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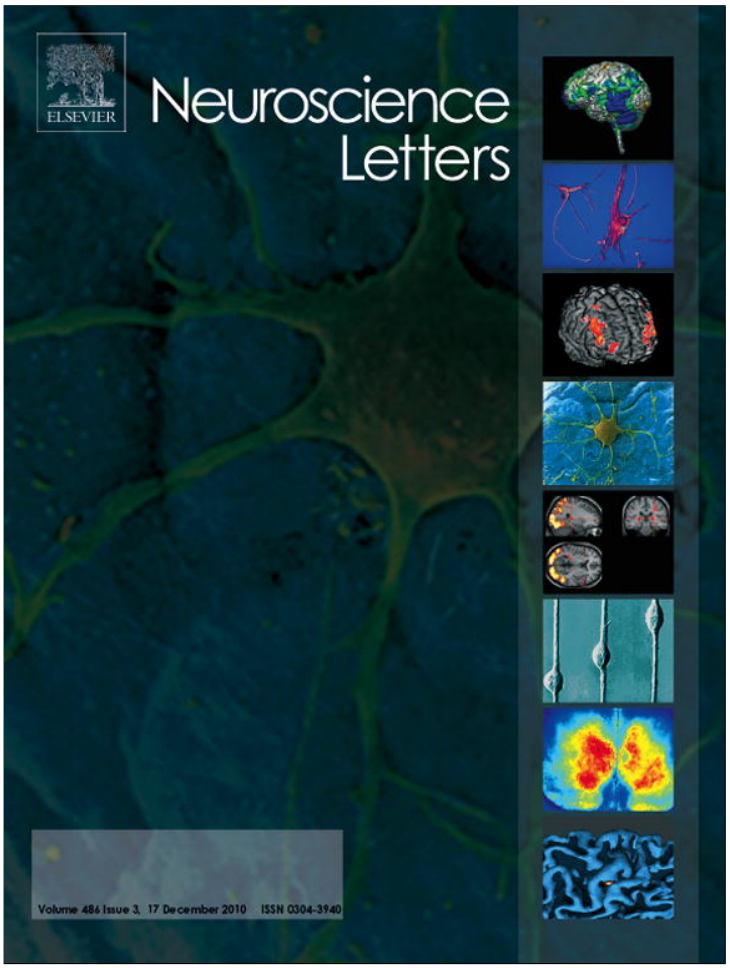

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Neuroscience Letters 486 (2010) 122–126

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Nicotine activation of neuronal nitric oxide synthase and guanylyl cyclase in the medulla increases blood flow of the common carotid artery in cats

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ARTICLE INFO

Article history:
Received 27 May 2010
Received in revised form 30 July 2010
Accepted 25 August 2010

Keywords:
Carotid artery
Cerebral blood flow
Nicotinic receptor
Nitric oxide synthase
Guanylyl cyclase

ABSTRACT

Individual activation of nicotinic acetylcholine receptor (nAChR) or nitric oxide (NO) synthase in the dorsal facial area (DFA) increases blood flow of common carotid artery (CCA) supplying intra- and extracranial tissues. We investigated whether the activation of nAChR initiated the activation of NO synthase and guanylyl cyclase to increase CCA blood flow in anesthetized cats. Microinjections of nicotine (a non-selective nAChR agonist), or choline (a selective α7-nAChR agonist) in the DFA produced increases in CCA blood flow ipsilaterally. These increases were significantly reduced by pretreatment with NG-nitro-arginine methyl ester (L-NAME, a non-specific NO synthase inhibitor), 7-nitroindazole (7-NI, a relatively selective neuronal NO synthase inhibitor) or methylene blue (MB, a guanylyl cyclase inhibitor) but not by that with N5-(1-iminoethyl)-L-ornithine (L-NIO, a potent endothelial NO synthase inhibitor). Control microinjection with D-NAME (an isomer of L-NAME), artificial cerebrospinal fluid or DMSO (a solvent for 7-NI) did not affect resting CCA blood flow, nor did they affect nicotine- or choline-induced response. In conclusion, activation of nAChR, at least α7-nAChR, led to the activation of neuronal NO synthase and guanylyl cyclase in the DFA, which induced an increase in CCA blood flow.

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Kuo et al. [15] for the first time reported that stimulation of the dorsal facial area (DFA) in the cat medulla induced an ipsilateral increase in blood flow of common carotid artery (CCA) without significant changes in other cardiovascular parameters. This effect is mediated by the parasympathetic branches of the 7th and 9th nerves [1] and is attenuated by atropine, a parasympathetic blocker [1]. The DFA thus is likely the rostral extension of the dorsal motor nucleus of the vagus nerve (DMN) [2]. Kuo and colleagues have serially reported about neurochemical actions of glutamate [1,2,9,10,15], serotonin [14,16], nicotine [8,13], and nitric oxide synthase (NOS) [7,12] in the DFA. It is established that activation in the DFA on either nicotinic acetylcholine receptor (nAChR) [8,13] or NOS [7,12] increases CCA blood flow. Yet it is not known whether activation of the nAChR in the DFA may thereby activate the NOS to induce the increase in CCA blood flow.

In the present study, we demonstrated that in the DFA, stimulation of the α7-nicotinic receptor activated neuronal NOS and guanylyl cyclase, which then led to the increase in CCA blood flow in anesthetized cats.

The experiments were carried out according to the guidelines of the China Medical University Ethical Committee for Animal Research. This study was approved by the Committees.

Cats (2.0–3.5 kg) of either sex were anesthetized with α-chloralose (40 mg/kg) and urethane (400 mg/kg) intraperitoneally, and paralyzed with atracurium with an initial dose of 0.05 mg/kg and a maintaining dose of 0.02 mg/kg (IV) every 20 min to eliminate interference in recording of blood flow. Tracheotomy was performed for artificial ventilation that maintained end expiratory CO_2 concentration at 3.5–4.5%. The rectal temperature was kept at 37.5 ± 0.5 °C by an electrical heating pad. The femoral artery and vein were cannulated with PE-90 polyethylene tubing for monitoring the systemic arterial pressure and supplying fluid, respectively. The ultrasound Doppler probes (diameter 1.5–2.0 mm), which were placed around the right and left CCA, were connected with a directional pulsed Doppler flowmeter (University of Iowa, Bioengineering, 545C-4, Iowa, USA) for monitoring CCA blood flow.

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0304-3940/$ – see front matter © 2010 Published by Elsevier Ireland Ltd.
doi:10.1016/j.neulet.2010.08.072

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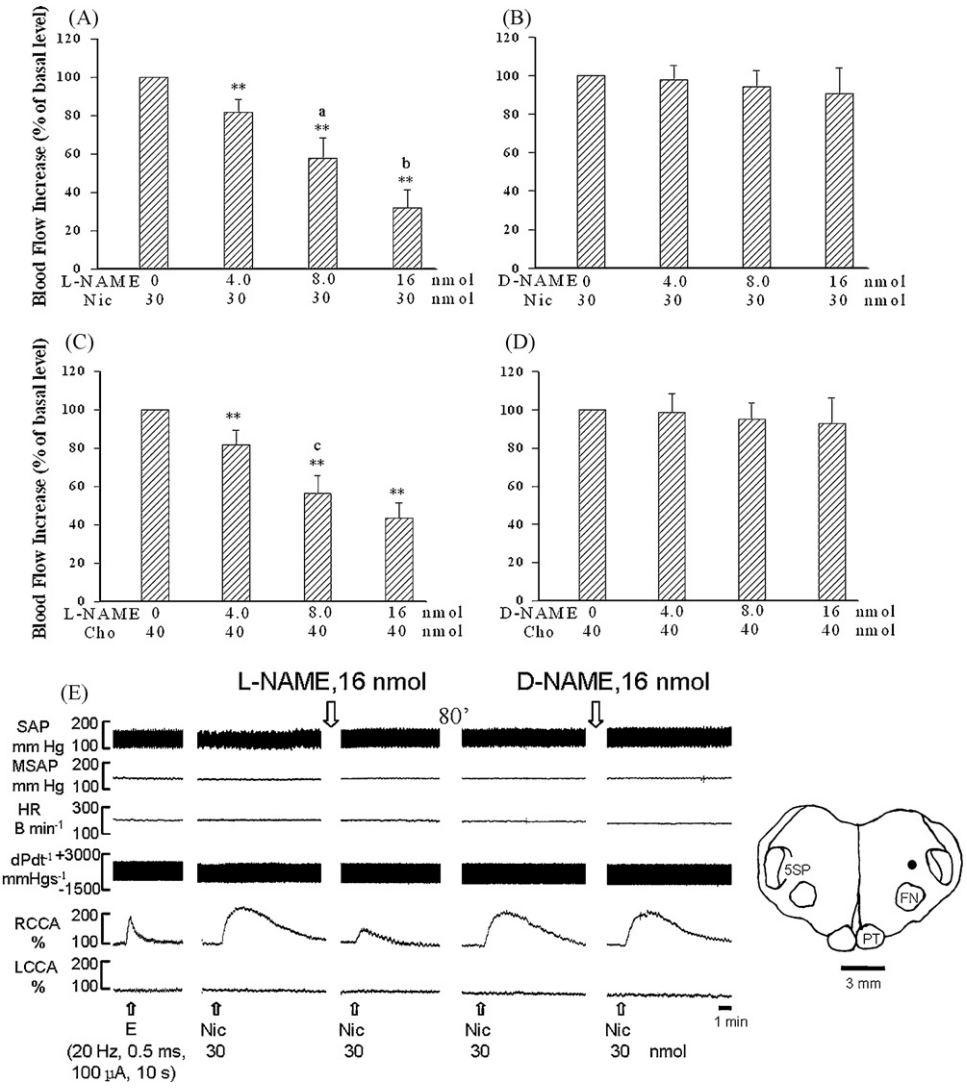

Fig. 1. Effects of pretreatment with non-specific NOS antagonist L-NAME (A, $n=7$; C, $n=5$) and its D-form isomer D-NAME (B, $n=4$; D, $n=4$) on nicotine- and choline-induced increases in the CCA blood flow. All chemicals were microinjected into the DFA. Original tracings are shown in E. Data are expressed as mean ± S.E.M. and analyzed by ANOVA and Tukey's test. For A, $^{**}P<0.01$ vs. L-NAME 0 nmol; $^{a}P<0.01$ vs. L-NAME 4.0 nmol; $^{b}P<0.01$ vs. L-NAME 8.0 nmol. For C, $^{**}P<0.01$ vs. L-NAME 0 nmol; $^{c}P<0.01$ vs. L-NAME 4.0 nmol. The dot on the drawing medullary section indicates injected locus. Abbreviations for this and following figures: B min^{-1}, beats per min; $dPdt^{-1}$, cardiac contractile force; Cho, choline; E, electrical stimulation; FN, facial nucleus; HR, heart rate; MSAP, mean systemic arterial pressure; Nic, nicotine; PT, pyramidal tract; RCCA or LCCA, right or left common carotid arterial blood flow; SAP, systemic arterial pressure; 5ST, spinal trigeminal nucleus.

Systemic arterial pressure, heart rate, cardiac contractile force, and CCA blood flows were routinely recorded on a Gould Recorder RS3800 (Cleveland, OH, USA) [7,8,10–13].

The head of the cat was immobilized in a David-Kopf stereotaxic instrument. The stereotaxic coordinates of the center of DFA were about 6.0 mm rostral to the obex, 3.5 mm lateral to the midline, and 3.5 mm ventral to the floor of the fourth cerebral ventricle [2,7,8,11–13]. Aiming at this point, a four-barrel microinjection tube [11,13] was placed at an angle of 34° from the vertical axis of the stereotaxic instrument. The DFA was confirmed by an increase of CCA blood flow in response to an electrical stimulation (20 Hz, 0.5 ms, 100 μA, 10 s) through the tubing. Misplaced injection of glutamate does not cause any response in CCA blood flow [2]. Each barrel was used for microinjecting 200 nl nicotine (a non-selective nAChR agonist), choline (an α7-nAChR agonist), L-NAME (a non-selective NOS inhibitor), D-NAME (an isomer of L-NAME), methylene blue (a guanylyl cyclase inhibitor), 7-NI (a neuronal NOS inhibitor), or L-NIO (an endothelial NOS inhibitor). All of these drugs were purchased from Sigma–Aldrich Inc. St. Louis, USA. Except 7-NI which was dissolved in DMSO, all drugs were dissolved in artificial cerebrospinal fluid (aCSF) containing the following chemicals in mM: NaCl 119, KCl 2.5, $MgCl_2$ 4, $CaCl_2$ 4, $NaHCO_3$ 26.2, NaH_2PO_4 1, and glucose 11. These solutions were gassed with 95% O_2 and 5% CO_2 at pH 7.4. They were microinjected at a rate of 1.2 μl/min into the DFA over 10 s with a microinjection pump (CMA/100, Carnegie Medicin, North Chelmsford, MA, USA).

At the end of the whole experiment, the animals were sacrificed by intravenous injection of saturated KCl. The brains were removed and frozen-sectioned by a microtome (2800 Frigocut) at 40 μm thickness. Only brain sections in which the injection point had been correctly positioned into the DFA were considered for data analysis.

Changes in systemic arterial pressure, heart rate, cardiac contractile force (dP/dt), and CCA blood flows in response to microinjections of chemicals were calculated as (response value − control value)/(control value) × 100% and then normalized with the control. The normalized data (means ± S.E.M.) were

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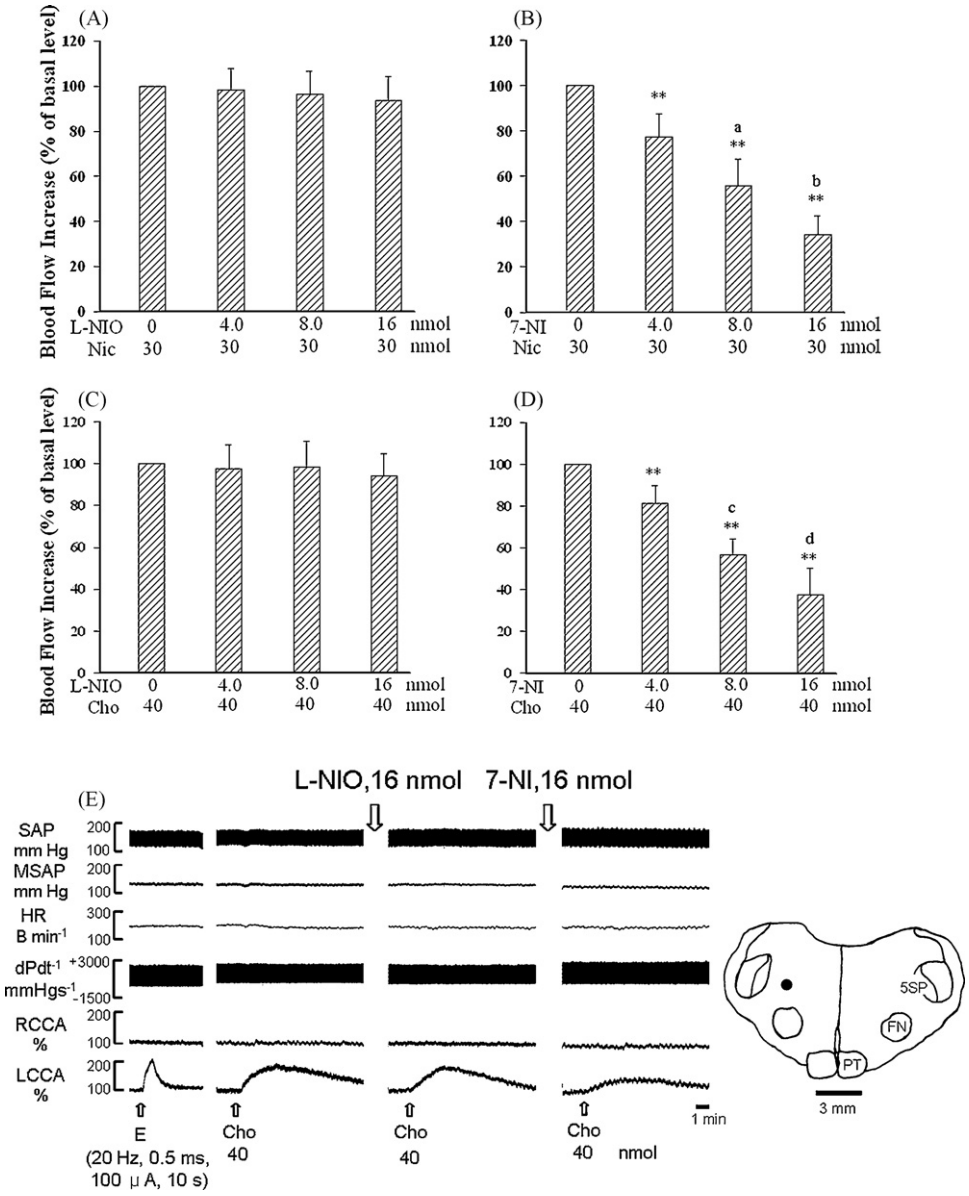

Fig. 2. Effects of pretreatment with eNOS antagonist L-NIO (A, $n=4$; C, $n=4$) and selective nNOS antagonist 7-NI (B, $n=5$; D, $n=5$) on nicotine- and choline-induced increases in the CCA blood flow. Original tracings are shown in E. Data are expressed as mean ± S.E.M. and analyzed by ANOVA and Tukey's test. For B, $^{**}P<0.01$ vs. 7-NI 0 nmol; $^{a}P<0.01$ vs. 7-NI 4.0 nmol; $^{b}P<0.01$ vs. 7-NI 8.0 nmol. For D, $^{**}P<0.01$ vs. 7-NI 0 nmol; $^{c}P<0.01$ vs. 7-NI 4.0 nmol; $^{d}P<0.05$ vs. 7-NI 8.0 nmol.

analyzed by ANOVA and Tukey's test. The probability level of a significant difference was $p<0.05$.

We examined whether the nicotine (a non-selective nAChR agonist) or choline (an α7-nAChR agonist) effects in the DFA were mediated through NOS activation. Because microinjection of 30 nmol nicotine or 40 nmol choline into the DFA induces a modest increase in CCA blood flow about 60–100% of the basal [8,13], these doses were chosen for the present experiment. The nicotine-induced blood flow increase was reduced dose-dependently by pretreatment with 4.0, 8.0 and 16 nmol L-NAME (a non-selective NOS inhibitor) to 81.7 ± 6.8, 57.7 ± 10.6, and 31.8 ± 9.4%, respectively (Fig. 1A), but not affected by pretreatment with D-NAME (an isomer of L-NAME) (Fig. 1B). The choline-induced blood flow increase was reduced dose-dependently by pretreatment with 4.0, 8.0 and 16 nmol L-NAME to 81.5 ± 7.8, 56.4 ± 9.1, and 43.4 ± 8.1%, respectively (Fig. 1C), but not affected by pretreatment with D-NAME (Fig. 1D). These findings suggest that an activation of the nAChR (primarily α7-nAChR) may mediate an activation of NOS in the DFA to increase CCA blood flow.

Neuronal NOS (nNOS) and endothelial NOS (eNOS) are enzymes that catalyze synthesis of NO from L-arginine primarily in neurons and endothelial cells, respectively. Although results in Fig. 1 have demonstrated involvement of NOS following activation in the DFA by nicotine or choline, the type of NOS involved was not addressed. Therefore, 7-NI (a relatively selective nNOS inhibitor) and L-NIO (a potent eNOS inhibitor) were used to intervene with nicotine- or choline-induced increase in CCA blood flow. The nicotine-induced blood flow increase was inhibited dose-dependently by pretreatment with 4.0, 8.0 and 16 nmol 7-NI to 77.2 ± 10.5, 55.7 ± 11.7, and 34.2 ± 8.4%, respectively (Fig. 2B), but not affected by pretreatment with L-NIO (Fig. 2A). The choline-induced blood flow increase was dose-dependently inhibited by pretreatment with 4.0, 8.0 and 16 nmol 7-NI to 81.1 ± 8.7, 56.4 ± 7.8, and 37.7 ± 12.4%, respectively (Fig. 2D), but not affected by pretreatment with L-NIO (Fig. 2C). These findings suggest that the effect of nAChR (primarily α7-nAChR) activation on CCA blood flow was mediated via an activation of nNOS in the DFA.

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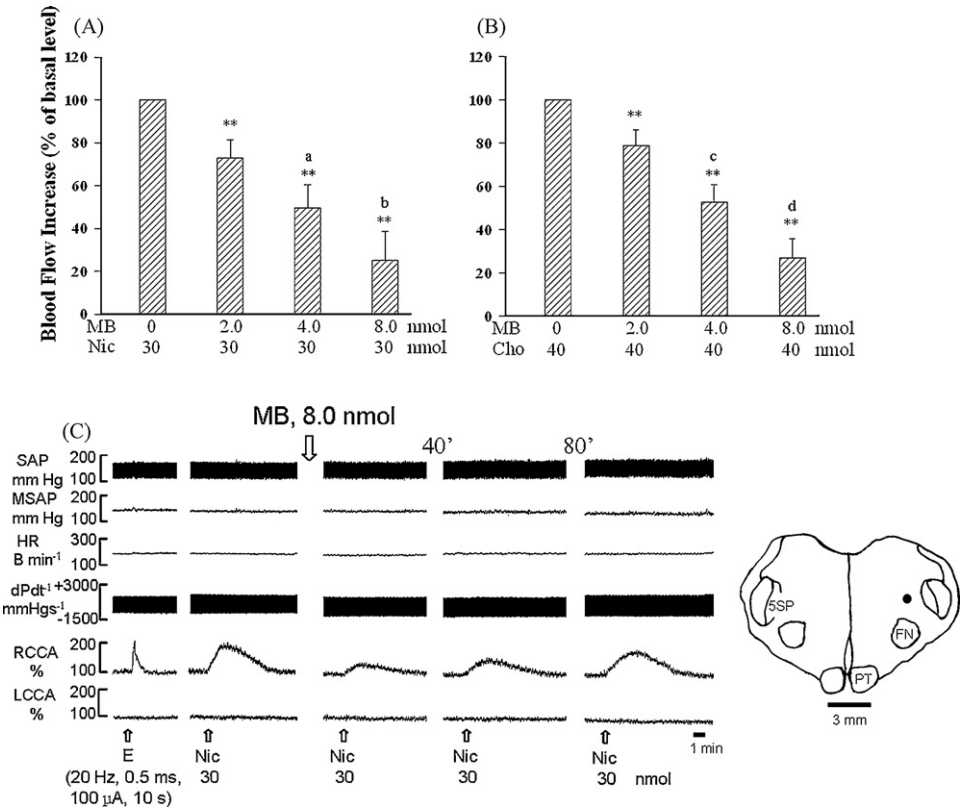

Fig. 3. Intra-DFA pretreatment with a guanylyl cyclase inhibitor MB dose-dependently attenuated the increase in CCA blood flow induced by intra-DFA microinjections of nicotine (A, $n=5$) or choline (B, $n=5$). (C) Original tracings. Data are expressed as mean ± S.E.M. and analyzed by ANOVA and Tukey's test. For A, $^{**}P<0.01$ vs. MB 0 nmol; $^{a}P<0.01$ vs. MB 2.0 nmol; $^{b}P<0.01$ vs. MB 4.0 nmol. For B, $^{**}P<0.01$ vs. MB 0 nmol; $^{c}P<0.01$ vs. MB 2.0 nmol. $^{d}P<0.01$ vs. MB 4.0 nmol. *Abbreviation*: MB, methylene blue.

We further examined whether nicotine or choline might cause guanylyl cyclase activation in the DFA to increase CCA blood flow. The nicotine-induced blood flow increase was dose-dependently inhibited by pretreatment with 2.0, 4.0 and 8.0 nmol methylene blue (MB, a guanylyl cyclase inhibitor) to 72.9 ± 8.5, 49.5 ± 10.9 and 25.2 ± 13.7%, respectively (Fig. 3A). The choline-induced blood flow increase was dose-dependently inhibited by pretreatment with 2.0, 4.0 and 8.0 nmol MB to 78.8 ± 7.2, 52.8 ± 8.1 and 26.9 ± 8.8%, respectively (Fig. 3B). This blockade was recovered by 90% in approximately 80 min (Fig. 3C). These findings suggest that activation of nAChR, primarily α7-nAChR, may induce an activation of guanylyl cyclase in the DFA to increase CCA blood flow.

Our major findings demonstrated that the nicotine- or choline-induced increases in CCA blood flow were dose-dependently reduced by prior administration with L-NAME (Fig. 1A and C), 7-NI (Fig. 2B and D), or MB (Fig. 3), but not by that with D-NAME (Fig. 1B and D) or with NIO (Fig. 2A and C). Central action of various doses of NOS blockades was suggested by the findings that these NOS blockades locally microinjected into the DFA (Figs. 1E, 2E, and 3E) or systemically injected via intravenous route (data not shown) did not affect basal cardiovascular parameters including systemic blood pressure, heart rate and cardiac contractility. Furthermore, pontamine blue (0.1%, 200 nl) microinjected into the DFA appears to diffuse within 1 mm^2 [7,12]. Our novel findings suggest that the activation of nAChR, primarily via α7-nAChR, induce the activation of nNOS/guanylyl cyclase system in the DFA, which then lead to the increase in CCA blood flow.

Our previous experiments have demonstrated that activation of nAChRs primarily via α7-nAChR in the DFA is responsible for the increase in CCA blood flow [8,13]. Alternatively, NOS-containing neurons (nitrergic neurons) are surrounded by profuse NOS-containing fibers (nitrergic fibers) in the DFA, while stimulation of these nitrergic fibers or/and neurons is also responsible for the increase in CCA blood flow [7,12]. In the present study, the increase in CCA blood flow by activation with choline or nicotine on the nAChR was blocked by L-NAME (Fig. 1A and C), 7-NI (Fig. 2B and D), and MB (Fig. 3). Therefore, we suggest that in the DFA, stimulation of the nAChR may activate nNOS and guanylyl cyclase of the nitrergic fibers or neurons, which then cause the increase in CCA blood flow. Whether nAChR is present in these nitrergic neurons or/and fibers is still unknown.

The above question can be answered by referring to our working hypothesis for the DFA [12]. In this hypothesis, glutamatergic fibers innate with a nitrergic mechanism (NOS/guanylyl cyclase/cGMP system) in the DFA are defined as nitrergic–glutamatergic fibers; the preganglionic neurons innate with a nitrergic system and bearing both NMDA and AMPA receptors in the DFA are the preganglionic nitrergic–cholinergic neurons that are presynaptically innervated by the former fibers. Our recent investigation, however, demonstrates that the nAChR-activated increase in CCA blood flow is prominently reduced by combined pretreatment of NMDA and AMPA receptor antagonists, indicating a release of glutamate from the nitrergic–glutamatergic fibers upon activation of nAChRs in the DFA [13]. Therefore, we suggest that the nAChR primarily resides in the nitrergic–glutamatergic fibers. However, the increase in CCA blood flow induced by activation of nAChRs could not be completely abolished by the antagonists of both NMDA and AMPA receptors; presence of less nAChRs on the preganglionic nitrergic–cholinergic neurons in the DFA may be possible.

The reports by Mifflin and colleagues, interestingly, indicate that NO facilitates AMPA-mediated neuronal transmission [5] and plays a modulatory role in the nucleus tractus solitarious (NTS) for cardiovascular regulation [6]. Furthermore, activation of AMPA [4] or acetylcholine [3] receptor stimulates NO production. Whether

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these findings may also suggest a nitrergic–glutamatergic fibers or nitrergic–cholinergic fibers in the NTS for cardiovascular regulation deserve further study.

In conclusion, stimulation of nAChRs, primarily α7-nAChR, caused an activation of the nNOS/guanylyl cyclase system in the DFA, resulting in an increase in CCA blood flow that supplies intra- and extra-cranial tissues. The nAChRs may primarily reside in the nitrergic–glutamatergic fibers. Results from our previous, present and future investigations may provide insights for better pharmacological interventions to the DFA or for developing therapeutic strategy in management of diseases involving CCA blood flow, such as migraine, hypertensive disease and cerebral ischemia.

Acknowledgment

This work was supported by grants from China Medical University (CMU96-241), and the National Science Council (NSC 99-2320-B-039-026-MY3), Taiwan, ROC.

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