

# The Nicotinic Receptor in the Rat Pineal Gland Is an $\alpha 3\beta 4$ Subtype

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## ABSTRACT

The rat pineal gland contains a high density of neuronal nicotinic acetylcholine 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 ([ $^3$ H]EB) binds with an affinity of  $\sim 100$  pM to nAChRs in the pineal gland, and the density of these sites is  $\sim 5$  times that in rat cerebral cortex. The affinities of nicotinic drugs for binding sites in the pineal gland are similar to those at  $\alpha 3\beta 4$  nAChRs heterologously expressed in human embryonic kidney 293 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 effica-

cies to activate or block  $^{86}\text{Rb}^+$  efflux in the cells expressing heterologous  $\alpha 3\beta 4$  nAChRs. Measurements of mRNA indicated the presence of transcripts for  $\alpha 3$ ,  $\beta 2$ , and  $\beta 4$  nAChR subunits but not those for  $\alpha 2$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\alpha 7$ , or  $\beta 3$  subunits. Immunoprecipitation with subunit-specific antibodies showed that virtually all [ $^3$ H]EB-labeled nAChRs contained  $\alpha 3$  and  $\beta 4$  subunits associated in one complex. The  $\beta 2$  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  $\alpha 3\beta 4$  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.

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated cation channels composed of  $\alpha$  and  $\beta$  subunits. Nine  $\alpha$  ( $\alpha 2$ – $\alpha 10$ ) and three  $\beta$  ( $\beta 2$ – $\beta 4$ ) subunits have been identified in vertebrates, and different subunit combinations define specific receptor subtypes. All of these subtypes pass  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Ca}^{2+}$ , but they exhibit distinct biophysical and pharmacological properties. Studies of nAChRs in *Xenopus laevis* oocytes and transfected mammalian cells have pro-

vided 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  $\alpha 4\beta 2$  subtype (Whiting and Lindstrom, 1987; Flores et al., 1992), which has high affinity for most agonists, and the  $\alpha 7$  subtype, which has high affinity for  $\alpha$ -bungarotoxin ( $\alpha$ -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 central nervous system (Marks et al.,

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**ABBREVIATIONS:** nAChR, nicotinic acetylcholine receptor; [ $^3$ H]EB, [ $^3$ H]( $\pm$ )-epibatidine;  $^{125}\text{I}$ -EB,  $^{125}\text{I}$ -( $\pm$ )-epibatidine; EB, ( $\pm$ )-epibatidine;  $^{125}\text{I}$ - $\alpha$ -BTX,  $^{125}\text{I}$ - $\alpha$ -bungarotoxin;  $\alpha$ -BTX,  $\alpha$ -bungarotoxin; EB, epibatidine; DH $\beta$ E, dihydro- $\beta$ -erythroidine; DMPP, 1,1-dimethyl-4-phenylpiperazinium; HEK, human embryonic kidney; mAb, monoclonal antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; A-85380, 3-[2(S)-azetidylmethoxy]pyridine dihydrochloride.

1998; Perry et al., 2002), and some of these less prevalent receptors may play critical roles because of their strategic location (e.g.,  $\alpha 6$ -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  $\alpha 4\beta 2$  and/or  $\alpha 7$  receptors are desensitized or inactivated by exposure to nicotine or during certain disease states that may involve the loss of specific nAChR subtypes, the less prevalent receptors may take on critical roles in mediating cholinergic signals. In addition,  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  nAChR subtypes are the predominant nAChRs in 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 to translate light stimuli into neuroendocrine responses. The main role of the pineal gland seems 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  $\beta$ -adrenergic receptors in the pineal gland (Axelrod, 1974). More recent studies have demonstrated a cholinergic innervation of the pineal gland 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 seems 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., 1998a).

The pineal expresses mRNA encoding the  $\alpha 3$ ,  $\beta 2$ , and  $\beta 4$  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.

## Materials and Methods

**Materials.** [ $^3\text{H}$ ]Epibatidine ([ $^3\text{H}$ ]EB) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [ $^{125}\text{I}$ ]Epibatidine ( $^{125}\text{I}$ -EB) used for autoradiography was a kind gift from Dr. John Musachio (National Institute of Mental Health, Bethesda, MD). [ $^{125}\text{I}$ ]- $\alpha$ -Bungarotoxin ( $^{125}\text{I}$ - $\alpha$ -BTX) and [ $\alpha$ - $^{32}\text{P}$ ]ATP were purchased from Amersham Biosciences Inc. (Piscataway, NJ). Tissue culture medium, fetal bovine serum, and antibiotics were purchased from Invitrogen (Carlsbad, CA). DNase1 was purchased from Roche Diagnostics (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 (Gaithersburg, MD). Other drugs and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific Co. (Fairlawn, NJ). The  $\alpha 3$  nAChR subunit-specific polyclonal antibody was raised in rabbits and has been described previously (Yeh et al., 2001). The  $\beta 4$  nAChR subunit specific polyclonal antibody was a

generous gift from Dr. Scott Rogers (University of Utah, Salt Lake City, UT). The  $\beta 2$  nAChR subunit-specific monoclonal antibody (mAb) 270 was produced from hybridoma stocks purchased from American Type Culture Collection (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  $\beta 2$  subunits in Western blots. Therefore, an nAChR  $\beta 2$  subunit-specific polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and used for Western blotting analysis.

**Tissues.** Frozen pineal glands and cerebral cortex from male Sprague-Dawley rats weighing 225 to 250 g were purchased from Zivic-Miller Laboratories (Portersville, PA) and stored at  $-80^\circ\text{C}$  until assayed.

**Primary Cell Culture.** Male Sprague-Dawley rats purchased from Taconic Farms (Germantown, NY) were group-housed with food and water ad libitum in a temperature- and light-controlled room ( $24^\circ\text{C}$ , lights on from 7:00 AM to 7:00 PM). At 30 to 35 days old (weighing 150 to 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 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 phosphate-buffered saline and incubated with gentle shaking for 10 min at  $37^\circ\text{C}$ . The tissues were triturated with a fire-polished pasture pipette and incubated for another 10 min at  $37^\circ\text{C}$ . 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 800g in culture media containing basal Eagle's medium supplemented with 10% fetal bovine serum, 25 mM glutamine, and 50  $\mu\text{g}/\text{ml}$  gentamycin. The dissociated pineal cells were resuspended in fresh culture media and plated onto poly-D-lysine coated coverslips in a 30-mm dish. Cultures were maintained at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  in a humidified incubator for 1 to 3 days. HEK 293 cells stably expressing the  $\alpha 3\beta 4$  nAChR (KX $\alpha 3\beta 4$  cells) and  $\alpha 3\beta 2$  nAChR (KX $\alpha 3\beta 2$  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  $\alpha 3\beta 4$  and  $\alpha 3\beta 2$  nAChRs were measured with [ $^3\text{H}$ ]EB. Tissues were homogenized with a Polytron homogenizer in 50 mM Tris-HCl, pH 7.4, and the homogenates were centrifuged at 35,000g for 10 min. Membrane pellets were washed twice and then resuspended in fresh buffer. Membrane aliquots ( $\sim 35$   $\mu\text{g}$  of protein) were incubated with [ $^3\text{H}$ ]EB ( $\sim 5$ –3000 pM) for 4 h at  $24^\circ\text{C}$  in a volume of 1 ml of Tris-HCl buffer. In competition binding experiments, a series of concentrations of each drug was incubated with  $\sim 500$  pM [ $^3\text{H}$ ]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  $\mu\text{M}$  (–)-nicotine, and specific binding was defined as the difference between total and nonspecific binding. In saturation binding experiments,  $B_{\text{max}}$  and  $K_d$  values were determined by nonlinear regression analysis (Prism software; GraphPad Software Inc., San Diego, CA). In competition binding experiments, inhibition curves and the  $\text{IC}_{50}$  values were determined by nonlinear regression analysis (GraphPad Prism). The affinities of drugs ( $K_i$  values) at nAChRs were calculated from the  $\text{IC}_{50}$  values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

**Autoradiography.** Autoradiography of  $^{125}\text{I}$ -EB and  $^{125}\text{I}$ - $\alpha$ -BTX binding sites was carried out in 20- $\mu\text{m}$  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  $\sim 500$  pM  $^{125}\text{I}$ -EB or  $\sim 5$  nM  $^{125}\text{I}$ - $\alpha$ -BTX and then rinsed, air-dried, and apposed to autoradio-

graphic film for 1 to 6 days. Nonspecific labeling was determined in the presence of 300  $\mu$ M nicotine.

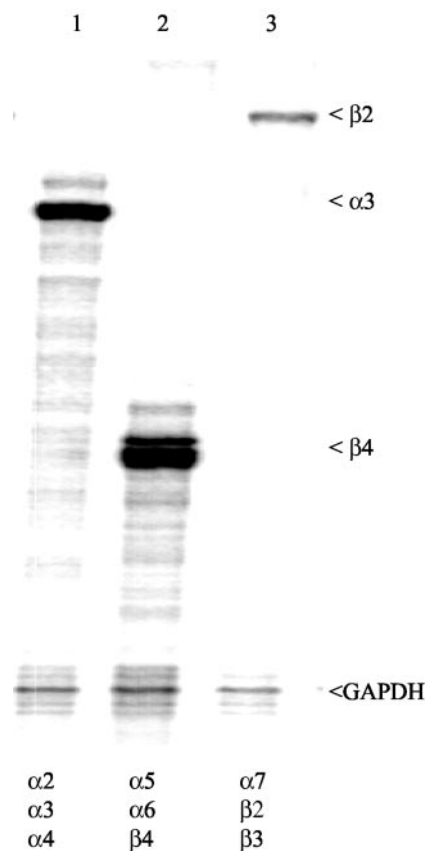
**Immunoprecipitation.** Rat pineal glands were prepared as for a radioligand binding assay, and the membrane pellet was resuspended in fresh buffer and incubated with  $\sim 3$  nM [ $^3$ H]EB for 2 h at room temperature. The tissue was then solubilized by the addition of Triton X-100 at a final concentration of 2% with gentle mixing for 2 h at room temperature. After solubilization, the mixture was centrifuged at 35,000g 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  $\beta 4$  subunit, affinity-purified antibody directed at the  $\alpha 3$  subunit, or an mAb directed at the  $\beta 2$  subunit. The  $\beta 4$  antiserum was tested at dilutions from 1:50 to 1:6 to determine the optimal concentration. The stock  $\alpha 3$  and  $\beta 2$  antibodies each contained 1  $\mu$ g/ $\mu$ l and were tested at dilutions from 2:100 to 15:100 to determine the optimal concentration. Tubes containing normal rabbit serum or an irrelevant monoclonal antibody served as controls. The mixtures were incubated for 4 to 16 h at 4°C with gentle rotation. After this incubation period, 50- $\mu$ l 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 14,000g for 30 s. The pellets were washed two times with 1 ml of Tris/EDTA buffer, dissolved in 400  $\mu$ l of 0.1 NaOH and 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 h. 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 described previously (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. In brief, total cellular RNA was isolated using RNA-STAT-60 (Tel-Test Inc., Friendswood, TX). Antisense riboprobes for the  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\beta 2$ ,  $\beta 3$ , and  $\beta 4$  nAChR subunits were generated from DNA templates using T7 RNA polymerase and [ $\alpha$ - $^{32}$ P]CTP. The RNase protection assays were carried out using the RPA II kit (Ambion, Austin, TX). Total RNA (20  $\mu$ g) from the tissue samples was hybridized overnight at 42°C with the subunit riboprobes and a riboprobe for rat GAPDH, which was used as an internal and loading control. Specific activities of [ $\alpha$ - $^{32}$ P]CTP used for synthesizing the probes of rat nAChR subunit genes and the probe of GAPDH were 800 and 40 Ci/mmol, respectively. After hybridization, nonprotected fragments were digested with a combination of RNase A and RNase T1 for 30 min at 37°C. The numbers of bases of the full-length probes and the protected fragments of the probes were as follows:  $\alpha 2$ , 416 and 332;  $\alpha 3$ , 306 and 230;  $\alpha 4$ , 496 and 408;  $\alpha 5$ , 411 and 380;  $\alpha 6$ , 462 and 396;  $\alpha 7$ , 450 and 376;  $\beta 2$ , 322 and 263;  $\beta 3$ , 430 and 394;  $\beta 4$ , 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  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ , and GAPDH; group 2 contained  $\alpha 5$ ,  $\alpha 6$ ,  $\beta 4$ , and GAPDH; and group 3 contained  $\alpha 7$ ,  $\beta 2$ ,  $\beta 3$ , 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 filmless autoradiographic analysis (PhosphorImager, 445 SI, Amersham Biosciences).

**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  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 5 mM glucose, and 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 1 ml/min. Ionic currents were monitored with an Axopatch 1-D patch amplifier (Axon Instruments Inc., Union City, CA). Recording patch electrodes were pulled from borosilicate glass capillaries to a resistance of 5 to 7 M $\Omega$  when filled with internal electrode solution containing 145 mM potassium gluconate, 5 mM EGTA, 5 mM  $\text{MgCl}_2$ , 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 or 3 days were noted. Drugs were applied using a gravity-fed Y-shaped tubing system positioned within 500  $\mu$ m of the cell under investigation. Drugs were applied and exchanged rapidly with an onset of <100 ms. For experiments requiring  $\alpha$ -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.



**Fig. 1.** Expression of nAChR subunit mRNA in the rat pineal gland. RNase protection assays were carried out as described under *Materials and Methods*. Total RNA from rat pineal gland was hybridized with a combination of  $^{32}$ P-labeled antisense probes corresponding to the nine rat nAChR subunit genes  $\alpha 2$ ,  $\alpha 3$ , and  $\alpha 4$  (lane 1);  $\alpha 5$ ,  $\alpha 6$ , and  $\beta 4$  (lane 2); and  $\alpha 7$ ,  $\beta 2$ , and  $\beta 3$  (lane 3). The probe corresponding to the GAPDH gene was used as an internal and loading control. The gel shown is representative of three to six independent experiments for the different subunits.



## Results

**Expressions of nAChR Subunit mRNAs in Rat Pineal Glands.** Expressions of mRNAs encoding nine nAChR subunits ( $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\beta 2$ ,  $\beta 3$ , and  $\beta 4$ ) 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 Fig. 1. The pineal gland expressed nAChR mRNAs encoding the  $\alpha 3$  (lane 1),  $\beta 4$  (lane 2), and  $\beta 2$  (lane 3) subunits only, and the intensity of the bands indicated that the expression levels of the  $\alpha 3$  and  $\beta 4$  subunit mRNAs were somewhat higher than that of the  $\beta 2$  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).

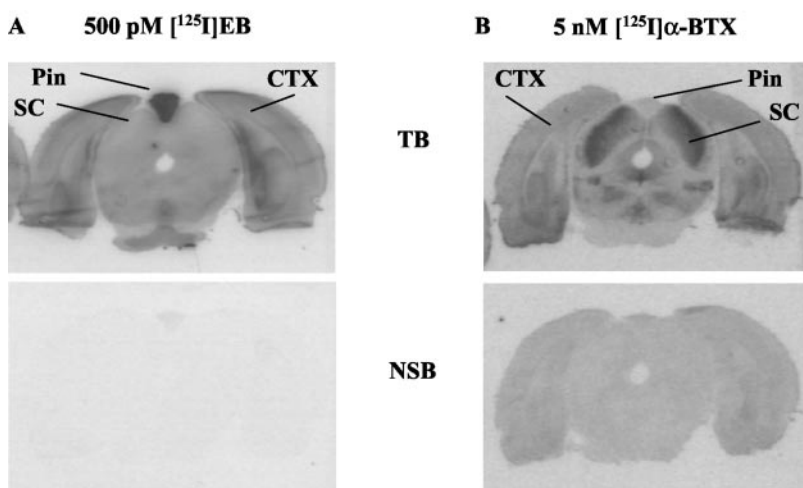
**nAChR Binding Sites in the Rat Pineal Gland.** Autoradiographic studies of nAChR binding sites in coronal brain sections at the level of the superior colliculus revealed dense  $^{125}\text{I}$ -EB binding sites in the pineal gland (Fig. 2A); in contrast, autoradiography of  $^{125}\text{I}$ - $\alpha$ -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  $\alpha 7$  receptors or other subtypes that bind  $^{125}\text{I}$ - $\alpha$ -BTX. As shown in Fig. 3, saturation binding measurements of  $^3\text{H}$ EB binding sites in membrane homogenates demonstrate that the pineal gland expresses a high density of nAChRs ( $\sim 300$  fmol/mg of protein) that is approximately five times the density found in the rat forebrain ( $\sim 60$  fmol/mg of protein). The  $^3\text{H}$ EB binding curves in the pineal fit a model for a single site, with a  $K_d$  of  $\sim 100$  pM.

From the nAChR subunit mRNA it expresses, the pineal could contain either an  $\alpha 3\beta 2$  or an  $\alpha 3\beta 4$  subtype, both subtypes, or a receptor composed of  $\alpha 3$  subunits in association with both  $\beta 2$  and  $\beta 4$  subunits (i.e., an  $\alpha 3\beta 2\beta 4$  subtype). To begin to determine the characteristics of the nAChR subtype(s) in the pineal gland, the affinities of drugs for the  $^3\text{H}$ EB-labeled sites were assessed in binding competition assays and compared with the affinities at defined  $\alpha 3\beta 4$  and  $\alpha 3\beta 2$  receptors heterologously expressed in HEK 293 cells. The agonists A-85380, cytosine, and nicotine all competed for  $^3\text{H}$ EB binding sites in the pineal gland with affinities between  $\sim 24$  and  $200$  nM, whereas the antagonist dihydro- $\beta$ -

erythroidine (DH $\beta$ E) displayed an affinity of  $\sim 65$   $\mu\text{M}$  (Fig. 4A and Table 1). These affinities are much closer to those found at the defined  $\alpha 3\beta 4$  receptor than at the  $\alpha 3\beta 2$  receptor, especially for A-85380 and DH $\beta$ E, two drugs that are good discriminators between these subtypes (Fig. 4, B and C; Table 1). In fact, the correlation line between the  $K_i$  values for the pineal and the  $\alpha 3\beta 4$  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  $\alpha 3\beta 4$  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  $\sim 3$  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  $\sim 1000$  times more potent than acetylcholine (Fig. 5, A and B; Table 2). Most of the agonists examined here, including cytosine, functioned as full agonists compared with acetylcholine, eliciting peak currents of 250 to 300 pA; the exception was DMPP, which seemed to be a partial agonist with approximately half the efficacy of acetylcholine (Fig. 5C). The receptors seemed to desensitize in a manner that was also concentration-dependent, but they recovered their sensitivity within approximately 2 min after removal of agonists (data not shown). At agonist concentrations  $\geq 500$   $\mu\text{M}$ , there was usually an obvious loss of receptor function, which probably reflects channel 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  $\text{EC}_{50}$  values for these six agonists to activate whole-cell currents via nAChRs in the pineal gland are very similar to their  $\text{EC}_{50}$  values to stimulate  $^{86}\text{Rb}^+$  efflux via defined  $\alpha 3\beta 4$  receptors heterologously expressed in HEK 293 cells. In fact, as shown in Fig. 6, comparison of the  $\text{EC}_{50}$  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



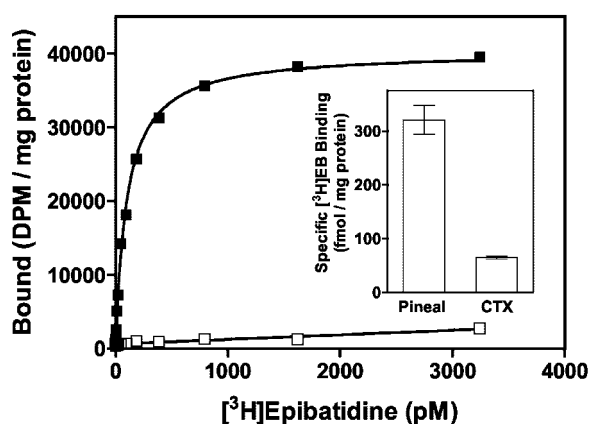
**Fig. 2.** Autoradiographic images of  $^{125}\text{I}$ -EB and  $^{125}\text{I}$ - $\alpha$ -BTX binding to rat pineal gland. Autoradiography was carried out as described under *Materials and Methods*. Coronal sections ( $20$   $\mu\text{m}$ ) of brain at the level of the superior colliculus with the pineal gland intact were mounted onto slides and incubated with  $500$  pM  $^{125}\text{I}$ -EB (A) or  $5$  nM  $^{125}\text{I}$ - $\alpha$ -BTX (B) to measure total binding (TB) to the sections. Nonspecific binding (NSB) was measured in parallel by incubating adjacent brain sections with the radioligands in the presence of  $300$   $\mu\text{M}$  nicotine. Slides were washed, dried, and apposed to autoradiographic X-ray film in cassettes for two to six days. CTX, cerebral cortex; SC, superior colliculus; Pin, pineal.

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  $\mu$ M nicotine alone followed at 2-min intervals by measurements after simultaneous application of 30  $\mu$ M nicotine and decreasing concentrations of mecamylamine, curare, or DH $\beta$ E in the same cell. Each of these antagonists blocked nicotine-activated currents (Fig. 7A), and the block was concentration-dependent (Fig. 7B). In contrast,  $\alpha$ -BTX, which is a highly selective  $\alpha 7$  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 min before application of nicotine (Fig. 7A).

As shown in Table 3, the  $IC_{50}$  values for the three 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  $\alpha 3\beta 4$  receptors heterologously expressed in HEK 293 cells. Moreover, for DH $\beta$ E, the only effective competitive antagonist examined here, its  $K_i$  value calculated from its  $IC_{50}$  to inhibit whole-cell 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 versus 22  $\mu$ M).

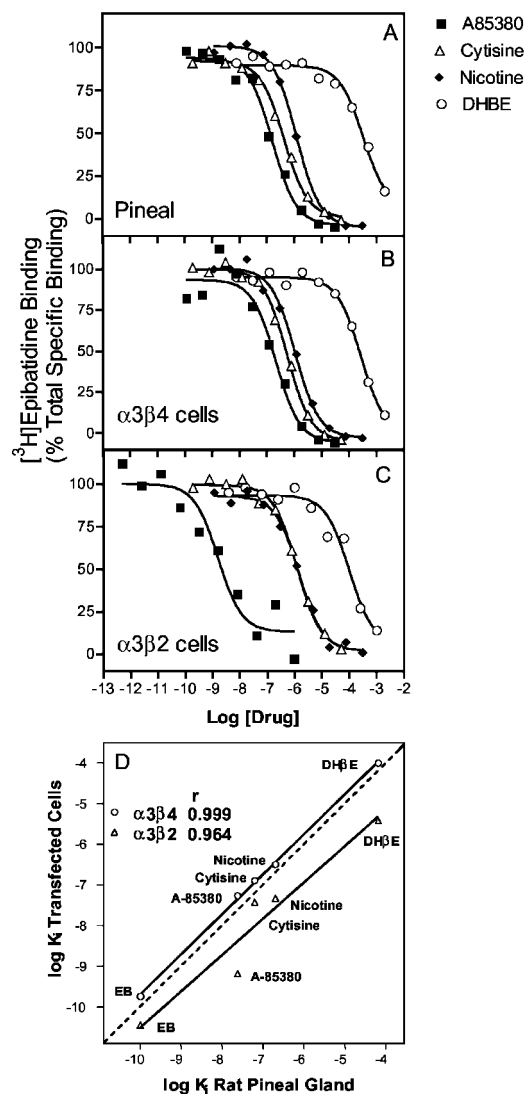
**Subunit Composition of nAChRs in Pineal Gland.** The pharmacological characteristics of the nAChRs in the pineal gland are consistent with an  $\alpha 3\beta 4$  receptor subtype, but pharmacology cannot prove subtype, which is defined by subunit composition. Therefore, we carried out immunoprecipitation assays to identify the subunits that comprise the pineal nAChRs. To do this, we used a polyclonal antibody selective for the  $\alpha 3$  subunit (Yeh et al., 2001), another directed at the  $\beta 4$  subunit (Flores et al., 1996), and the monoclonal antibody mAb 270, which is selective for the  $\beta 2$  subunit (Whiting and Lindstrom, 1987). As shown in Fig. 8, the polyclonal antibodies directed at the  $\alpha 3$  and  $\beta 4$  subunits each immunoprecipitated [ $^3H$ ]EB-la-



**Fig. 3.** Saturation Binding of [ $^3H$ ]EB to membrane homogenates from rat pineal gland. Binding curves represent specific binding (■) and nonspecific binding (□) of [ $^3H$ ]EB to membrane homogenates from rat pineal gland. Inset, a comparison of the  $B_{max}$  value for [ $^3H$ ]EB-specific binding to membrane homogenates from rat pineal gland versus 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 (GraphPad). 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  $103 \pm 15$  pM and  $321 \pm 37$  fmol/mg of protein, respectively.

beled nAChRs from detergent-solubilized extracts of pineal tissue in a concentration-dependent manner. In contrast, the  $\beta 2$  subunit-selective mAb270 did not immunoprecipitate any [ $^3H$ ]EB-labeled receptors from the pineal extracts (Fig. 8). To confirm that mAb270 was, in fact, capable of immunoprecipitating nAChRs that contained  $\beta 2$  subunits, it was tested in similarly prepared extracts from rat cerebral cortex, where it effectively immunoprecipitated the  $\alpha 4\beta 2$  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



**Fig. 4.** Comparison between ligand binding competition profiles of nAChRs in membrane homogenates from rat pineal gland and HEK 293 cells expressing  $\alpha 3\beta 4$  or  $\alpha 3\beta 2$  nAChRs. Competition of nicotinic drugs for [ $^3H$ ]EB binding sites in pineal gland (A),  $\alpha 3\beta 4$  cells (B), and  $\alpha 3\beta 2$  cells (C). The membrane homogenates were incubated with 500 pM [ $^3H$ ]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 three to six independent experiments. D, correlation between  $K_i$  values for nAChRs in the pineal gland and the  $\alpha 3\beta 4$  and  $\alpha 3\beta 2$  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 broken line represents the line of identity for the data and indicates that the pineal gland  $K_i$  values corresponded most closely to the  $\alpha 3\beta 4$  transfected cells. Data shown are the mean values of three to six independent experiments.

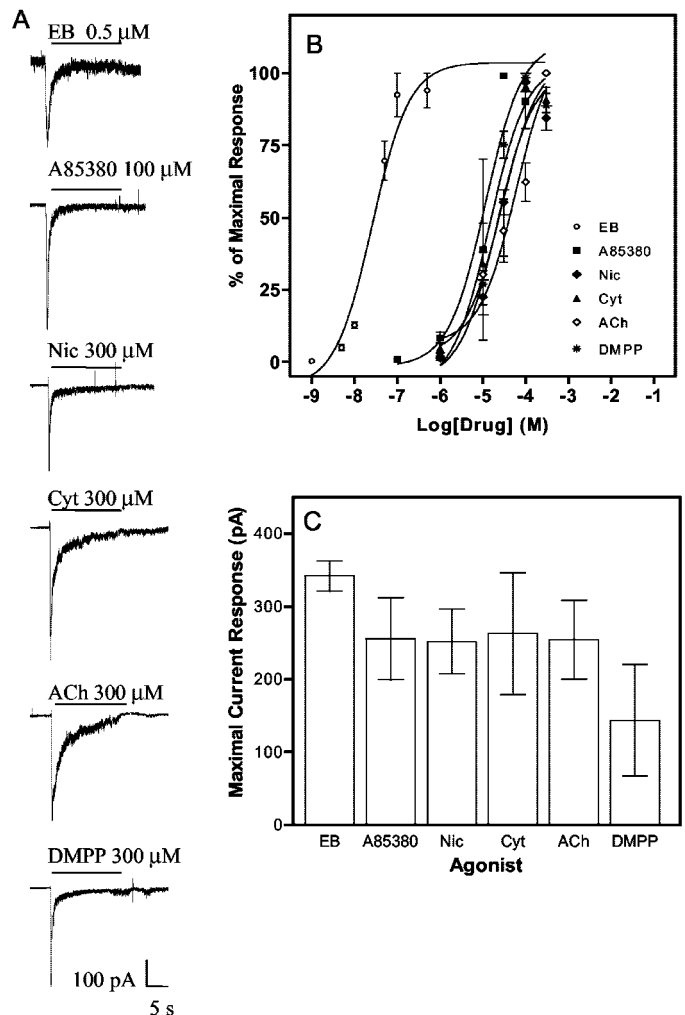
only with antibodies directed at  $\alpha 3$  and  $\beta 4$  subunits indicates that these two subunits probably comprise the nAChR in the pineal gland. To further test this, sequential immunoprecipitation assays were carried out to determine whether the  $\alpha 3$  and  $\beta 4$  subunits are physically associated with each other in the pineal gland. In these studies, [ $^3\text{H}$ ]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 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 Fig. 9. When the pineal extracts were first immunoprecipitated with the  $\alpha 3$  subunit-specific antibody, subsequent immunoprecipitations of the supernatant with the antibodies directed at the  $\alpha 3$ ,  $\beta 4$ , or  $\beta 2$  subunits yielded essentially no additional immunoprecipitation (Fig. 9A). Nearly identical results were found when the extracts were first immunoprecipitated with the  $\beta 4$  subunit-specific antisera (Fig. 9B). In contrast, the  $\beta 2$  subunit-specific mAb 270 antibody did not immunoprecipitate significant amounts of [ $^3\text{H}$ ]EB-labeled receptors from the pineal extracts; thus, after a first exposure to mAb 270, subsequent exposure to the antibodies directed at either the  $\alpha 3$  or  $\beta 4$  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  $\alpha 3$  and  $\beta 4$  subunits, but no signal was detected for the  $\beta 2$  subunit (data not shown).

## 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 eight  $\alpha$  and three  $\beta$  subunits known to be expressed in mammalian tissues, but because 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 in which the subunit composition of native nAChRs is known with some certainty. The  $\alpha 7$  subtype is identifiable in brain by its rapid kinetics and especially by its sensitivity to  $\alpha$ -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  $\alpha 4 \beta 2$  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 seem to contain both  $\alpha 3 \beta 2$  and  $\alpha 3 \beta 4$  receptors, possibly incorporating an  $\alpha 5$  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.



**Fig. 5.** Concentration response of different nicotinic agonists at nAChRs in dissociated rat pinealocytes. A, sample traces of currents activated by EB, A-85380, nicotine (Nic), cytosine (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 concentrations. B, concentration-response curves for currents activated by increasing concentrations of EB ( $\circ$ ), A-85380 ( $\blacksquare$ ), NIC ( $\blacklozenge$ ), Cyt ( $\blacktriangle$ ), ACh ( $\diamond$ ), and DMPP ( $\ast$ ). Increasing concentrations of each agonist were applied at two mini-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, peak currents activated by each drug. Data are the mean  $\pm$  S.E.M. for five to seven independent experiments.

TABLE 1

Comparison of  $K_i$  values of nAChR ligand binding in membrane homogenates from rat pineal gland,  $\alpha 3 \beta 4$  cells, and  $\alpha 3 \beta 2$  cells

Competition binding assays were carried out as described under *Materials and Methods* using  $\sim 500$  pM [ $^3\text{H}$ ]EB. The  $K_i$  values were calculated using the Cheng-Prusoff equation. Values shown are the mean  $\pm$  S.E.M. of three to six independent experiments.

Drug	$K_i \pm$ S.E.M.		
	Rat Pineal Gland	$\alpha 3 \beta 4$	$\alpha 3 \beta 2^a$
		nM	
A-85380	$24 \pm 2.8$	$54 \pm 4.8$	$0.21 \pm 0.04$
Cytisine	$63 \pm 11$	$128 \pm 15$	$37 \pm 9.0$
Nicotine	$202 \pm 26$	$318 \pm 42$	$47 \pm 11$
DH $\beta$ E	$64,833 \pm 22,190$	$98,700 \pm 22,124$	$3800 \pm 1300$

<sup>a</sup> Data for transfected cells expressing  $\alpha 3 \beta 2$  receptors were taken from Xiao and Kellar (2004).



as well as a somewhat weaker mRNA signal for the  $\beta 2$  subunit in the rat pineal gland. 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  $\beta 2$  subunits; in fact, we found no evidence for the

**A**

Nic 30  $\mu\text{M}$

Nic 30  $\mu\text{M}$

+ Mec 10  $\mu\text{M}$

+ Curare 100  $\mu\text{M}$

+ DH $\beta$ E 100  $\mu\text{M}$

+ 500 nM  $\alpha$ -BTX

100 pA

5 s

Comparison of EC<sub>50</sub> values of nicotinic agonists for activation of currents through nAChRs in rat pineal cells and stimulation of <sup>86</sup>Rb<sup>+</sup> efflux from α3β4-transfected cells

**B**

○ Mec  
□ Curare  
▼ DHBE

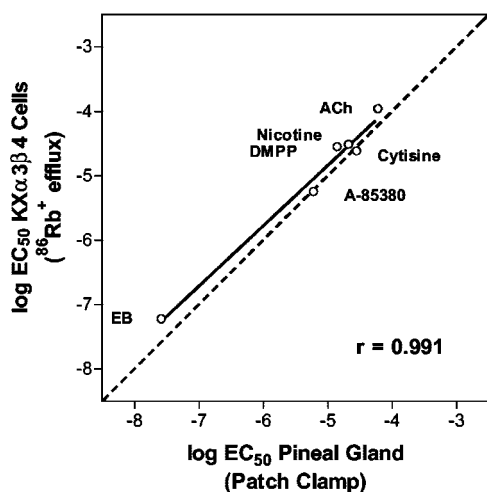
% of Control Current

Log [Antagonist] M

Log [Antagonist] M	Mec (% of Control Current)	Curare (% of Control Current)	DHBE (% of Control Current)
-8.0	98	-	-
-7.0	90	100	-
-6.0	52	92	100
-5.0	22	88	92
-4.5	18	55	70
-4.0	12	45	25
-3.5	8	10	10
-3.0	5	2	5

**Fig. 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  $\mu\text{M}$  nicotine in rat pinealocytes in the absence and presence of mecamylamine (Mec), curare, DH $\beta$ E, and  $\alpha$ -BTX. The cells first received an initial control application of 30  $\mu\text{M}$  nicotine followed by simultaneous application of 30  $\mu\text{M}$  nicotine plus the indicated concentration of antagonist, except for  $\alpha$ -BTX, which was present 2.5 min before and during the second nicotine application. **B**, concentration-response inhibition curves for Mec ( $\circ$ ), curare ( $\square$ ), and DH $\beta$ E ( $\blacktriangledown$ ). Before curve-fitting, the peak currents were normalized to the maximal current induced by 30  $\mu\text{M}$  nicotine and expressed as a percentage of control. Data were fit to a sigmoidal concentration-response relationship using GraphPad. Data shown are mean  $\pm$  S.E.M. of three to five independent experiments.

**Fig. 6.** Correlation between EC<sub>50</sub> values for agonist-stimulated whole-cell currents in pineal cells and agonist-stimulated <sup>86</sup>Rb<sup>+</sup> 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 broken line represents the line of identity and indicates a close correspondence between the EC<sub>50</sub> values derived from the two different types of assays.



presence of the  $\beta 2$  protein via Western blots. From the sensitivity of our binding and immunoprecipitation assays, we estimate we could have detected  $\beta 2$ -containing receptors if they constituted  $\sim 5\%$  or more of the pineal receptors. The presence of the  $\beta 2$  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. Therefore, pineal function and melatonin have been implicated in reproductive cycles, gonad size (in some species), and sleep-wake cycles

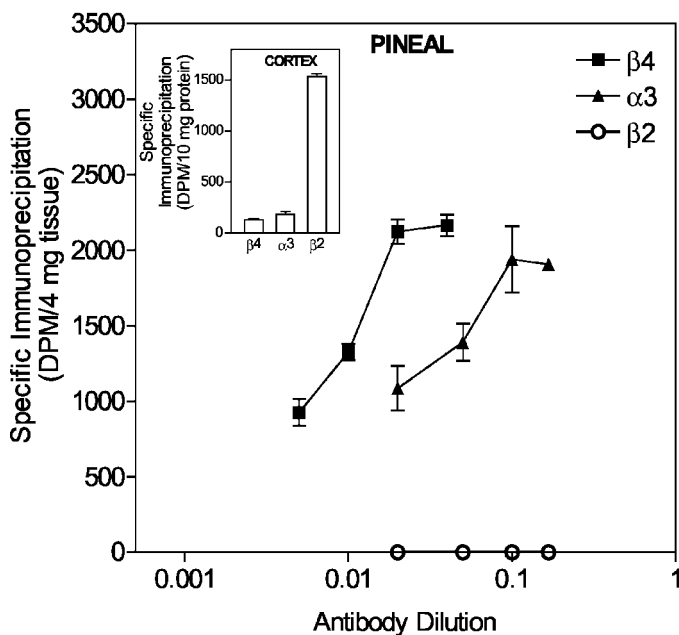
TABLE 3

Comparison of  $IC_{50}$  values of nicotinic antagonists for inhibition of nicotine (30 mM)-activated currents through nAChRs in rat pineal cells and inhibition of nicotine (100 mM)-stimulated  $^{86}Rb^+$  efflux from  $\alpha 3\beta 4$  cells

Dissociated pineal cells were prepared from  $\sim 30$ -day-old male Sprague-Dawley rats as described under *Materials and Methods*. See Fig. 5 for description of data analyses and curve-fittings of pineal whole-cell recordings. Values shown are the mean  $\pm$  S.E.M. from four to five whole-cell recordings and at least three  $^{86}Rb^+$  efflux assays.

Antagonist	$IC_{50} \pm$ S.E.M.	
	Rat Pineal Gland (Whole-Cell Patch Clamp)	$\alpha 3\beta 4$ HEK Cells <sup>a</sup> ( $^{86}Rb^+$ Efflux)
	$\mu M$	
Mecamylamine	$0.9 \pm 0.2$	$1.2 \pm 0.4$
Curare	$16 \pm 14$	$9.6 \pm 1.4$
DH $\beta$ E	$50 \pm 10$	$100 \pm 6$
	$(K_i = 20 \mu M \pm 4.0)$	$(K_i = 22 \mu M \pm 1.3)$

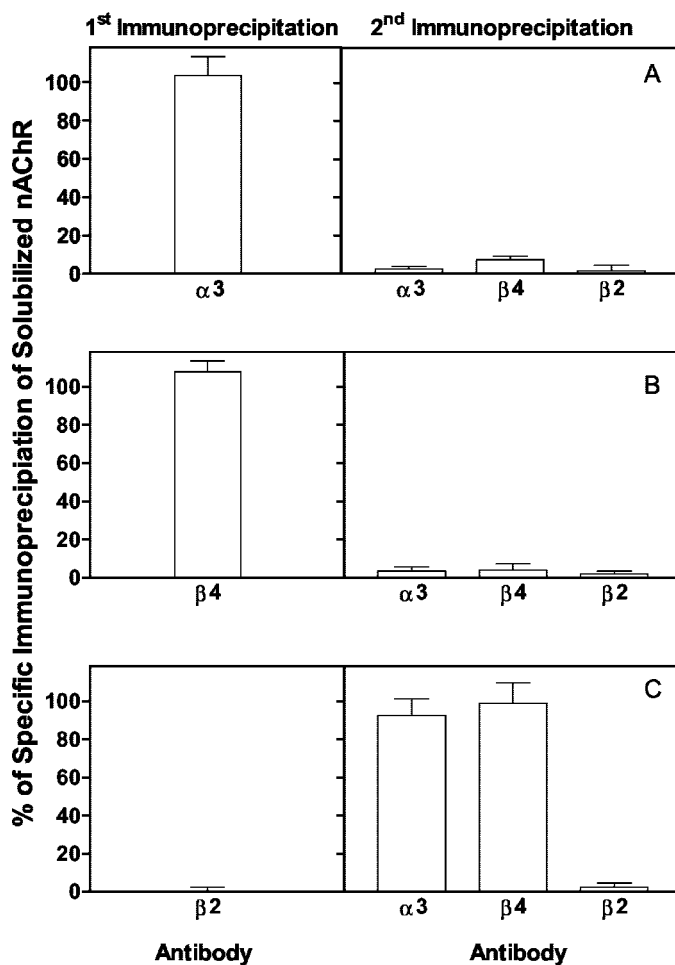
<sup>a</sup> Data for transfected cells expressing  $\alpha 3\beta 4$  receptors are from Xiao et al. (1998).



**Fig. 8.** Concentration-dependence of antibodies for immunoprecipitation of solubilized nAChRs from the rat pineal gland. Pineal gland membrane homogenates were solubilized, and [ $^3H$ ]EB-labeled nAChRs equivalent to 4 mg of rat pineal gland tissue was incubated with the indicated dilutions of anti- $\beta 4$  antiserum ( $\blacksquare$ ), anti- $\alpha 3$  antibody ( $\blacktriangle$ ), or anti- $\beta 2$  monoclonal antibody ( $\circ$ ) (mAb 270), and specific immunoprecipitation was measured as described under *Materials and Methods*. As shown, only the anti- $\alpha 3$  antibody and anti- $\beta 4$  antiserum immunoprecipitated [ $^3H$ ]EB-labeled nAChRs from the pineal gland. Inset, in rat cerebral cortex, in contrast to the pineal gland, the anti- $\beta 2$  monoclonal antibody immunoprecipitated [ $^3H$ ]EB-labeled nAChRs. Data shown are mean  $\pm$  S.E.M. of three independent experiments.

(including jet lag). The pineal gland seems 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  $\beta$ -adrenergic receptors, which leads to an increase in cAMP and activation of melatonin synthesis (Axelrod, 1974). Parasympathetic cholinergic axons to the pineal gland 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 gland from the medial habenula in some species (Schafer et al., 1998). Previous studies have found [ $^3H$ ]EB binding sites in the pineal gland, 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 the



**Fig. 9.** Association of  $\alpha 3$  and  $\beta 4$  subunits in the rat pineal gland nAChR. Sequential immunoprecipitations were performed as described under *Materials and Methods*. Aliquots of solubilized, [ $^3H$ ]EB-labeled nAChRs from rat pineal gland were incubated sequentially with anti- $\alpha 3$  antibody, anti- $\beta 4$  antiserum, or anti- $\beta 2$  monoclonal antibody (mAb 270). Initial immunoprecipitation with  $\alpha 3$  antibody (A),  $\beta 4$  antiserum (B), or  $\beta 2$  monoclonal antibody (C) was followed in each case by a second immunoprecipitation with  $\alpha 3$  antibody,  $\beta 4$  antiserum, or the  $\beta 2$  monoclonal antibody. Results are expressed as the percentage of specific immunoprecipitation of total solubilized receptors. Data are the mean  $\pm$  S.E.M. from three independent experiments.



inhibition of melatonin synthesis in rat pineal explants (Stankov et al., 1993; Yamada et al., 1998a). In an elegant series of studies, Yamada et al. (1996a,b, 1998a,b) established a link between pineal nAChRs, L-type calcium channels, glutamate release, metabotropic glutamate receptors, and inhibition of cAMP in a signaling pathway leading to the 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  $\text{Ca}^{2+}$  levels trigger exocytotic release of glutamate, which activates mGluR3 metabotropic glutamate receptors and leads to decreased cAMP. 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  $\text{EC}_{50}$  values for activation of whole-cell currents by nicotine and acetylcholine in dissociated pinealocytes are similar to the values reported for release of glutamate and inhibition of melatonin synthesis (Yamada et al., 1998a). Moreover, both nicotine-activated whole-cell currents (present data) and acetylcholine-induced release of glutamate (Yamada et al., 1998a) in pinealocytes are blocked by *d*-tubocurarine but not by  $\alpha$ -BTX.

A second major finding from our studies reported here is the remarkably close correspondence between the function of  $\alpha 3\beta 4$  nAChRs measured by whole-cell patch-clamp analysis in pinealocytes and  $^{86}\text{Rb}^{+}$  efflux in transfected HEK 293 cells that heterologously express these receptors. Thus, the  $\text{EC}_{50}$  values for the activation of whole-cell currents in pinealocytes by six nicotinic agonists were nearly indistinguishable from their  $\text{EC}_{50}$  values for the activation of  $^{86}\text{Rb}^{+}$  efflux measured in HEK 293 cells expressing the  $\alpha 3\beta 4$  nAChR subtype. Moreover, cytosine, which is a full agonist at  $\beta 4$ -containing receptors but a weak partial agonist at  $\beta 2$ -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  $\alpha 3\beta 4$  receptor (Meyer et al., 2001). Likewise, DMPP seems to be a partial agonist at the pineal receptors, as it is at the heterologously expressed  $\alpha 3\beta 4$  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 DH $\beta$ E derived from functional studies in pineal cells and the heterologously expressed  $\alpha 3\beta 4$  receptors are nearly identical.

Nevertheless, although the pharmacological evidence that the pineal nAChR is an  $\alpha 3\beta 4$  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  $\alpha 3\beta 2\beta 4$  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  $\alpha 3$  and  $\beta 4$  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  $\alpha 3$  and  $\beta 4$  subunits are physically associated. In contrast, the  $\beta 2$ -directed antibody did not immunoprecipitate nAChRs from the pineal extracts, although it

efficiently immunoprecipitated receptors from the cerebral cortex, which is known to contain nAChRs with the  $\beta 2$  subunit, namely the  $\alpha 4\beta 2$  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  $\alpha 3\beta 4$  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  $\alpha 3\beta 4$  nAChR require a native tissue that expresses a high level of receptor. The pineal gland is such a tissue and should be useful for this purpose.

#### Acknowledgments

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