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Estradiol increases $\alpha 7$ nicotinic receptor in serotonergic dorsal raphe and noradrenergic locus coeruleus neurons of macaques

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Abstract

Acetylcholine, acting on presynaptic nicotinic receptors (nAChRs) modulates the release of neurotransmitters in the brain. The rat dorsal raphe nucleus (DR) and the locus coeruleus (LC) receive cholinergic input and express the $\alpha 7$ nAChR. In previous reports, we demonstrated that estradiol (E) administration stimulates DR serotonergic and LC noradrenergic function in the macaque. In addition, it has been reported that E induces the expression of the $\alpha 7$ nAChR in rats. We questioned whether E increased the expression of the $\alpha 7$ nAChR in the macaque DR and LC. We utilized double immunostaining to study the effect of a simulated preovulatory surge of E on the expression of the $\alpha 7$ nAChR in the DR and the LC and to determine whether $\alpha 7$ nAChR colocalizes with serotonin and tyrosine hydroxylase (TH) in macaques. There was no difference in the number of $\alpha 7$ nAChR-positive neurons between ovariectomized (OVX) controls and OVX animals treated with a silastic capsule containing E (Ecap). However, supplemental infusion of E for 5–30 h to Ecap animals (Ecap+inf) significantly increased the number of $\alpha 7$ nAChR-positive neurons in DR and LC. In addition, supplemental E infusion significantly increased the number of neurons in which $\alpha 7$ nAChR colocalized with serotonin and TH. These results constitute an important antecedent to the study of the effects of nicotine and ovarian steroid hormones in the physiological functions regulated by the DR and the LC in woman.

Keywords

Nicotinic Receptors; Steroid Hormones; Acetylcholine; Serotonin; Noradrenaline

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels permeable to cations (Lindstrom, 2000). The different subtypes of nAChRs are defined by the composition of their subunits (Lukas et al., 1999). Seventeen nAChR subunit genes have been cloned from vertebrates: $\alpha 1$ to $\alpha 10$, $\beta 1$ to $\beta 4$, γ , ϵ and δ (Le Novère and Changeux, 1995; Elgoyhen et al., 2001). One of the most widely expressed nAChR in the central

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nervous system is constituted by five $\alpha 7$ subunits ($\alpha 7$ nAChR), and has a high affinity for α -bungarotoxin (α -BTX) (Seguela et al., 1993). Presynaptic nAChRs in the central nervous system modulate the release of neurotransmitters (Wonnacott, 1997) like ACh, glutamate (McGehee et al., 1995), serotonin (Seth et al., 2002) noradrenaline (NE) (Cucchiari and Commons, 2003; Yoshida et al., 1980), GABA (Alkondon et al., 2000) and dopamine (DA) (Champtiaux et al., 2003). However, postsynaptic nAChRs also play important roles; the most clearly demonstrated being the control of ganglionic transmission and fast $\alpha 7$ nAChR-mediated synaptic transmission in the hippocampus and in the sensory cortex (Jones et al., 1999).

There is evidence that brain expression of nAChRs may be induced by estradiol (E). There are higher basal levels of nAChRs in the cerebellum of female rats compared to males (Koyle et al., 1997). Furthermore, ovariectomy decreases (Miller et al., 1982) and administration of E increases the densities of α -BTX binding sites (Miller et al., 1984), suggesting that E induces the expression of the $\alpha 7$ nAChR in the rat hypothalamus. Moreover, the motivation to self-administer nicotine is greater in female rats than in males (Donny et al., 2000), suggesting a potential interaction of ovarian steroids with nicotine.

Cholinergic neurons of the pedunculopontine tegmental nucleus in the brainstem project widely to numerous targets, including the midbrain dorsal raphe nucleus (DR) and the brainstem locus coeruleus (LC) (Woolf and Butcher, 1989). Both, the DR and the LC express the $\alpha 7$ nAChR (Lena et al., 1999; Bitner and Nikkel, 2002; Vincler and Eisenach, 2003). Electrophysiological and pharmacological studies suggest that this receptor stimulates serotonin and NE secretion in the DR (Li et al., 1998), as well as NE secretion in the hippocampus (Vizi and Lendvai, 1999). Moreover, there is evidence that E modulates the stimulatory effect of ACh on serotonin release. Subcutaneous infusion of nicotine increased the concentration of serotonin in the ventromedial nucleus of the rat hypothalamus, but only in presence of E (Zhang et al., 2000). Serotonin neurons contain the β isoform of the E receptor (ER β) (Gundlach et al., 2001), and E regulates pivotal genes and proteins in serotonin neurons (Betha et al., 2002). The LC also expresses ER β (Pau et al., 1998), and E increases the expression of the mRNA for tyrosine hydroxylase (TH, the rate-limiting enzyme in the synthesis of NE) and the release of NE in the LC (Pau et al., 2000).

Hence, there is a body of evidence that both E and ACh modulate serotonin and NE neurotransmission. We questioned whether E could increase the sensitivity of serotonin and NE neurons to ACh by increasing the expression of the $\alpha 7$ nAChR. In the present study, we determined whether the expression of the $\alpha 7$ nAChR is altered by E treatment in serotonergic neurons of the DR and in NE neurons of the LC of female ovariectomized macaques using fluorescence-based immunocytochemistry.

MATERIALS AND METHODS

Animals

Ovarian intact rhesus macaques (*Macaca mulatta*), 7–12 years of age and 5.5–6.5 kg in body weight, were individually housed and maintained in quarters kept at temperatures between 21 °C and 25 °C with a 12 L:12 D photoperiod in accordance with NIH guidelines. Monkey chow (Ralston Purina, St. Louis, MO, USA) was provided twice daily and fresh fruit was provided once daily; fresh water was available *ad libitum*.

Experimental design

Eleven ovarian-intact animals were bilaterally ovariectomized (OVX) and immediately received a 3-cm silastic capsule (0.132 in inner diameter, 0.183 in outer diameter) containing either nothing (OVX, n=4) or crystalline estradiol (Ecap, n=7), implanted

subcutaneously. The E implants maintained circulating E levels between 30 and 120 pg/ml, similar to serum E levels during the mid-follicular phase of the monkey menstrual cycle. Four of the seven Ecap rhesus females were injected (1 ml) with 400 ng E/kg body weight followed by 5–30 h of continuous i.v. infusion (1 ml/h) of 320 ng E/kg body weight/h through a femoral vein catheter as described previously (Pau et al., 2000). This E infusion maintained circulating E levels near 300 pg/ml, and was designed to mimic the preovulatory surge of E. The circulating concentrations of E and LH in these animals were previously published (Pau et al., 1993). The E infusion elicited a LH surge at 24 h after initiation of the E infusion. Animals were killed at 0 (OVX and Ecap), 5, 10, 18 and 30 h after initiation of the E infusion (Ecap + 5–30 h inf, one animal per infusion time).

Tissue preparation

Brains were perfused through the ventricular/aorta with 1 liter of 0.9% sodium chloride at room temperature, followed by 3 liters of cold (4 °C) paraformaldehyde (4% in 0.1 M borate, pH 9.5). The midbrains and brainstems were post-fixed and cryoprotected in 20% sucrose-4% paraformaldehyde-0.1 M borate, pH 9.5, for 48–60 h at 4 °C with continuous gentle agitation before they were snap-frozen in a isopentane bath cooled to the temperature of dry ice-ethanol and stored at –80 °C. Frozen brainstems were sectioned on a microtome in a caudal to rostral direction from the caudal end of the LC to the caudal linear nucleus at the level of the substantia nigra. Sections were 20 microns in thickness and serial sections were collected in a cryoprotecting buffer (30% ethylene glycol, 20% glycerol in 0.05 M PBS) as described previously (Pau et al., 2000). Every sixteenth section through the largest extent of the DR and LC (at 320 μ m intervals) was selected for this study and care was taken to insure that there was similar representation of the areas from all animals. The region of the DR sampled began at the most rostral tip and extended caudally until the disappearance of the decussation of the cerebellar peduncles. Animals with larger brains yielded slightly more sections. Therefore, in the data analysis, the average number of cells per section was obtained to equalize the analysis. The minimal variance within the groups suggests that the morphology between animals was well matched. All sections were stored at –20 °C until they were double immunostained by specific antibodies against α 7nAChR and TH or α 7nAChR and serotonin.

Antibody characterization

To localize the α 7nAChR, we used the monoclonal antibody mAb306 (gift from Dr. Jon M. Lindstrom, University of Pennsylvania, Philadelphia, PA) that was generated in mice using affinite purified native chicken and rat α -BTX binding proteins (Schoepfer et al., 1990). Previous studies of epitope mapping reported that the antibody mAb306 recognizes the sequence corresponding to the cytoplasmic loop, residues 380–400, of the α 7 subunit from rat (DSGVVCGRLACSPTHDEHLMH) and chicken (DSGVICGRMTCSPTTEENLLH) (Schoepfer et al., 1990; McLane et al., 1992). The specificity of mAb306 was demonstrated initially in rat by Western blot of brain extract, resulting in the staining of a single band of 59 kD molecular weight (Dominguez del Toro et al., 1994).

However, the recent report of cross-reactivity in the α 7 knockout mouse raised new questions regarding specificity. Therefore, to further verify the specificity of mAb306 in macaques, we performed a Western blot on an extract of the DR and an adsorption control with a newly synthesized peptide corresponding to the antigenic site (DSGVVCGRLACSPTHDEHLMH). In addition, we examined α 7nAChR staining with a new commercial antibody and blocking peptide from Santa Cruz Biotechnology (Santa Cruz, CA).

For Western blot, a crude membrane fraction of the DR region from two monkeys was prepared and solubilized as previously described (Smith et al., 2004). The protein concentration of the solubilized membrane preparation was determined with the Bio-Rad protein determination reagent according to the method of Bradford (1976). Samples from one OVX and one Ecap animal containing 25 µg of membrane protein were dissolved in 5% sodium dodecyl sulfate (SDS) containing 4% beta-mercaptoethanol at 80 °C for 15 min and heated at 90 °C for 10 min before being loaded onto a 10% SDS polyacrylamide gel (Jule Biotechnologies). Molecular weight markers (Kalidescope, Biorad) were also included. Transfer to nitrocellulose was performed as published previously (Smith et al., 2004).

The nitrocellulose membrane was blocked in 8% aqueous Carnation nonfat dry milk (Nestle Food Company, Glendale, CA) for 90 min before incubating with the mouse monoclonal anti- $\alpha 7$ nAChR antibody mAb306 at a dilution of 1:500 in buffer containing 50 mM Tris and 150 mM NaCl (pH 7.5) at 4 °C overnight. The blots were rinsed in saline and 0.05% Tween-20 (Bio-Rad) and incubated with goat anti-mouse IgG (Sigma, St. Louis, MO) conjugated with horseradish peroxidase at a dilution of 1:5000 in 5% nonfat milk at room temperature for 60 min. Signal was detected by exposing the blot to chemiluminescent film after developing in Supersignal substrate reagents (Pierce, Rockford, IL) followed by exposure to Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY). The bands on the film were captured using an XC-77 CCD video camera (Sony, Towada, Japan) and a framegrabber board on a Mac G4.

To further verify the specificity of the anti- $\alpha 7$ nAChR antibody in macaques, an adsorption control with a new antigenic peptide was executed. The peptide antigen to which the antibody is directed was synthesized (Global Peptides, Fort Collins, CO) using the sequence corresponding to the residues 380–400 of the rat $\alpha 7$ subunit (DSGVVCGRLACSPTHDEHLMH).

Then, 1 µl of the anti- $\alpha 7$ subunit antibody mAb306 was incubated overnight with either nothing (non-adsorbed) or 1 mg of the blocking peptide in 975 µl of potassium phosphate-buffered saline (KPBS). Sections of one Ecap+inf macaque were washed in 0.02 M KPBS and exposed for 30 min in a 0.1% hydrogen peroxide solution to eliminate endogenous peroxidase activity. After blocking of nonspecific binding sites with 2% normal donkey serum (NDS, Jackson ImmunoResearch Laboratories, West Grove, PA) in 0.4% Triton X-100 for 1 h, sections were incubated for 48 h at 4 °C with either the non adsorbed or the pre-adsorbed anti- $\alpha 7$ subunit antibody mAb306 plus 20 µl NDS and 4 µl Triton X-100. Additional, control sections were processed using BSA (1 mg/ml) in substitution of the primary antibody. Following KPBS washes, sections were incubated in biotin-SP donkey anti-mouse IgG (1:200 in 0.4% Triton X-100, Jackson ImmunoResearch Laboratories) for 1 h at room temperature. Tissues were then incubated for 1 h in avidin-biotin-HRP complex (1:50 in 0.4% Triton X-100, Vector Laboratories, Burlingame, CA). Visualization was performed using 0.5% diaminobenzidine in 0.02 M KPBS as the chromogen and 0.003% hydrogen peroxide as the substrate, which produces a brown precipitate. Sections were mounted onto Fisher superfrost slides (Fisher Scientific), dehydrated, cleared with Hemo-De (Fisher Scientific) and coverslipped with DPX (Electron Microscopy Sciences, Washington, PA). Sections were examined using a Zeiss Axioplan microscope equipped with bright field illumination and images were captured using a Cool Snap digital camera linked to a Micron PC/Pentium 3 computer.

Evaluation of $\alpha 7$ nAChR expression using the $\alpha 7$ nAChR antibody sc-1447

To further corroborate the expression of the $\alpha 7$ nAChR in the areas of interest, we performed an immunofluorescence study using the $\alpha 7$ nAChR antibody sc-1447 (Santa Cruz

Biotechnology, Santa Cruz, CA) in the presence and absence of blocking peptide provided by Santa Cruz.

The antibody sc-1447 is an affinity purified goat polyclonal antibody raised against a peptide mapping at the C-terminus of the $\alpha 7$ nAChR of human origin. The epitope recognized by this antibody was determined by Edmand sequencing of the Santa Cruz blocking peptide (Global Peptides) and corresponds to the residues 482–500 of the $\alpha 7$ nAChR of human origin (PubMed accession #NP000737; CTIGILMSAPNFVEAVSKDF). The antiserum stains a single band of approximately 70 kD molecular weight on Western blot (mouse and rat brain SDS extracts electrophoresed through 10% SDS-PAGE gels and electrophoretically transferred to PVDF membranes under reducing conditions by Santa Cruz). All staining was abolished when the primary antibody was preincubated with the immunizing peptide.

Free-floating sections from the LC and DR of one Ecap+inf macaque were washed in 0.02 M KPBS and nonspecific tissue antibody binding sites were blocked by incubating the tissue in 2% NDS in 0.4% Triton X-100 for 1 h at room temperature. Sections were then incubated with a cocktail containing the goat polyclonal anti- $\alpha 7$ -subunit antibody sc-1447 at a dilution of 1:100 in 2%NDS in 0.4% Triton for 48 h at 4 °C. The antibody sc-1447 recognizes the sequence corresponding to the residues 482–500 of the human $\alpha 7$ subunit. After KPBS washes, sections were incubated with Alexa Fluor 488 donkey anti-goat IgG (Molecular Probes, Eugene, OR) at a dilution of 1:200 in 0.4% Triton X-100 for 1 h at room temperature and darkness. Sections were mounted on slides and coverslipped with PVA-DABCO solution (2.5% polyvinyl alcohol-1,4-diazabicyclo[2.2.2]octane in glycerol) (Valnes and Brandtzaeg, 1985).

Control sections were processed by incubating the tissue with the antibody that was pre-adsorbed with a five-fold (by weight) excess of blocking peptide (sc-1447P, Santa Cruz Biotechnology). Additional negative controls were processed by omitting the primary antibody.

$\alpha 7$ nAChR/serotonin and $\alpha 7$ nAChR/TH double immunofluorescence

Double immunofluorescence was performed to determine whether serotonin or NE neurons express the $\alpha 7$ nAChR. Free-floating sections were washed in 0.02 M KPBS and nonspecific tissue antibody binding sites were blocked by incubating the tissue in 2% NDS in 0.4% Triton X-100 for 1 h at room temperature. For serotonin determination, the tissue was incubated after the NDS, with 3% BSA for 1 h. Sections were then incubated with a cocktail containing the mouse monoclonal anti- $\alpha 7$ -subunit antibody mAb306 at a dilution of 1:1000, and either the rabbit polyclonal anti-serotonin antibody S5545 (Sigma) at a dilution of 1:1000, or the sheep polyclonal anti-TH antibody AB1542 (Chemicon International, Temecula, CA) at a dilution of 1:2000 in 2% NDS for 48 h at 4 °C. After KPBS washes, sections were incubated with a cocktail of Alexa Fluor 488 donkey anti-mouse IgG and either Alexa Fluor 594 donkey anti-rabbit or Alexa Fluor 594 donkey anti-sheep IgG (Molecular Probes) at a dilution of 1:200 in 0.4% Triton X-100 for 1 h at room temperature and darkness. Following KPBS washes, the tissue was counterstained with Hoechst 3342, trihydrochloride, trihydrate (Molecular Probes). Sections were mounted on slides and coverslipped with PVA-DABCO solution.

Control sections were processed by omitting the primary antibody, and tissue sections from all animals were immunostained together to ensure that the immunocytochemistry was performed identically for all the groups.

Data processing and statistics

The slides were coded so that the status (OVX, Ecap and Ecap + 5–30 h inf) of the subjects was unknown to the investigator. Sections were examined using a Zeiss Axioplan microscope equipped with fluorescence filters. Serotonin and TH immunoreactivity was identified in the perikarya with the TRITC filter (EX=528–553, DM=565, BA=600–660) as a red immunofluorescence in the cytoplasm. $\alpha 7$ nAChR was identified in the perikarya with the FITC filter (EX=465–495, DM=505, BA=515–555) as a green fluorescence in the cytoplasm. The cell nucleus was identified with the DAPI filter (EX=330–380, DM=400, BA=435–485) as a blue immunofluorescence in the nucleus. Immunofluorescence images (TH or serotonin in red, $\alpha 7$ nAChR in green, and cell nuclei in blue) across the extent of the DR and LC were captured using a Cool Snap digital camera linked to a Micron PC/Pentium 3 computer. The settings for image capture were standardized by subtraction of background from the control tissue sections (none showed any significant level) and the same exposure time was used for all the groups. Each image was enlarged and the cells were counted from the images on the computer screen. For illustration, images were optimized by adjusting only the brightness and contrast, to the same level in all the groups, before they were overlaid using Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, CA). Of note, the immunofluorescence is not visible at lower magnifications, thus our ability to show the entire DR required construction of a collage of numerous higher magnification photographs. A collage of sequential overlaid pictures of $\alpha 7$ nAChR+serotonin double-labeled neurons in one section of the DR of two representative monkeys was constructed using the software Adobe Illustrator 8.0 (Adobe Systems Inc., San Jose, CA).

The cytoarchitectonic identification of brainstem regions was based on the stereotaxic atlas of the monkey brain (Paxinos et al., 2000). The number of single-labeled and double-labeled cells was counted bilaterally in the two areas that also correspond to the following plates of the monkey atlas: plates 80–84 for the DR/B7-B6 (rostral areas), and plates 89–97 for the LC/A6. Each area was represented and counted manually bilaterally in 9 ± 3 sections of the DR/B7-B6 and 16 ± 2 sections of the LC/A6 as described previously (Centeno et al., 2004). The area counted in the DR was approximately 640 mm^2 , and the area counted in the LC area was approximately 770 mm^2 . The area sampled was the same in all groups, and was controlled with the aid of an ocular micrometer. The distance between adjacent sections was $320 \mu\text{m}$. Thus, it is unlikely that the same cell would be represented in two sections. We observed that some cells differed in the intensity of immunolabeling, which may indicate an increment in the expression of the antigen. However, in this work we considered only the number of cells that expressed the antigen and not the density of antigen. The cell was counted as a positive cell if immunostaining (lightly or darkly) was present in the cytoplasm, even if the cell nucleus was not visible in the plane of section. The total number of cells expressing each marker or markers was counted on each section. Then, the section counts were averaged for each animal. Finally, the individual animal means were averaged to obtain the group mean. Thus, the within group variance represents the difference between animals. Changes in cell numbers among groups (OVX, Ecap and Ecap + 5–30 h inf) were determined by one-way analysis of variance (ANOVA), with post-hoc comparison of group means in the Tukey test. Significance was accepted with a 95% confidence level.

RESULTS

Specificity of the anti- $\alpha 7$ subunit antibody mAb306

A recent report that mAb306 cross-reacts with a protein in the $\alpha 7$ nAChR knockout mouse raised a question regarding its specificity. However, a similar observation was made in the serotonin transporter knock out mouse that was later shown to be a truncated nonfunctional transporter protein (Ravary et al., 2001). Nonetheless, to confirm that the mAb306

recognizes only the $\alpha 7$ nAChR subunit in the macaque, we initially performed Western blot analysis on the solubilized membrane fraction of extracts of the DR region from OVX and Ecap animals. Figure 1 shows that the antibody mAb306 stains only one robust band at approximately 60 kDa molecular weight in both animals with no marked difference between treatments.

To further corroborate the antibody specificity in immunocytochemistry, we contracted for the synthesis of a peptide corresponding to the reported antigenic epitope of mAb306 (Global Peptides). We then pre-adsorbed the antibody mAb306 with this peptide. Figure 2 illustrates the immunostaining for $\alpha 7$ nAChR obtained after incubating sections of one Ecap +inf (5 h) macaque with non-adsorbed antibody (Figure 2A) and the immunoreactivity obtained after exposing the tissue to the antibody pre-adsorbed with 1 mg of its blocking peptide (Figure 2B), versus incubation with BSA instead of the antibody mAb306 (Figure 2C). All staining was abolished when 1 μ l of the diluted primary antibody mAb306 was pre-incubated with 1 mg of the immunizing peptide.

Distribution of $\alpha 7$ nAChR in the monkey midbrain and brainstem

With antibody mAb306, we observed that $\alpha 7$ nAChR immunoreactivity was widely distributed among several midbrain and brainstem areas. Figure 3 contains immunofluorescent images of different representative areas of one Ecap+inf monkey (10 h) that expressed the receptor. The dorsal nucleus of the inferior olive (IOD) localized at the level of the lateral paragigantocellular nucleus (LPGi) showed some positive cells, but not the rostral medial accessory olive (RMAO, Figure 3A). $\alpha 7$ nAChR immunoreactivity was also observed in the oral dorsal paramedian nucleus (ODPMn) (Figure 3B) and the lateral parabrachial nucleus (LPB), indicated at the level of the superior cerebellar peduncle (scp, Figure 3C). All the levels of the locus LC showed numerous $\alpha 7$ nAChR-positive cells, as shown in Figure 3D, which represents this area at the level of the mesencephalic 5 tract (me5). In a similar manner, all the levels of the DR exhibited numerous $\alpha 7$ nAChR-positive cells. Figure 3E shows an image of the DR at the level of the trochlear nucleus (4N), which also showed $\alpha 7$ nAChR immunoreactivity (Figure 3F).

The same immunoreactivity pattern was observed using the polyclonal antibody anti- $\alpha 7$ nAChR, sc-1447 (Santa Cruz Biotechnology). Figure 4 shows immunofluorescent images of the LC (4A) and DRN (4B) of one Ecap+inf monkey, followed by their respective pre-adsorbed controls (4C and D). The $\alpha 7$ nAChR immunostaining is completely blocked by pre-adsorption of the antibody with the antigenic peptide. The sequence of the blocking peptide was determined by Global Peptides, and corresponded to amino acids 482–500 of the C-terminus of the $\alpha 7$ nAChR of human origin.

Effect of E on the expression of $\alpha 7$ nAChR and serotonin on the monkey DR

Statistical variance within the infused animals was minimal and significantly less ($p > 0.1$) than the variance when compared with the non-infused animals ($p < 0.01$). Hence, the Ecap + 5–30 h inf animals were grouped together (Ecap+inf) and compared to OVX and Ecap animals with ANOVA.

Figure 5 illustrates the distribution of $\alpha 7$ nAChR, 5-HT and $\alpha 7$ +5-HT double-labeled neurons in one section of the rostral DR of one representative OVX (Figure 5A) and one Ecap+inf monkey (10 h, Figure 5B). The location of the area that was subjected to cell counting is outlined. No apparent difference in the number of $\alpha 7$ nAChR-positive cells was observed between treatment groups in other areas adjacent to the DR, like the 4N or the ventrolateral periaqueductal gray (VLPAG).

Figure 6 illustrates double-immunofluorescent images showing $\alpha 7$ nAChR (green) and serotonin (5-HT, red) immunoreactivity in OVX (Figure 6A–C), Ecap (Figure 6D–F) and Ecap+inf (Figure 6G–I) female macaques. About 70% of 5-HT-positive cells in OVX and Ecap animals also expressed the $\alpha 7$ nAChR (Figure 5 and 6, shown in yellow). The mean number of neurons expressing $\alpha 7$ nAChR or $\alpha 7$ nAChR+5-HT (80% of serotonergic neurons) was higher ($p<0.01$ and $p<0.05$, respectively; ANOVA and Tukey's post-hoc pairwise comparison) in the Ecap+inf group compared to the OVX and Ecap groups (Figure 8)

There was a significant difference in the number of $\alpha 7$ nAChR-positive neurons with treatment ($p<0.0013$, ANOVA and Tukey's post-hoc pairwise comparison). The number of neurons expressing $\alpha 7$ nAChR equaled 355 ± 44 in the OVX group and 397 ± 23 in the Ecap group (ANOVA and Tukey's, not different). However, the number of $\alpha 7$ nAChR-expressing cells increased to 593 ± 15 in the Ecap+inf group ($p<0.01$, ANOVA and Tukey's post-hoc pairwise comparison). The number of serotonin-positive neurons equaled 222 ± 26 , 262 ± 34 and 305 ± 16 in the OVX, Ecap and Ecap+inf groups, respectively (ANOVA and Tukey's, not different). There was a significant difference in the number of neurons co-expressing $\alpha 7$ nAChR and 5-HT with treatment ($p<0.0248$, ANOVA and Tukey's post-hoc pairwise comparison). The number of $\alpha 7$ nAChR+5-HT neurons equaled 163 ± 20 in the OVX group and 188 ± 16 in the Ecap group (ANOVA and Tukey's, not different). However, the number of $\alpha 7$ nAChR+5-HT neurons increased to 245 ± 14 in the Ecap+inf group ($p<0.05$, ANOVA and Tukey's post-hoc pairwise comparison). As shown in Figure 5, there was also an apparent increase in the number of serotonin-positive fibers between OVX and Ecap+inf groups, however this was not quantified. Neighboring areas were not counted, although we did not observe any obvious differences between the groups (see VLPAG and 4N in Figure 5).

Effect of E on the expression of $\alpha 7$ nAChR and TH on the monkey LC

The number of immunoreactive $\alpha 7$ nAChR ($p<0.0293$, ANOVA and Tukey's post-hoc pairwise comparison) or $\alpha 7$ +TH ($p<0.05$, ANOVA and Tukey's post-hoc pairwise comparison) neurons was higher in the A6 noradrenergic area of the LC (LC/A6) after E infusion (Figure 7 and 8) as compared with non-infused animals. The number of neurons expressing $\alpha 7$ nAChR was 63 ± 4 in the OVX group and 45 ± 1 in the Ecap group (ANOVA and Tukey's, not different). However, the number of $\alpha 7$ nAChR-expressing cells increased to 96 ± 19 in the Ecap+inf group ($p<0.05$, ANOVA and Tukey's post-hoc pairwise comparison). The number of TH-positive neurons equaled 65 ± 1 , 72 ± 4 and 65 ± 6 in the OVX, Ecap and Ecap+inf groups, respectively (ANOVA and Tukey's, not different). The number of $\alpha 7$ nAChR+TH neurons equaled 43 ± 3 in the OVX group and 32 ± 0.25 in the Ecap group (ANOVA and Tukey's, not different). However, the number of $\alpha 7$ nAChR+TH neurons increased to 60 ± 10 in the Ecap+inf group ($p<0.05$, ANOVA and Tukey's post-hoc pairwise comparison). There was no difference in the number of single- or double-labeled neurons in the OVX and Ecap animals. 50% of TH-positive neurons in the OVX and Ecap groups and 90% in the Ecap+inf group expressed $\alpha 7$ nAChR. There was no difference in the number of TH-positive neurons in any group and there was no apparent difference in the number of positive cells at the different levels of the LC.

DISCUSSION

The goal of this work was to determine the effect of E treatment on the expression of the $\alpha 7$ nAChR in serotonergic neurons of the DR and in noradrenergic neurons of the LC in OVX monkeys. We found that administration of E in a concentration similar to that present in the preovulatory phase of the monkey menstrual cycle, significantly increased the number of serotonergic, noradrenergic and other phenotypically un-identified neurons that express the $\alpha 7$ nAChR in the DR and LC of OVX macaques.

The two main nAChRs in the brain are the $\alpha 4\beta 2$ and the $\alpha 7$. The $\alpha 7$ nAChR has a high affinity for α -BTX and it is widely distributed throughout the nervous system. In this study, we used a monoclonal antibody that recognizes the $\alpha 7$ subunit, named mAb306. A recent report showing cross-reactivity of the mAb306 in a $\alpha 7$ nAChR knockout mouse raised the possibility that this antibody exhibits non-specific binding (Herber et al., 2004). Thus, to ensure that mAb306 does not cross-react with proteins other than the $\alpha 7$ nAChR subunit in the rhesus macaque brain; we (1) examined a solubilized membrane fraction of the monkey DR region on Western blot and found one robust band, (2) blocked mAb306 labeling with a newly synthesized peptide corresponding to the published antigenic epitope of mAb306, (3) duplicated the staining pattern of mAb306 with a commercial antibody against $\alpha 7$ nAChR, (4) completely blocked the staining of the commercial antibody with its blocking peptide, and (5) sequenced the commercial blocking peptide and determined that it corresponds to 18 amino acids in the C-terminal region of human $\alpha 7$ nAChR.

We observed one clear band at approximately 60 kD molecular weight indicating that mAb306 does not bind to additional proteins present in our tissue. Moreover, the molecular weight of the $\alpha 7$ subunit ranges in size from 45 to 70 kD, depending on the species studied. There are no reports of the molecular weight of the macaque $\alpha 7$ nAChR, but the one band in our monkey DR extracts recognized by mAb306 migrated at a molecular weight similar to that reported for the rat $\alpha 7$ nAChR (Dominguez del Toro et al., 1994), further suggesting that the band recognized by mAb306 corresponds to the $\alpha 7$ nAChR. Pre-adsorption of mAb306 with a newly synthesized antigenic peptide reduced the intensity of immunolabeling in E-treated monkey DR sections to a level observed in the absence of antibody. Finally, the commercial antibody to $\alpha 7$ nAChR yielded the same pattern of staining as mAb306 and this staining was blocked by the respective antigenic peptide, which upon sequencing maps to the C-terminal of the human receptor. Together, these results indicate that mAb306 recognizes one protein, which is likely $\alpha 7$ nAChR, in the macaque brain.

We found that the $\alpha 7$ nAChR is expressed in several areas, the DR and LC included, of the monkey midbrain and brainstem. This observation is consistent with previous reports of the distribution of this receptor in the brain of several species (Dominguez del Toro et al., 1994; Lena et al., 1999; Quik et al., 2000; Bitner and Nikkel, 2002; Vincler and Eisenach, 2003; Tribollet et al., 2004). The expression of the $\alpha 7$ nAChR appears to be regulated by E. In rats, ovariectomy reduces α -BTX binding sites in the suprachiasmatic nucleus (Miller et al., 1982), and the reduction was reversed by E replacement (Miller et al., 1984). In addition, it has been demonstrated that E plays an important role in the hypothalamic expression of nAChRs during development (Block and Billiar, 1979) and puberty (Morley et al., 1983). In this work, we analyzed the effect of E replacement in OVX macaques on the number of serotonin and NE neurons that express the $\alpha 7$ nAChR in the DR and LC, respectively. We found that the number of serotonin and NE neurons expressing the $\alpha 7$ nAChR increased only after E infusion for 5 to 30 h in a concentration that mimics the preovulatory surge of E, but not in OVX animals with an implant of E. This result suggests that E, in a concentration equivalent to that present in the preovulatory phase of the monkey menstrual cycle, may be participating in the stimulation of the expression of the $\alpha 7$ nAChR in serotonergic and NE neurons of the monkey DR and LC, respectively.

In the present work, we observed an increase in serotonergic fibers after E infusion that was not quantified. However, previous work indicates that E increases serotonin synthesis in the DR (Bethea et al., 2002), which may be reflected by the increase in serotonin fiber staining. Also consistent with an earlier report (Bethea, 1994), there was no difference in the number of serotonin-positive neurons with this paradigm of E treatment.

In other studies, we observed that E caused an increase in the mRNA for TH, determined by in situ hybridization (Pau et al., 2000). In this work, we did not find differences in the number of cells expressing the TH protein. Thus, the increase in TH mRNA may be due to regulation of TH expression within the LC neurons and not due to cell recruitment.

It is interesting that the expression of the $\alpha 7$ nAChR is induced by E infusion as early as 5 h after the treatment was initiated. There are no reports of time-related changes in the expression of the $\alpha 7$ nAChR with E treatment. However, we reported previously that coitus induced the expression of nAChRs in rabbits one hour after mating (Centeno et al, 2004). Whether this expression is induced by E or ACh activity is unknown. Other rapid changes in expression have been noted. For example, acute intermittent nicotine treatment induced fibroblast growth factor-2 mRNA and protein in the rat brain 4 h after the first injection (Belluardo et al., 1998). The molecular mechanism involved in the induction of $\alpha 7$ nAChR by E infusion remains to be elucidated and is the aim of future experimentation.

The DR and LC receive cholinergic innervation from the pedunculopontine and laterodorsal tegmental nuclei (McGeer et al., 1986; Woolf and Butcher, 1989), and it has been demonstrated that both the DR and LC express the $\alpha 7$ nAChR in the rat (Bitner and Nikkel, 2002). Presynaptic $\alpha 7$ nAChRs have been implicated in the modulation of neurotransmitter release, by increasing calcium influx in a concentration sufficient to stimulate vesicular neurotransmitter release in presynaptic terminals from the hippocampal mossy-fibers (Gray et al., 1996). It is possible that $\alpha 7$ nAChRs expressed by serotonin neurons in the monkey DR play an important role in the regulation of serotonin release controlled by ACh. There is evidence that serotonin released from the DR is controlled by ACh acting on the $\alpha 7$ nAChR (Li et al., 1998; Tucci et al., 2003). In earlier studies, an interaction between brainstem ACh and serotonin neurons was implicated in the control of sleep cycles (Steriade and McCarley, 1990). Zhang et al. (2000) reported that infusion of nicotine increases the concentration of serotonin in the ventromedial nucleus of the hypothalamus in sham-operated but not in OVX rats, suggesting that the stimulatory effect of acetylcholine on serotonin secretion may be affected by E. These reports support the results obtained in this work, indicating that E induces the $\alpha 7$ nAChR expression, thereby potentially increasing the sensitivity of serotonin neurons to the stimulatory effect of ACh.

No reports were found suggesting that E acts on ACh-induced NE release. However, it has been reported that nicotine increases the release of NE from rat hypothalamic synaptosomes *in vitro* (Yoshida et al., 1980). Moreover, cholinergic antagonists attenuated the cold stress-induced increase of TH mRNA in the rat adrenal medulla (Stachowiak et al., 1988) and nicotine inhaled from smoking stimulates NE release from the LC neurons that project to the hippocampus (Vizi and Lendvai, 1999). It has been proposed that ACh may activate presynaptic $\alpha 7$ nAChRs located on NE neurons from the LC to facilitate release of NE onto DR neurons (Li et al., 1998). The monkey LC expresses ER β (Pau et al., 1998), and administration of E to OVX monkeys, in a concentration similar to that found in the preovulatory phase of the monkey menstrual cycle, increased TH mRNA levels in the LC and stimulated NE release in the mediobasal hypothalamus (Pau et al., 2000). Based upon these results, we speculate that the stimulatory effect of E on NE release in the monkey LC may involve the participation of the cholinergic system via the $\alpha 7$ nAChR.

We also found that the number of phenotypically un-identified neurons expressing the $\alpha 7$ nAChR increased in both the DR and the LC. There are reports of the expression of the $\alpha 7$ nAChR in GABAergic neurons of the rat DR and LC (Bitner and Nikkel, 2002). Additional studies reported the presence of non-serotonergic interneurons within local raphe circuits (Aghajanian et al., 1978; Charara and Parent, 1998). It is possible that E is also

participating in the ACh regulation of serotonin and NE release via these interneurons. The phenotype of these un-identified neurons is of interest for future study.

Both, the DR and the LC are associated with the regulation of several physiological functions, like food intake (Leibowitz et al., 1984; Schwartz et al., 1989), response to stress (Abercrombie and Jacobs, 1987; Fujino et al., 2002) and reproduction (Lynch et al., 1984, Martins-Afferri et al., 2003). In humans, these functions can be affected by E and nicotine. For example, women appear to benefit more from the weight-control benefits of smoking than do men (Dicken, 1978). There is a relationship between exposure to tobacco smoke and dysmenorrhea (Chen et al., 2000), and between depression and smoking in woman (Weg et al., 2004). Due to the similarities between humans and non-humans primates, the results obtained in the present work constitute an important antecedent to the study of the interactive effects of nicotine and ovarian steroid hormones on mental health and addiction in women.

In summary, E increased the expression of the $\alpha 7$ nAChR in serotonergic, noradrenergic and other non-identified neurons. Thus, the present work provides evidence of the possible participation of E in the sensitivity to ACh in the DR and the LC of the OVX macaque. This could provide an important link between sex steroids and nicotine dependent mood alterations or behaviors.

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Figure 1.

Digitized photograph of the autoradiographic film from the Western blot of solubilized membrane preparations of the DR region probed with antibody mAb306. Membrane-bound proteins were extracted from the DR of one OVX and one Ecap macaque. The antibody mAb306 produced only one positive band at approximately 60 kDa molecular weight in both OVX and Ecap macaques that is similar in density.

Figure 2.

Immunocytochemistry images showing the immunoreactivity for the $\alpha 7$ nAChR after the pre-adsorption of the anti- $\alpha 7$ nAChR antibody mAb306 with nothing (A) or with a newly synthesized antigenic blocking peptide (B), as well as the nonspecific immunostaining after substitution of the antibody mAb306 with 1 mg/ml BSA (C). Compared with the immunoreactivity obtained with the non pre-adsorbed antibody mAb306 (A), pre-adsorption with the blocking peptide reduced the immunoreactivity (B) to a level observed in the absence of primary antibody (C). Scale bar = 50 μ m.

Figure 3.

Distribution of $\alpha 7$ nAChR-immunoreactivity (green) in various areas of the midbrain and brainstem of one E-treated monkey. 4N, trochlear nucleus; DR, dorsal raphe nucleus; IOD, dorsal nucleus of the inferior olive; LC, locus coeruleus; LPB, lateral parabrachial nucleus; LPGi, lateral paragigantocellular nucleus; me5, mesencephalic 5 tract; ODPMn, oral dorsal paramedian nucleus; RMAO, rostral medial accessory olive; scp, superior cerebellar peduncle. Scale bar = 50 μ m.

Figure 4. Immunofluorescence images showing immunoreactivity for $\alpha 7$ nAChR in the LC (A) and DR (B) using the polyclonal antibody sc-1447. Followed by the immunostaining obtained after pre-adsorption of the antibody with its blocking peptide (C and D). Scale bar = 50 μ m.

Figure 5.

Collage illustrating the distribution of $\alpha 7$ nAChR (green), serotonin (red) and $\alpha 7$ nAChR +serotonin co-localization (yellow) in one section corresponding to the rostral part of the DR of one representative OVX (A) and one Ecap+inf monkey (10 h, B). These images were constructed by joining 10x independent overlaid pictures using the software Adobe Illustrator 8.0. Outlined is the representative area in which cells were counted. DR, dorsal raphe nucleus; me5, mesencephalic 5 tract; VLPAG, ventrolateral periaqueductal gray.

Figure 6.

Double immunofluorescence images that show immunoreactivity for $\alpha 7$ nAChR (green; A, D and G), serotonin (5-HT; red; B, E and H) and $\alpha 7$ nAChR/serotonin co-localization (yellow; C, F and I) in the DR of a representative OVX (A–C), Ecap (D–F) and Ecap+inf (G–I) monkey. Scale bar = 50 μ m.

Figure 7.

Double immunofluorescence images that show immunoreactivity for $\alpha 7$ nAChR (green; A, D and G), TH (red; B, E and H) and $\alpha 7$ nAChR+TH co-localization (yellow; C, F and I) in the LC/A6 area of a representative OVX (A–C), Ecap (D–F) and Ecap+inf monkey (G–I). Scale bar = 50 μ m.

Figure 8. Histogram illustrating the mean $\pm$ S.E.M. of $\alpha 7$ nAChR, serotonin (5-HT) or TH immunoreactive cells, and $\alpha 7$ nAChR+5-HT or $\alpha 7$ nAChR+TH double-labeled neurons in the DR and in the LC of OVX (n=4), Ecap (n=3) and Ecap+inf (n=4) macaques. There was a significant difference in the number of $\alpha 7$ nAChR-positive neurons between the treatment groups in both the DR (ANOVA, $p < 0.0013$) and LC (ANOVA, $p < 0.0293$). There was also a significant difference in the number of $\alpha 7$ nAChR+5-HT (ANOVA, $p < 0.0248$) and $\alpha 7$ nAChR+TH (ANOVA, $p < 0.05$) between treatment groups. ANOVA was followed by Tukey's post-hoc pairwise comparison. Asterisks (*) represent a significant increase at $p < 0.01$, and hatch signs (#) represent a significant increase at $p < 0.05$, when compared with the OVX animals.