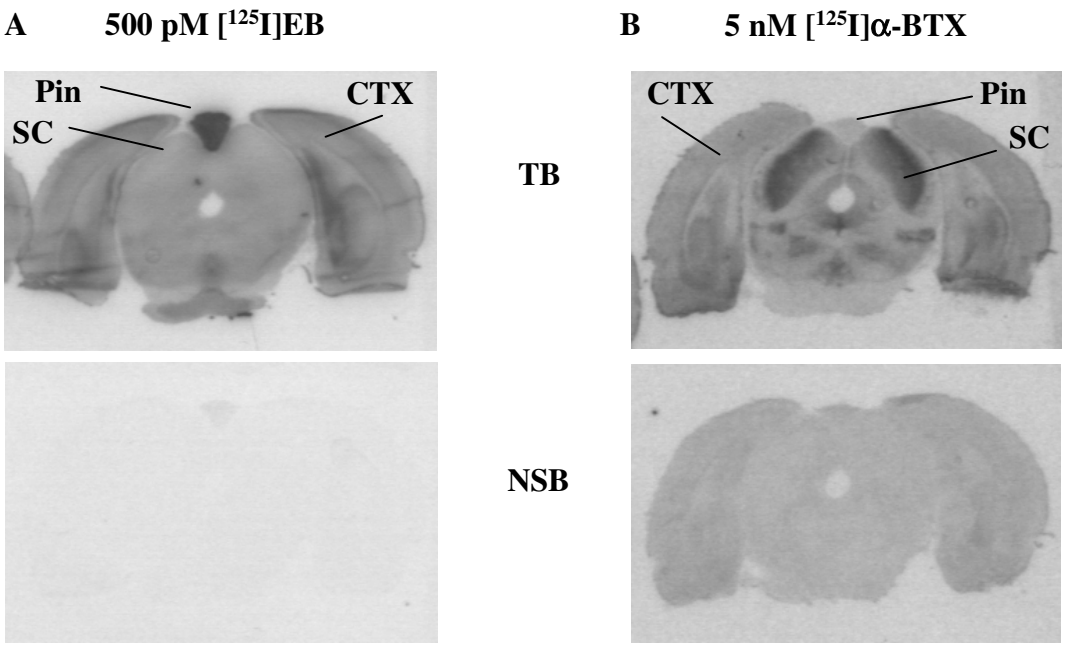

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Figure 3

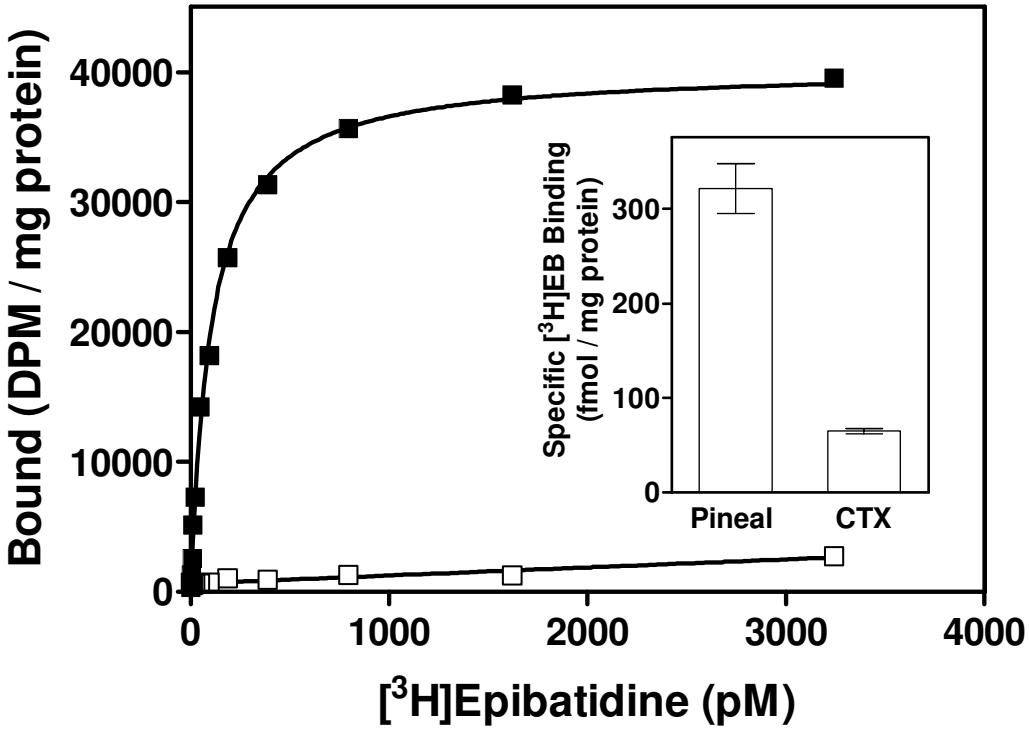

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Figure 4

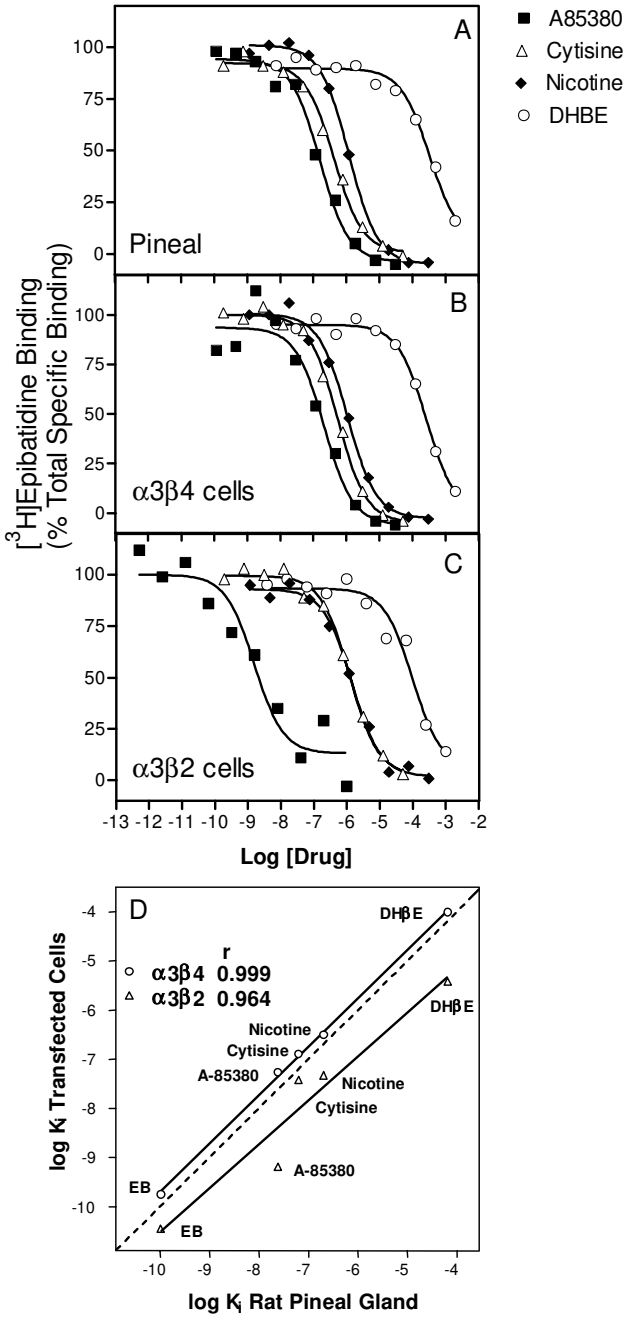

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Figure 5

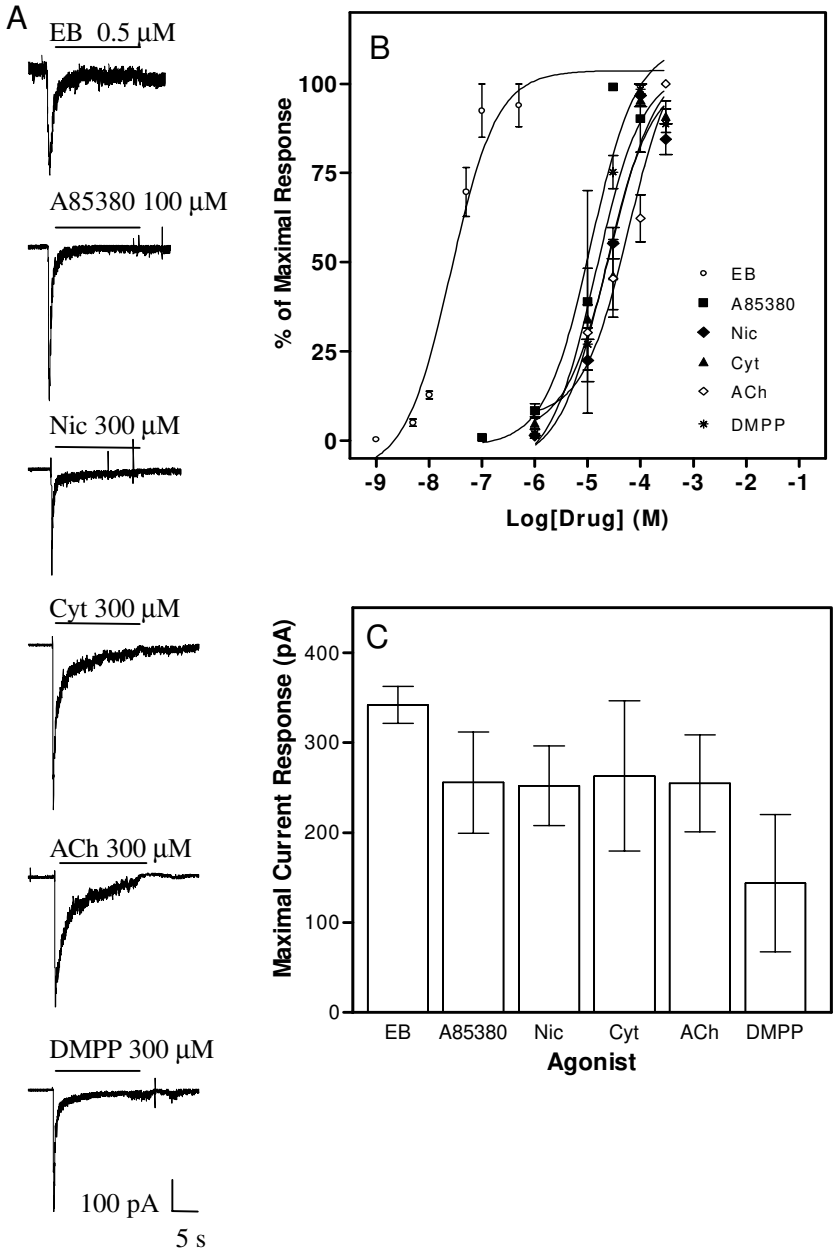

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Figure 6

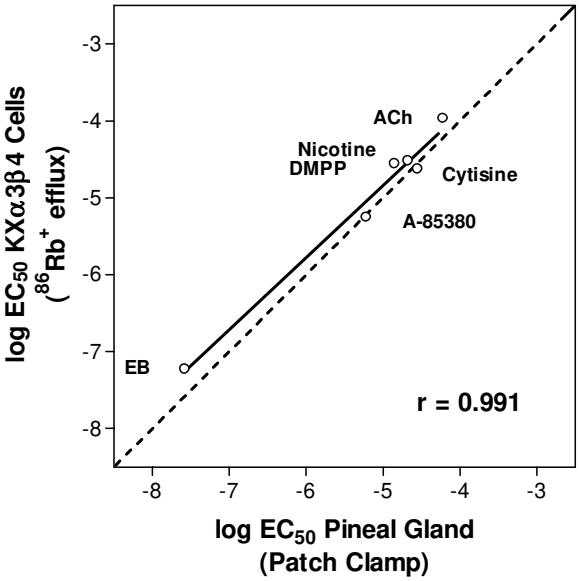

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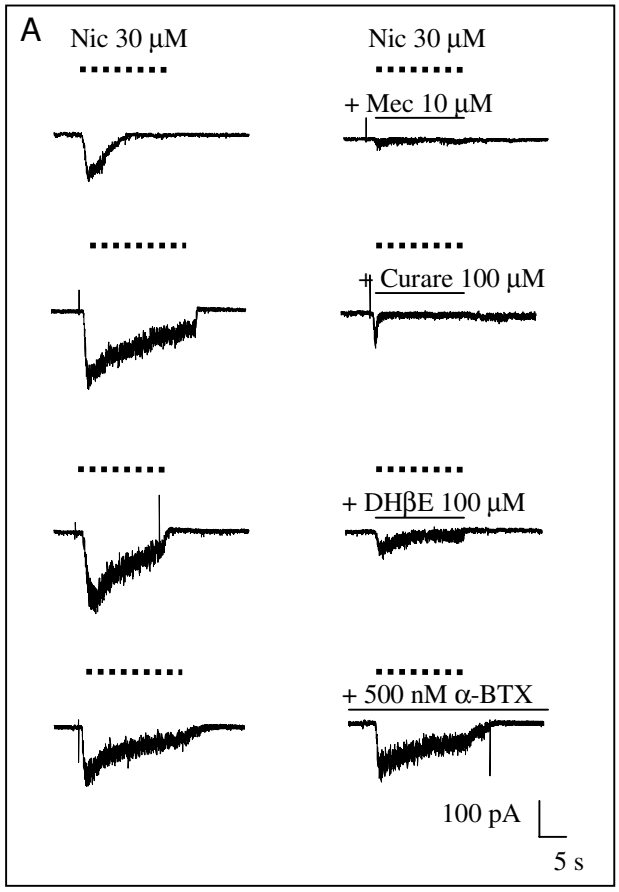
Figure 7



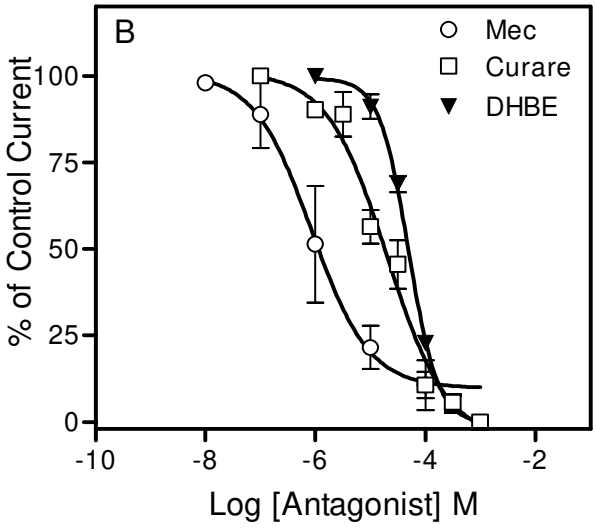


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Figure 8

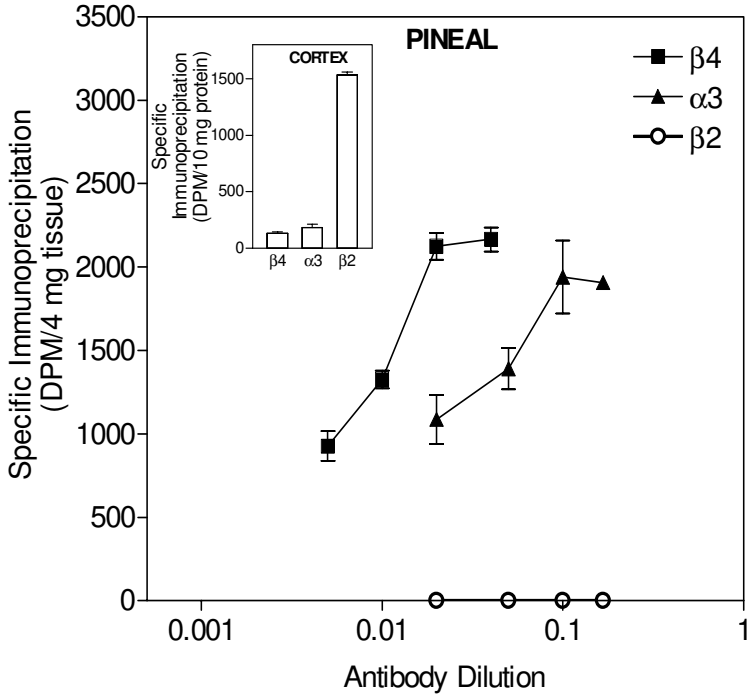

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Figure 9