Sympathetic $\alpha_3\beta_2$-nAChRs mediate cerebral neurogenic nitrergic vasodilation in the swine

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Lee RH, Liu YQ, Chen PY, Liu CH, Chen MF, Lin HW, Kuo JS, Premkumar LS, Lee TJ. Sympathetic $\alpha_3\beta_2$-nAChRs mediate cerebral neurogenic nitrergic vasodilation in the swine. Am J Physiol Heart Circ Physiol 301: H344–H354, 2011. First published May 2, 2011; doi:10.1152/ajpheart.00172.2011.—The $\alpha_3$-nicotinic ACh receptor ($\alpha_3$-nAChR) on sympathetic neurons innervating basilar arteries of pigs crossed bred between Landrace and Yorkshire (LY) is known to mediate nicotine-induced, $\beta$-amyloid (A$\beta$)-sensitive nitrergic neurogenic vasodilation. Preliminary studies, however, demonstrated that nicotine-induced cerebral vasodilation in pigs crossed bred among Landrace, Yorkshire, and Duroc (LYD) was insensitive to A$\beta$ and $\alpha$-bungarotoxin ($\alpha$-BTGTX). We investigated nAChR subtype on sympathetic neurons innervating LYD basilar arteries. Nicotine-induced relaxation of porcine isolated basilar arteries was examined by tissue bath myography, inward currents on nAChR-expressing oocytes by two-electrode voltage recording, and mRNA and protein expression in the superior cervical ganglion (SCG) and middle cervical ganglion (MCG) by reverse transcription PCR and Western blotting. Nicotine-induced basilar arterial relaxation was not affected by A$\beta$, $\alpha$-BTGTX, and $\alpha$-conotoxin IMI ($\alpha_2\delta$-nAChR antagonists), or $\alpha$-conotoxin AuIB ($\alpha_3\beta_2$-nAChR antagonist) but was inhibited by tropinone and tropane ($\alpha_3$-containing nAChR antagonists) and $\alpha$-conotoxin MII (selective $\alpha_2\delta$-nAChR antagonist). Nicotine-induced inward currents in $\alpha_2\delta$-nAChR-expressing oocytes were inhibited by $\alpha$-conotoxin MII but not by $\alpha$-BTGTX, A$\beta$, or $\alpha$-conotoxin AuIB. mRNAs of $\alpha_2$, $\alpha_3$, $\beta_2$, and $\beta_3$-subunits were expressed in both SCGs and MCGs with significantly higher mRNAs of $\alpha_3$, $\beta_2$, and $\beta_3$-subunits than that of $\alpha_2$-subunit. The A$\beta$-insensitive sympathetic $\alpha_3\beta_2$-nAChR mediates nicotine-induced cerebral neurogenic vasodilation in LYD pigs. The different finding from A$\beta$-sensitive $\alpha_2\delta$-nAChR in basilar arteries of LY pigs may offer a partial explanation for different sensitivities of individuals to A$\beta$ in causing diminished cerebral nitrergic vasodilation in diseases involving A$\beta$.

$\alpha_3\beta_2$-nicotinic ACh receptor; axo-axonal interaction; $\beta$-amyloid; cerebral perivascular sympathetic nerves; superior cervical ganglion

IT IS WELL ESTABLISHED THAT CEREBRAL ARTERIES RECEIVE DENSE SYMPATHETIC INNERVATION ORIGINATING IN THE SUPERIOR CERVICAL GANGLION (SCG) (24, 50) AND THAT NICOTINIC ACh RECEPTORS (nAChRs) ON THE NERVE TERMINALS REGULATE NOREPINEPHRINE (NE) RELEASE (50). IN THE PIGS CROSSED BETWEEN LANDRACE AND YORKSHIRE IN THE UNITED STATES, ACTIVATION BY NICOTINIC AGONISTS OF THE nAChRs ON PERIVASCULAR SYMPATHETIC NERVES INNERVATING CEREBRAL ARTERIES RESULTS IN RELEASE OF NE, WHICH THEN CAUSES RELEASE OF NITRIC OXIDE (NO) FROM THE NEARBY PARASYMPATHETIC NITRERGIC NERVES AND DILATION OF THE ARTERS (22, 39). THIS NICOTINIC AGONIST-INDUCED AXO-AXONAL INTERACTION LEADING TO NITRERGIC VASODILATION IS MEDIATED BY THE $\alpha_7$-nAChR SUBTYPE (39). THE VASODILATION IS BLOCKED BY $\beta$-AMYLLOID PEPTIDE (A$\beta$) VIA INHIBITION OF THE $\alpha_7$-nAChR (40). BECAUSE A$\beta$ IS A KEY CAUSATIVE FACTOR IN PATHOGENESIS OF ALZHEIMER’S DISEASE (AD), A$\beta$-INHIBITION OF CEREBRAL NITRERGIC VASODILATION IS CONSISTENT WITH THE REPORTS THAT AD IS CLOSELY ASSOCIATED WITH REDUCTION IN REGIONAL CEREBRAL BLOOD FLOW THAT MAY ACCELERATE THE PATHOLOGICAL PROGRESS (5, 14). ACCORDINGLY, INHIBITION BY A$\beta$ OF $\alpha_7$-nAChRs ON THE CEREBRAL PERIVASCULAR SYMPATHETIC NERVES MAY PLAY A ROLE IN DIMINISHED CEREBRAL ARTERIAL FUNCTION IN PATHOGENESIS OF AD (39).

OUR PRELIMINARY RESULTS INDICATED THAT NICOTINIC AGONISTS INDUCED AXO-AXONAL INTERACTION-MEDIATED NEUROGENIC NITRERGIC VASODILATION IN BASILAR ARTERIES FROM PIGS CROSSED BREED AMONG LANDRACE, YORKSHIRE, AND Duroc (LYD) FOUND IN TAIWAN. THE VASODILATION, HOWEVER, IS INSENSITIVE TO A$\beta$ AND $\alpha$-BUNGAROTOXIN ($\alpha$-BTGTX), SUGGESTING THAT $\alpha_7$-nAChR MAY NOT BE THE FUNCTIONAL SUBTYPE MEDIATING NICOTINIC-INDUCED CEREBRAL NITRERGIC VASODILATION IN THE LYD PIGS. THE SUBTYPES OF nAChR ON PERIVASCULAR SYMPATHETIC NERVES INNERVATING BASILAR ARTERIES IN THE LYD PIGS, THEREFORE, WERE EXAMINED USING MULTIFACETED APPROACHES. FOR THIS PURPOSE, THE nAChR SUBTYPE ON THE SCG, WHICH IS THE ORIGIN OF CEREBRAL PERIVASCULAR SYMPATHETIC NERVES, AND THE MIDDLE CERVICAL GANGLION (MCG), WHICH IS ON THE SAME CERVICAL SYMPATHETIC TRUNK, WAS EXAMINED. THE RESULTS INDICATED THAT $\alpha_3$- AND $\beta_2$-SUBUNITS WERE PRESENT IN THE SCG AND MCG AND THAT $\alpha_3\beta_2$-nAChR WAS THE FUNCTIONAL SUBTYPE EXPRESSED ON CEREBRAL PERIVASCULAR SYMPATHETIC NEURONS MEDIATING NICOTINIC AGONIST-INDUCED CEREBRAL NITRERGIC NITRERGIC VASODILATION IN THE LYD PIGS.

MATERIALS AND METHODS

General procedure. Fresh heads of adult pigs (90–110 kg) of either sex, crossed bred among LYD, were collected at a local packing company (Hsien Meat Market Limited, Hualien, Taiwan). The entire brain, with dura mater attached, was removed and placed in Krebs’ solution equilibrated with 95% O$_2$ balanced with 5% CO$_2$ at room temperature. The composition of the Krebs’ solution was as follows (in mM): 122.0 NaCl, 5.16 KCl, 1.2 CaCl$_2$, 1.22 MgSO$_4$, 25.6 NaHCO$_3$, 0.03 ethylenediamine-tetraacetic acid, 0.1 l-ascorbic acid, and 11.0 glucose with a final pH of 7.4. The basilar artery was dissected and cleaned of any connective tissue under a dissecting microscope.

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microscope and then processed for tissue bath myography study (50). Freshly dissected SCGs and MCGs were placed in cold Krebs’ solution. Connective and fat tissues on the ganglia were removed carefully under a dissecting microscope and were immediately chilled in liquid nitrogen. The SCGs and MCGs were then stored in a refrigerator set at −80°C for further investigation. All protocols were approved by the Animal Experimentation Committee of the Tzu Chi University.

**Tissue bath myography.** The endothelial cells of all arterial ring segments were mechanically denuded by a standard, brief, gentle rubbing of the intimal surface with a stainless-steel rod having a diameter (22–30 gauge) equivalent to the lumen of the arteries (25, 50). An arterial ring segment (4 mm long) was cannulated with a stainless-steel rod (30-gauge hemispherical section) and a short piece of platinum wire and mounted horizontally in a plastic tissue bath containing 9 ml Krebs’ solution. The platinum wire was bent into a U shape and anchored to a gate. The stainless-steel rod was connected to a strain-gauge transducer for isometric recording of changes in force (24). The temperature of the Krebs’ solution was maintained at 37°C. The ring was equilibrated in the Krebs’ solution for an initial 30 min and then mechanically stretched to resting tension of 750 mg (25, 50). The ring segments were then contracted with U-46619 (0.3 to 3 μM) to induce an active muscle tone of 0.5–0.75 gm. Vasorelaxation was induced by nicotine at 100 μM or transmural nerve stimulation (TNS) at 8 Hz in frequency, 0.6 ms in pulse duration, and 200 mA in intensity for 25 s. After establishment of TNS- or nicotine-induced relaxation, the arteries were washed with prewarmed Krebs’ solution. A similar magnitude of active muscle tone was induced with U-46619 again, and TNS was repeated to serve as a control for comparison with the TNS-induced relaxation before wash. Different concentrations of nAChR antagonists were administered 15 min before repeating the TNS and nicotine application. To avoid the possible development of tachyphylaxis on repeated applications of nicotine, at least five washes every 15 min for 75 min were performed before the next application of nicotine (25, 39). At the end of each experiment, the complete removal of endothelial cells was verified by lack of changes in basal tone upon application of N⁵-nitro-L-arginine (L-NNA), an NO synthase (NOS) inhibitor. At the end of each experiment, papaverine (100 μM) was added to induce maximum relaxation. The magnitude of a vasodilator response was expressed as a percentage of the maximum.

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**Fig. 1.** Effects of tetrodotoxin (TTX), N⁵-nitro-L-arginine (L-NNA), and guanethidine on transmural nerve stimulation (TNS)- and nicotine-induced neurogenic vasodilation in porcine basilar arteries. All experiments were carried out in endothelium-denuded porcine basilar arteries in the presence of active muscle tone induced by U46619 (0.2 μM). A: representative tracing showing nicotine-induced concentration-dependent vasodilation, which is summarized in B. C: representative tracing showing vasodilation induced by TNS at different frequencies with maximum dilation obtained at 8 Hz, which is summarized in D. Effects of TTX, L-NNA, and guanethidine are summarized in E. Vasodilation is estimated as percent papaverine (PPV; 100 μM)-induced maximum relaxation. Values are means ± SE; n, number of experiments. *P < 0.01, significantly different from the respective control.
response induced by papaverine (50). IC_{50} values (the concentration that inhibits 50% of the maximum relaxation) were determined. From these values, the geometric means of EC_{50} or IC_{50} values with 95% confidence intervals (12) were calculated.

**RT-PCR.** AllPrep DNA/RNA/Protein Mini Kit (Qiagen) was used to extract total RNA from the SCG and MCG. For PCR, single-stranded cDNA as PCR template from 0.5 μg total RNA was synthesized by SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Due to lack of information of cDNA sequences of porcine nAChR subunits, primers used in the present study were designed according to reports from previous studies (27, 41, 43, 46). The primer for the β2-subunit was designed according to human β2-subunit cDNA sequence. Primers for PCR are indicated in Supplemental Table S1. A total reaction volume of 20 μl was used for PCR amplification (0.8 μl of cDNA from reverse transcription, 18 μl of Platinum PCR SuperMix High Fidelity (Invitrogen), 0.4 μl 10 μM both sense and antisense primer, and 0.4 μl diethyl pyrocarbonate (DEPC)-treated, distilled, and deionized water), and the PCR was completed in a MJ Mini gradient thermal cycler (Bio-Rad, Hercules, CA). Thermal cycle profile was as follows: 94°C (2 min); then 35 cycles at 94°C (1 min), 50–62°C (1 min; the annealing temperatures were determined according to the sequence of the primers), 72°C (30 s), and 4°C at the end of reactions. RT-PCR without SuperScript III reverse transcriptase was used as a negative control. PCR products were electrophoresed in 2% agarose gel containing 0.002% ethidium bromide. Bands were visualized and photographed.

**Western blotting analysis.** Freshly dissected porcine SCGs and MCGs were stored in −80°C for further investigation. SCGs were first homogenized in ice-cold (4°C) PRO-PREP Protein Extraction Solution (iNtRON) containing a protease inhibitor and thereafter centrifuged at 12,000 rpm for 60 min at 4°C. The protein contents of supernatant were measured employing the Protein Assay kit (Bio-Rad). Protein contents of supernatant (50 μg) were subjected to separation by 10% SDS-PAGE and then blotted onto polyvinylidene difluoride (PVDF) membranes using a Semi-Dry Transfer Unit (Amersham Biosciences). After these membranes were blocked with 5% dry milk, the PVDF membranes were incubated with primary antibodies at 4°C overnight. The primary antibodies and their dilution used in this experiment were as follows: rat monoclonal anti-α2- and α3-nAChRs (1:1,000), 7-nAChR (1:500), 2-nAChR (1:1,000), 3-nAChR (1:1,000), 7- and 2-nAChRs expression, 50 nl aliquots of α2- and α3-nAChR subunits cRNA (0.5 μg/μl) were injected into the Xenopus oocytes with a nanoinjector (Drummond, Broomall, PA); for α2/β2-nAChRs expression, 50 nl aliquots of α2- and β2-subunits cRNA (0.5 μg/μl) with the ratio of 1:1 were injected into the oocytes. These oocytes were maintained in ND96 solution at 18°C. The bath solution, ND96, contained (in mM) 96 NaCl, 2 KCl, 1.0 MgCl2, 1.8 CaCl2, and 2.5 glucose.

**Fig. 2.** Effects of β-adrenoceptor antagonists on nicotine (Nic)-induced vasodilation in porcine basilar arteries. All experiments were carried out in endothelium-denuded porcine basilar arteries in the presence of active muscle tone induced by U46619 (0.2 μM). A representative tracing in A shows that butoxamine (10 μM) abolished nicotine-induced vasodilation without affecting that induced by TNS (8 Hz). Propranolol (3 μM) abolished nicotine-induced relaxation without affecting that elicited by TNS. These results are summarized in B. Vasodilation was estimated as percent PPV (100 μM)-induced maximum vasodilation. Arrowheads in A indicate repeated washings (W). Values are means ± SE; n, number of experiments. *P < 0.01, significantly different from the respective control.
5.0 HEPES (pH 7.5). Stock solution of drugs was diluted in ND96 solution.

Two-electrode voltage clamp recording. A modified two-electrode voltage clamp recording was used in the present study (33). Two days after the injection of human α7, α3, and β2-subunit cRNAs, membrane currents of the oocytes were recorded in a chamber consisting of 1.3 ml cylindrical well at room temperature. During the recording, the oocytes were continuously perfused with the bath solution at a rate of 10 ml/min.

Two-electrode voltage clamp for the whole oocyte recording was performed by an amplifier (model OC-725C; Warner Instruments, Hamden, CT). The borosilicate glass capillaries (1.5 mm OD; World Precision Instruments, Sarasota, FL) were pulled by a microelectrode puller (model P-97; Sutter, Novato, CA). The resistance of an electrode filled with 3 M KCl was 0.2–1 MΩ. The membrane potential was held at −60 mV. Data acquisition and analysis were performed with pClamp 9.0 and Digidata 1322A (Axon Instruments, Union City, CA). The input signals were filtered at 1 kHz and sampled at 2 kHz.

The magnitude of inward current induced by nicotine was determined by the current amplitude. To compensate for the difference in the nAChR expression level, the current amplitudes were normalized and expressed as percentage of nicotine (100%).

RESULTS
Nicotine- and TNS-elicited neurogenic vasorelaxation of basilar arterial rings. The endothelium-denuded basilar arterial rings of LYD pigs in the presence of U46619-induced active muscle tone relaxed upon applications of nicotine (3–100 μM) in a concentration-dependent manner (Fig. 1, A and B) with a maximum relaxation at 100 μM. TNS (4–32 Hz) also elicited a frequency-dependent vasorelaxation with the maximum relaxation at 8 Hz (Fig. 1, C and D). Therefore, 100 μM nicotine and TNS at 8 Hz were used in subsequent experiments. The vasodilation induced by TNS was completely blocked by 0.3 μM TTX (a sodium channel blocker) and 10 μM ruthenium red (an ATPase inhibitor).

Drugs and statistical analysis. The following drugs were used: U46619, (-)-nicotine, tetrodotoxin (TTX), Nω-nitro-L-arginine (L-NNA), papaverine (PPV), guanethidine, propranolol, butoxamine, α-BGTX, tropane, tropine, and (±)-isoproterenol (all from Sigma-Aldrich, St. Louis, MO); (RS)-atenolol, hexamethonium, mecamylamine, methyllycaconitine (MLA), and Aβ1-40 (all from Tocris, Ellisville, MO); guanethidine (Novartis, formerly CIBA Pharmaceuticals, Basel, Switzerland); sodium nitroprusside (Riedel-de haén, Seelze, Germany); α-conotoxin IMI (α-CTX IMI) (American Peptide, Sunnyvale, CA); and α-conotoxin AuIB (α-CTX AuIB) and α-conotoxin MMI (α-CTX MMI) were synthesized by Genemed Synthesis (San Antonio, TX) based on reported peptide sequences (6, 28). All drugs were dissolved in deionized water and added directly into tissue baths. The drug concentrations were the final concentrations in the bath. Shapiro-Wilk test was used to verify that all data sets were normally distributed. The sigmoidal dose-response curve fitting was measured and analyzed using GraphPad Prism (GraphPad software). Data were expressed as means ± SE; number of experiments.
µM L-NNA (a NOS inhibitor) (Fig. 1E). The nicotine-induced vasodilation, however, was not significantly blocked by TTX, although it was largely inhibited by L-NNA (Fig. 1E). These results are consistent with previous findings in the Landrace and Yorkshire (LY) porcine cerebral arteries that TNS- and nicotine-induced vasodilations were due to release of neurogenic NO and were endothelium independent (39).

Nicotine-induced cerebral vasodilation via the axo-axonal interaction mechanism. Nicotine-induced vasodilation in isolated basilar arteries of LYD pigs was completely inhibited by 10 µM guanethidine (a NE release inhibitor; Fig. 1E), 10 µM butoxamine (a selective β2-adrenergoreceptor antagonist; Fig. 2A and B), and 3 µM propanolol (a nonselective β-adrenergoreceptor antagonist; Fig. 2B) but was not affected by 10 µM atenolol (a selective β1-adrenoreceptor antagonist; Fig. 2B). These receptor antagonists at the concentrations used did not affect the TNS-induced vasodilation, indicating that blockade of the nicotine-induced vasodilation was not due to any possible local anesthetic or nonspecific effects of these antagonists (39, 50). These results are consistent with previous findings in LY pigs that nicotine-induced cerebral neurogenic vasodilation is dependent on release of NE from perivascular sympathetic nerves. The released NE then stimulates β2-adrenergoreceptors located on perivascular parasympathetic nitrergic nerves, causing release of NO and relaxation of the smooth muscle cells (39, 50). Thus the axo-axonal interaction mechanism underlying nicotine-induced nitrergic vasodilation is similar in the LYD and the LY pigs.

Failure of α7-nAChR selective antagonists in blocking nicotinic agonist-induced cerebral neurogenic vasodilation. We have reported that nicotine-induced vasodilation of the LY basilar arteries is mediated by activation of α7-nAChRs on perivascular sympathetic nerves (39), and this vasodilation is blocked by AB via inhibiting the α7-nAChR (40). The nicotine-induced vasodilation of the LYD basilar arteries in the presence of active muscle tone induced by U46619, however, was not affected by α7-nAChR selective antagonists, α-BGTX (up to 2 µM), α-CTX IMI (5 µM; Fig. 3, A and B), and Aβ (up to 3 µM; Fig. 3B). The successful inhibition of nicotine-induced inward currents on α7-nAChR-expressing oocytes by 30 nM α-BGTX was used as the positive control (Supplemental Fig. S1A). Similar to those found in the LY pigs, nicotine-induced cerebral vasodilation in the LYD pigs was inhibited by mecamylamine and hexamethonium (nonselective nAChR antagonists), and MLA (a preferential α7-nAChR antagonist) in a concentration-dependent manner, with the IC50 values of 0.23, 1.69, and 0.47 µM, respectively (Fig. 3C).

Effects of α3, α6, α3β2-, and αβ3- nAChR antagonists on nicotine-induced neurogenic vasodilation. In the presence of active muscle tone induced by U46619, basilar arterial rings relaxed upon application of nicotine. The relaxation was inhibited by α3-, and α6-preferential nAChR antagonists tropine (Fig. 4B) and tropinone (Fig. 4C) (35) and a selective α3/α6β2-nAChR antagonist α-CTX MII (6, 30) in a concentration-dependent manner (Fig. 4, A and D), but not by α-CTX AuIB (a selective α3β2-nAChR antagonist) (28) (Supplemental Fig. 6).
S2, A and B). The IC<sub>50</sub> values for tropane and α-CTX MII in blocking nicotine-induced relaxation were 4.11 (0.3–54.8) and 0.61 (0.14–2.67) μM, respectively (Fig. 4, B and D). In addition, tropane and tropinone, which are reported as α<sub>7</sub>-nAChR partial agonists (35), in concentrations up to 200 μM and 1 mM, respectively, did not induce nAChR-mediated relaxation of basilar arteries (Supplemental Fig. S3, A and B).

Tropane, tropinone, and α-CTX MII in concentrations used in the present studies did not affect vasorelaxation induced by TNS (Supplemental Fig. S3C), suggesting that inhibition of nicotine-induced vasorelaxation by these drugs is not due to any possible local anesthetic or nonspecific effects. These drugs did not affect the vasorelaxation induced by sodium nitroprusside (an NO donor) or isoproterenol (a β-adrenergic receptor agonist; Supplemental Fig. S4, A–F) either. These latter findings further suggest that inhibition of nicotine-induced vasorelaxation is not due to blockade of NO release or its coupling pathway or β<sub>2</sub>-adrenergic receptor on the nitrergic, cholinergic neurons.

**Effects of nAChR antagonists on α<sub>2</sub>β<sub>2</sub>- and α<sub>7</sub>-nAChR-mediated inward currents.** In two-electrode voltage clamp recording, α<sub>3</sub>β<sub>2</sub>-nAChR-mediated inward currents in the oocytes were inhibited by hexamethonium (10 μM) and mecamylamine (1 μM) (nonselective nAChR antagonists), α-CTX MII (0.5 μM, a selective α<sub>3</sub>/α<sub>2</sub>β<sub>2</sub>-nAChR antagonist; Fig. 5A), and tropane (10 μM) and tropinone (300 μM; preferential α<sub>3</sub>- and α<sub>7</sub>-nAChR antagonists) by 61.22 ± 3.10%, 32.69 ± 1.11%, 76.13 ± 9.02%, 42.32 ± 4.44%, and 35.32 ± 6.28%, respectively (Fig. 5, B and C). These inward currents were not affected by Aβ, α-BGTX, or α-CTX AuIB (a

![Fig. 5. Effects of different nAChR antagonists on nicotine-induced inward currents in Xenopus oocytes expressing α<sub>2</sub>β<sub>2</sub>-nAChRs or α<sub>7</sub>-nAChRs. A representative tracing in A from 2-electrode voltage clamp studies shows that α-CTX MII (0.5 μM) blocks α<sub>2</sub>β<sub>2</sub>-nAChRs-mediated inward currents elicited by nicotine (100 μM). Short horizontal black column above the tracing in A denotes application of nicotine for 1 s. Oocytes were continuously perfused with nAChR antagonist for 5 min as indicated by the long horizontal white column. The blockade was completely reversed after wash. A summary of α-CTX MII blockade in a concentration-dependent manner of α<sub>2</sub>β<sub>2</sub>-nAChRs or α<sub>7</sub>-nAChRs. In E, MLA in a concentration-dependent manner blocked α<sub>2</sub>β<sub>2</sub>- and α<sub>7</sub>-nAChRs-mediated inward currents elicited by nicotine (100 μM) with the IC<sub>50</sub> values of 31.87 (1.48–687.1) nM and 0.32 (0.07–1.39) nM, respectively. Inward currents were estimated as a percentage of that induced by 100 μM nicotine. Values are means ± SE; n, number of experiments. *P < 0.05, significantly different from respective control.
selective α3β4-nAChR antagonist) in concentrations up to 1 μM (Fig. 5C). The IC50 value for α-CTX MII in inhibition of α3β2-nAChR-mediated inward currents was 0.15 (0.008–2.81) μM (Fig. 5B). Furthermore, nicotine-induced inward currents in α2-nAChR-expressing oocytes were inhibited by 0.5 μM α-CTX MII and 10 μM tropane by only 6.90 ± 2.67% and 18.44 ± 3.57%, respectively (Fig. 5D). MLA, a reported preferential α7-nAChR antagonist (29), in a concentration-dependent manner inhibited both α3β2- and α7-nAChR-mediated inward currents with IC50 values of 31.87 (1.48–687.1) nM and 0.32 (0.07–1.39) nM, respectively (Fig. 5E).

**Expression of α3-, α7-, β2-, and β4-subunits of nAChR in the SCG and the MCG neuronal cells.** The mRNA transcripts for nAChR subunits on SCGs were detected by using RT-PCR. The primers for α3-, α7-, β2-, and β4-subunits yielded products of expected sizes of 290, 300, 166, and 290 bp, respectively (Fig. 6A). The primers for α1-, α2-, α4-, α5-, α6-, α9-, and α10-subunits, however, did not yield PCR products (Fig. 6A). The successful yields of PCR products from human α6-nAChR plasmids by the primers for α6-subunits were used as the positive control (Fig. 6C). The presence of proteins expressing α3- and β2-subunits in the SCGs (Fig. 6D) was confirmed by Western blotting. Results from using real-time RT-PCR analysis further indicated that the mRNA levels for α3-, α7-, and β4-subunits were significantly greater than that of α7-subunit (Fig. 6F).

As for the results from the MCGs, which are on the same cervical sympathetic trunk of the SCGs, α3-, α7-, β2-, and
DISCUSSION

Consistent to previous reports in the LY pigs (39, 50), TNS- and nicotine-induced vasorelaxations of endothelium-denuded basilar arteries of the LYD pigs were blocked by NOS inhibitor, suggesting that both TNS- and nicotine-induced vasorelaxation is due to release of neuronal NO. The vasorelaxation induced by nicotine but not by TNS was blocked by certain nAChR antagonists, guanethidine and β2-adrenoceptor antagonists, whereas vasorelaxation induced by both was insensitive to β1-adrenoceptor antagonists. These are consistent with the axo-axonal interaction hypothesis (Fig. 7) in regulating neurogenic nitric vasodilation in basilar arteries reported in the LY pigs (39, 50). Nicotinic agonists do not act directly on the nitricergic nerve terminals to release NO (39, 50). Accordingly, nicotine-induced nitric vasodilation in LYD pigs is dependent on release of NE from perivascular sympathetic nerves. The released NE then activates β2-adrenoceptors located on the neighboring parasympathetic nitric nerves, causing NO release and vasorelaxation (Fig. 7) (39, 50). Although it may be questioned that NE has low affinity for β2-adrenoceptors, NE influences immune cell function by binding to the β2-adrenergic receptor expressed on the surface of a variety of immune cells (19).

In the LY pig basilar arteries, the nAChR on sympathetic neurons mediating nicotinic agonist-induced neurogenic nitric vasodilation is of α7-subtype (22, 39). This conclusion is based on the findings that the nicotinic agonist-induced vasorelaxation is blocked by α-BGTX (a highly selective α7-nAChR antagonist) (39) and Aβs (40). α-BGTX, Aβs, and α-CTX IMI (a selective α7-nAChR antagonist), however, failed to affect nicotine-induced nitric vasodilation in basilar arteries of the LYD pigs even when concentrations of these blockers were in the micromolar ranges, which is about 10-fold higher than the reported IC50 value of α-CTX IMI in blocking α7-nAChR-mediated inward currents (10). Furthermore, tropane and tropinone, which in high concentrations possess α7-nAChR agonistic activity (35, 35), did not elicit vasorelaxation of isolated basilar arteries (Supplemental Fig. S3, A and B). Finally, Aβs, which have been shown to inhibit α7-, α2β2-, α2β2-, and α3α4β2-nAChRs (13, 21, 37) but not α2β2-nAChR (37), did not affect nicotine-induced, nAChR-mediated vasorelaxation of LYD basilar arteries or αβ2-nAChR-mediated inward currents in Xenopus oocytes. These results suggest that the sympathetic nAChR mediating nicotine-induced basilar arterial relaxation of the LYD pigs is different from α7-, α2β2-, α2β2-, and α3α4β2-nAChRs. The possibility of α3β4-nAChR being the subtype was unlikely either, since nicotine-induced vasorelaxation was not inhibited by α-CTX AuIB (a α3β4-nAChR antagonist; Supplemental Fig. S2, A and B) in concentrations known to block ACh-induced inward currents in α3β1-nAChR-expressing oocytes (28).

The nicotine-induced vasorelaxation, however, was blocked by preferential α3- or α6-nAChR antagonists tropane and tropinone and α-CTX MII (a selective α3/α6β2-nAChR antagonist) in a concentration-dependent manner. It is likely that the nAChR mediating nicotine-induced cerebral neurogenic nitric vasodilation in the LYD pigs is the α6β2- and/or αβ2-subtype. The close structural similarities of the α-subunits (2) make it difficult to distinguish the αβ2-nAChR from the αβ2-nAChR pharmacologically by α-CTX MII. The α6β2- subunit, however, was not expressed in the SCGs or MCG. The mRNA levels of the α3- or β2-subunit also were significantly higher than that of α2-subunit in the SCG and MCG of the LYD pig. These results indicate that two different ganglia of the same sympathetic trunk contain similar qualitative and quantitative expression of nAChR-subunits. These results suggest that the αβ2-nAChR is the functional subtype located on cerebral perivascular sympathetic nerves mediating cerebral neurogenic nitric vasorelaxation in the LYD pig. This suggestion is supported by the parallel IC50 values of α-CTX MII in blocking nicotine-induced αβ2-nAChR-mediated inward currents on the oocytes and relaxation of the basilar arteries.

Furthermore, nicotine-induced neurogenic vasorelaxation was sensitive to MLA, which was reported to block α3/α6β2β3-nAChRs (31) and αβ2-nAChRs (29). The IC50 values for inhibiting both nicotine-induced, αβ2-nAChR-mediated inward currents in oocytes and relaxation of basilar arteries are significantly higher than those for blocking α2-nAChR-mediated responses (Fig. 5E). Failure of several α-nAChR antagonists to inhibit nicotine-induced changes in vascular tone and inward currents, therefore, suggests that MLA blocks nicotine-induced
vasorelaxation of LYD basilar arteries by inhibiting the α3β2-nAChR.

The perivascular sympathetic nerves to brain blood vessels originate in the SCG (22, 24). The transcript levels of α3-, α5-, α7- and β2- but not β2-subunits in the SCG neurons have been shown to decrease in chronically denervated, but not denervated, rat SCGs (11, 48, 51, 52), suggesting that α3β2-nAChR and other subunits including α4β2- and α7- nAChRs are located mainly on the postganglionic neurons of the SCGs. This finding allows a reasonable prediction that α3β2-nAChRs are present on the sympathetic nerve terminals on the arterial wall, mediating nicotine-induced cerebral neurogenic nitrergic vasodilation.

The physiological significance of α3β2-nAChRs is relatively unknown. It has been reported that α3β2-nAChRs mediate tonic inhibition of the spinal transmission of nociceptive mechanical stimuli during peripheral injury (49), modulation of dopamine release in the striatum of the rat (18, 20, 36), and visual processing and ocular pathologies such as neovascularization in the retina (26, 32). In peripheral nervous system, most α3-subunits knockout mice have defects in the autonomic nervous system and are usually lethal. The surviving mice have substantially retarded growth with abnormal development of ocular globes and ocular autonomic innervations (1, 47). In addition, patients with auto-antibodies to ganglionic α3-nAChR display syndromes consisting of idiopathic or paraneoplastic autonomic neuropathy, postural tachycardia, and other autonomic disorders (3, 45). These results suggest that α3β2-nAChRs may play important physiological roles in regulation of the peripheral autonomic nervous system.

Our present findings add new information on the role of perivascular sympathetic α3β2-nAChR, via axo-axonal interaction mechanism (Fig. 7), in regulating cerebral neurogenic nitrergic vasodilation. The axo-axonal interaction mechanism appears to play a role in regulating the cerebral circulation in vivo. Inhalation of cigarette smoke in anesthetized rats caused pial arteriolar vasodilation, which was attenuated by pretreatment of mecamylamine, propranolol, and Nω-nitro-L-arginine methyl ester (L-NAME) (16). Similar results on nicotine-induced cortical vasodilation were found in the rat (44). Our preliminary results further indicated that electrical stimulation of sympathetic nerves originating in the SCG caused a frequency-dependent increase in basilar arterial blood flow in anesthetized rats (23). Topical application of nicotine (10 and 30 μM) onto the basilar artery also significantly increased the basilar arterial blood flow, which was inhibited by 7-nitroindazole (a neuronal NOS inhibitor) and ICI 118,551 (a β2-adrenoceptor antagonist) (23). These results are consistent with those found in in vitro studies (39, 50), supporting that the axo-axonal interaction mechanism plays a role in regulating the cerebral circulation. From a physiological point of view, the sympathetic enhancement of nitrergic vasodilation and blood flow in the brain stem via axo-axonal mechanism is expected to meet the need in the acute stress response or fight-or-flight response in normal subjects (23).

Direct evidence for the axo-axonal interaction in regulating human cerebral circulation remains to be demonstrated. Acute cigarette smoking, however, has been shown to increase regional cerebral blood flow in areas containing nAChRs, such as the thalamus and cerebellum (9, 53). Smoking also caused short-lasting pial vasodilation in pentobarbital-anesthetized rats, and the vasodilation is mediated by sympathetic activation and NO production (16). These findings are reasonably explained by the effect of nicotine in the cigarette in increasing cerebral nitrergic vasodilation, possibly, via the axo-axonal interaction mechanism.

Accordingly, modulation of cerebral perivascular sympathetic nAChR activity is expected to alter regional blood flow, and different subtypes of nAChRs may lead to different degrees of modulation by the same substances such as Aβ. Although many studies have demonstrated that aggregated Aβs are pathogenic in the human (38), recent results have indicated that soluble Aβs are also pathogenic in isolated peripheral and cerebral arteries (7, 15, 42). Likewise, freshly prepared soluble Aβs also inhibit α7-nAChRs and cerebral nitrergic vasodilation (39, 40).

It is interesting to know from the present finding that Aβ did not inhibit the α3β2-nAChR and related relaxation of basilar arteries. This is different from the reported findings that Aβ inhibits the α7-nAChRs (13, 37) and α2- nAChR-mediated cerebral vasodilation in LY pigs (40). This inhibition of α7- nAChR-mediated vasodilation by Aβ (40) may provide a partial explanation for decreased cerebral blood flow in the early phase of AD (5, 14, 40), since Aβs play a key role in pathogenesis of AD. This inhibition by Aβ may also explain the upregulation of α7-nAChR subunit protein in animal models of AD, as well as in human brain samples from AD patients (4, 8, 17). Aβ inhibition of nAChR-mediated cerebral nitrergic vasodilation, however, may not occur if α3β2-nAChRs are the predominant functional subtype located on the sympathetic nerves originating in the SCG. Indeed, α3-subunit is not upregulated in the brain tissue of AD patients (34). Although nAChR subtypes expressed on sympathetic nerves innervating brain arteries in human are not clarified, results of the present findings may offer an insight to further our understanding of the sympathetic regulation of cerebral nitrergic vasodilation in normal and pathological conditions such as the AD; that is, different subtypes or possible genetic variations of nAChRs expressed in cerebral perivascular sympathetic nerves may influence the drug response of the circulation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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