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RESEARCH PAPER

Regulation of the common carotid arterial blood flow by nicotinic receptors in the medulla of cats

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Background and purpose: Actions of glutamate and serotonin on their respective receptors in the dorsal facial area (DFA) of the medulla are known to regulate common carotid arterial (CCA) blood flow in cats. Less is known about acetylcholine action on its nicotinic receptor (nAChR) subtypes in the DFA for regulation of CCA blood flow and this aspect was investigated.
Experimental approach: Nicotinic and muscarinic agonists and antagonists were microinjected into the DFA through a three-barrel tubing in anesthetized cats.
Results: CCA blood flow was dose-dependently increased by nicotine (a non-selective nAChR agonist) and choline (a selective α7-nAChR agonist). These effects of nicotine were attenuated by α-bungarotoxin (an α7-nAChR antagonist), methyllycaconitine (an α7-nAChR antagonist), mecamylamine (a relatively selective α3β4-nAChR antagonist) and dihydro-β-erythroidine (a relatively selective α4β2-nAChR antagonist). The choline-induced flow increase was attenuated by α-bungarotoxin and mecamylamine, but not by dihydro-β-erythroidine. Muscarinic agonists (muscarine and methacholine) and antagonist (atropine) affected neither the basal nor the nicotine-induced increase in the CCA blood flow.
Conclusions and implications: Functional α7, α4β2, and α3β4 subunits of the nAChR appear to be present on the DFA neurons. Activations of these receptors increase the CCA blood flow. The present findings do not preclude the presence of other nAChRs subunits. Muscarinic receptors, if any, on the DFA are not involved in regulation of the CCA blood flow. Various subtypes of nAChRs in the DFA may mediate regulation of the CCA and cerebral blood flows.
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Keywords: cholinergic receptor; carotid artery; medulla; nAChR; parasympathetic; vascular regulation

Abbreviations: A-BT, α-bungarotoxin; CCA, common carotid artery; CVLM, caudal ventrolateral medulla; DBE, dihydro-β-erythroidine; DFA, dorsal facial area; DMNV, dorsal motor nucleus of the vagus; Glu, glutamate; HR, heart rate; Mec, mecamylamine; Met, methyllycaconitine; MSAP, mean systemic arterial pressure; nAChRs, nicotinic acetylcholine receptors; nTS, nucleus tractus solitarius; RVLM, rostral ventrolateral medulla; SAP, systemic arterial pressure

Introduction

Kuo *et al*. (1987) first identified the dorsal facial area (DFA), a reticular area just dorsal to the facial nucleus in the cat. Stimulation of the DFA with glutamate evoked mainly an ipsilateral increase in blood flow of the common carotid artery (CCA) without significant changes in systemic arterial blood pressure (Kuo *et al*., 1987; Chyi *et al*., 1995). Glutamate and serotonin, tonically released in the DFA, induce an increase and a decrease, respectively, in the CCA blood flow (Li *et al*., 1996; Kuo *et al*., 1999; Gong *et al*., 2002). The DFA is a parasympathetic nucleus (Kuo *et al*., 1987, 1992, 1995; Chyi *et al*., 1995, 2005). It may be functionally and anatomically equivalent to the rat parasympathetic cerebrovasodilator center (Nakai *et al*., 1993) that is located dorsolaterally to the facial nucleus. Therefore, both areas are likely to be the rostral extension of the dorsal motor nucleus of the vagus (DMNV).

Cholinergic nerves are widely distributed in the cortex (Sato *et al*., 2001; Hotta *et al*., 2002), hippocampus (Sato and Sato, 1995), striatum (Kaiser and Wonnacott, 2000; Zhou *et al*., 2002), hypothalamus (Hatton and Yang, 2002) as well as medulla oblongata such as the rostral ventrolateral medulla (RVLM) (Kubo *et al*., 2000, 2002), DMNV

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and nucleus tractus solitarius (nTS) (Reynolds *et al*., 1994). Stimulation of nicotinic receptors promotes glutamate release that modulates dopamine releases in a rat striatal slice (Kaiser and Wonnacott, 2000). Cholinergic inputs to the RVLM play a vasopressor effect through muscarinic action (Kubo *et al*., 2000, 2002). The cholinergic fibers to the rat cortex release acetylcholine to increase the cerebral blood flow via both muscarinic and nicotinic acetylcholine receptors (nAChRs) in the parenchyma of the cortex (Sato *et al*., 2001). In slice preparations of the rat medulla oblongata, application of acetylcholine to the preganglionic neurons of the DMNV results in marked depolarization through nicotinic action (Ito *et al*., 1989). Nicotinic receptors in specific medullary regions, such as the nTS (Dhar *et al*., 2000; Ferguson *et al*., 2000; Ferreira *et al*., 2001), the DMNV (Ferreira *et al*., 2001), the RVLM and caudal ventrolateral medulla (Huangfu *et al*., 1997; Aberger *et al*., 2001), play important roles in cardiovascular regulation. The DFA as a parasympathetic nucleus or the rostral extension of the DMNV (Kuo *et al*., 1987, 1992, 1995; Chyi *et al*., 1995, 2005), therefore, may quite possibly share some nature of the above-mentioned nuclei that regulate cardiovascular function. Whether nicotinic and/or muscarinic actions and their receptors in the DFA were involved in regulation of the CCA blood flow was not known.

Nicotinic receptors are abundant and play diverse roles in the central nervous system (Decker *et al*., 1995; Colquhoun and Patrick, 1997). The α7-nAChRs are present in the DMNV (Ferreira *et al*., 2000, 2001), the chick sympathetic ganglia (Du and Role, 2001) on the striatal glutamatergic terminals (Kaiser and Wonnacott, 2000) and in the hypothalamic supraoptic nucleus (Hatton and Yang, 2002). Both the α7- and $\alpha3\beta4$-nAChRs are present in the nTS (Dhar *et al*., 2000). The $\alpha3\beta4$-nAChRs are present in the hippocampus (Alkondon and Albuquerque, 2002; Giocomo and Hasselmo, 2005; Cao *et al*., 2005). The $\alpha4\beta2$-nAChRs are present in the substantia gelatinosa (Kiyosawa *et al*., 2001). Both the $\alpha3\beta2$- and $\alpha4\beta2$-nAChRs have been found in the striatum (Kaiser and Wonnacott, 2000). Nevertheless, nAChRs containing $\alpha7$, $\alpha3\beta4$ and $\alpha4\beta2$ subunits are most commonly present in the central nervous system. Hence, we focused on these three subunits on the neurons of the DFA, which have not yet been investigated so far as we know.

Our novel findings demonstrate that at least three different subtypes of nAChRs, $\alpha7$, $\alpha3\beta4$ and $\alpha4\beta2$ subunits, are present on the DFA neurons, and that their activations increase the CCA blood flow. Muscarinic receptors, if any, on the DFA neurons are not involved in regulation of the CCA blood flow.

Materials and methods

General procedures

The experiments were carried out in accordance with the guidelines of the Tzu-Chi University Institutional Animal

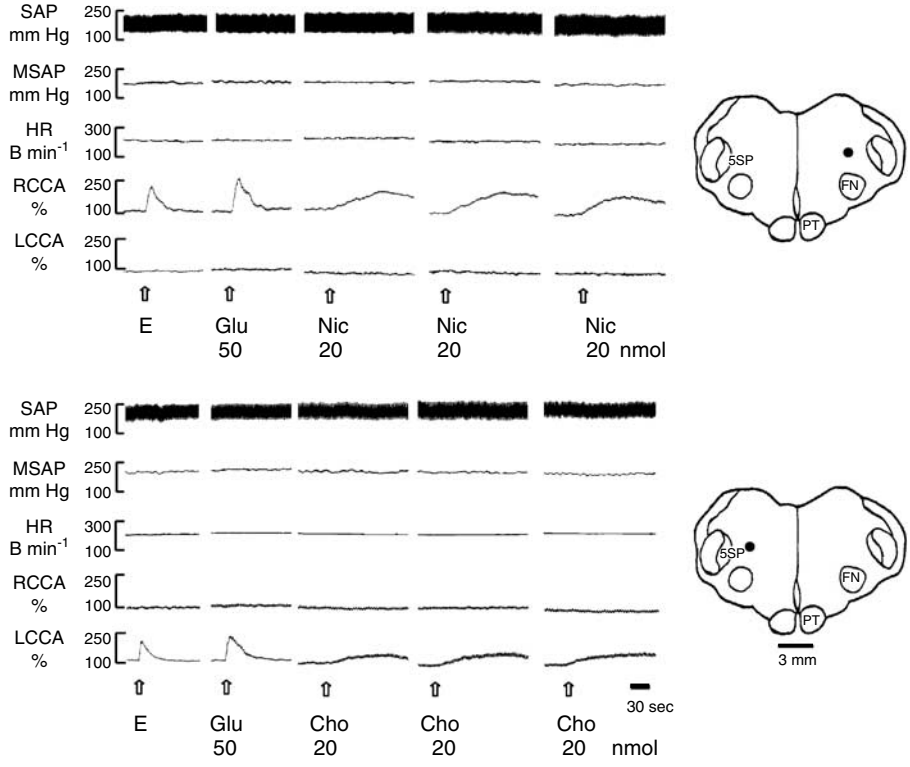

Figure 1 Typical tracings show the reproducible increase in the CCA blood flow induced by repeating microinjections (20 nl s^{-1} for 5 s) of nicotine and choline into the DFA at an interval of 30 min. The DFA was first identified by electrical stimulation (E) and then confirmed by glutamate stimulation. Note the ipsilateral increase in the CCA blood flow without changes in HR, SAP and MSAP. The dots on the drawing of medullary sections indicate the injected loci. Abbreviations for this and the following figures: B min^{-1}, beats per min; Cho, choline; DFA, dorsal facial area; E, electrical stimulation (20 Hz, 0.5 ms, 100 μA, 10 s); FN, facial nucleus; Glu, glutamate; 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.

Care and Use Committee and of the China Medical University Ethical Committee for Animal Research, and were approved by both committees.

Cats (2.0–3.5 kg) of either sex were anesthetized intraperitoneally with α-chloralose (40 mg kg^{-1}) and urethane (400 mg kg^{-1}). End expiratory CO_2 concentration was maintained at 3.5–4.5% by artificial ventilation. The rectal temperature was measured and kept at 37.5±0.5°C by an electrical heating pad. Right femoral artery and vein were cannulated with PE-90 polyethylene tubing for measurement of the systemic arterial pressure (SAP) and supplement of fluid, respectively. The ultrasound Doppler probes (diameter 1.5–2.0 mm) were placed around the right and left CCA and monitored with a Directional Pulsed Doppler Flowmeter (University of Iowa, Bioengineering, 545C-4, Iowa, USA). The SAP, heart rate (HR) and CCA blood flows were routinely recorded on a Gould Recorder RS3800 (Cleveland, OH, USA) as described in our previous papers (Li *et al*., 1996; Gong *et al*., 2002).

Microinjection technique

The head of cats was immobilized in a David-Kopf stereotaxic instrument. The stereotaxic coordinates of the DFA were about 6 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. The three-barrel electrode tubing was constructed with three stainless-steel tubings (0.3 mm in diameter) glued together and insulated, except the tip at one end. It was inserted into the DFA at an angle of 34° from the vertical axis of the stereotaxic instrument. This facilitated the tubing insertion to be perpendicular to the floor of the fourth cerebral ventricle. Each barrel of this tubing was filled with one of the following chemicals: sodium glutamate, nicotine (a non-selective nAChR agonist), choline (an α7-nAChR agonist), α-bungarotoxin (an α7-nAChR antagonist), methyllycaconitine (an α7-nAChR antagonist), mecamylamine (an α3β4-nAChR antagonist), dihydro-β-erythroidine (an α4β2-nAChR antagonist), muscarine (a muscarinic receptor agonist) and methacholine (a muscarinic receptor agonist). All these drugs were dissolved in artificial cerebrospinal fluid (aCSF) containing the chemicals (mM) NaCl 119, KCl 2.5, $MgCl_2$ 4, $CaCl_2$ 4, $NaHCO_3$ 26.2, NaH_2PO_4 1 and glucose 11, and gassed with 95% O_2 and 5% CO_2 at pH 7.4. The aCSF was used as a vehicle control. Each chemical with a volume of 100 nl was microinjected into the DFA in 5 s with the microinjection pump (CMA/100, Carnegie Medicin, North Chelmsford, MA, USA). All chemicals were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA).

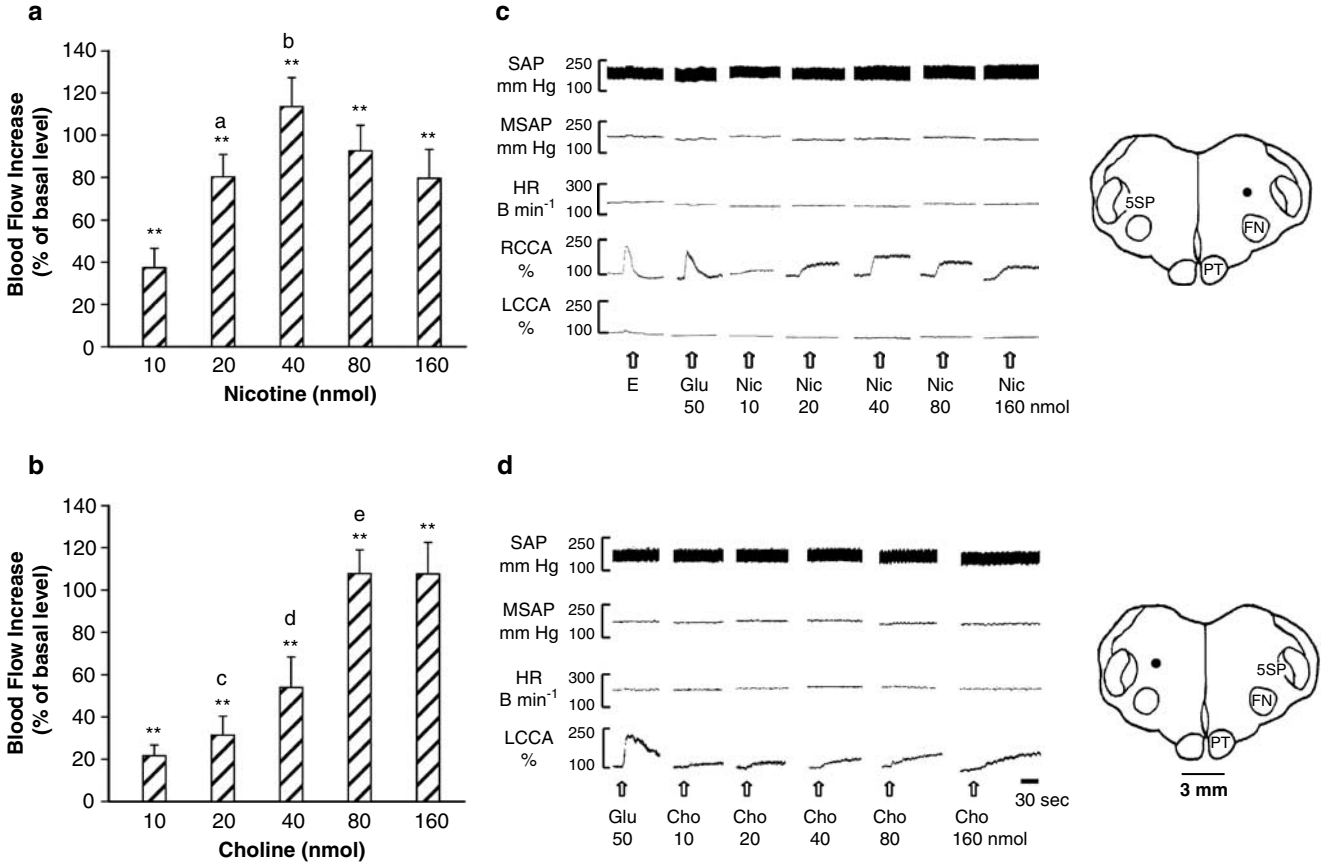

Figure 2 The increase of the CCA blood flow was dose-dependently induced by microinjections into the DFA of nicotine ($n=4$) (**a**, **c**) or choline ($n=4$) (**b**, **d**). (**a**, **b**) Statistical analysis; (**c**, **d**) original tracings and injection loci (indicated by dots) in the DFA. Data are expressed as means±s.e.m. and analyzed by Student's *t*-test. $^{**}P<0.01$ vs vehicle; $^{a}P<0.01$ vs Nic 10 nmol; $^{b}P<0.01$ vs Nic 20 nmol; $^{c}P<0.05$ vs Cho 10 nmol; $^{d}P<0.05$ vs Cho 20 nmol; $^{e}P<0.01$ vs Cho 40 nmol.

Experimental designs

For localization of the DFA by electrical stimulation of it, cats were further paralyzed with atracurium (GlaxoSmithKline S.p.A., Parma, Italy), initially 0.05 mg kg^{-1} and 0.02 mg kg^{-1} intravenously every 20 min, to eliminate interference in recording of blood flow owing to stimulation-induced muscle contraction. The DFA was identified by an increase of the CCA blood flow first induced by the electrical stimulation (20 Hz, 0.5 ms, 100 μA, 10 s) and then by glutamate (50 nmol) stimulation of the DFA through the electrode tubing. The electrode tubing was then maintained there throughout the whole course of the experiment.

The interval for reproducible increases in the CCA blood flow by repeated microinjections of nicotine (20 nmol) and choline (20 nmol) was 30 min (Figure 1, $n=3$ for each drug). For the subsequent experiments, each drug was also injected at an interval of 30 min. Dose–responsive effect of nicotine and choline was determined at doses of 10, 20, 40, 80 and 160 nmol ($n=5$ for each drug).

We determined nAChR subunits in the DFA for regulation of the CCA blood flow. Nicotine (40 nmol) and choline (80 nmol) were microinjected into the DFA to increase the CCA blood flow. This effect was then subjected to the effects of nicotinic antagonists, including α-bungarotoxin (2.0, 4.0 and 8.0 pmol), methyllycaconitine (0.025, 0.05 and 0.1 nmol), mecamylamine (1.0, 2.0 and 4.0 nmol) and dihydro-β-erythroidine (0.25, 0.5 and 1.0 nmol) ($n=4$ for each antagonist). In detail, nicotine (40 nmol) was microinjected to induce an increase in the CCA blood flow. After 30 min, α-bungarotoxin (2 pmol) was injected. After 5 min, the same dose of nicotine was repeated. After 30 min, the same process was repeated for 4.0 and 8.0 pmol α-bungarotoxin. Examination of other nAChR antagonists followed the same procedure as α-bungarotoxin. A similar protocol for choline (80 nmol) was followed.

Whether muscarinic receptors in the DFA might regulate the CCA blood flow was examined in nine animals. Muscarine (10 nmol), methacholine (20 nmol) and atropine (20 nmol) were microinjected into the DFA ($n=3$ for each drug) to determine if these agonists or antagonist affected the basal CCA blood flow.

Histology

The stimulated site of the DFA was marked with a pontamine blue (0.1%, 200 nl) microinjection or a lesion produced by DC current of 2 mA for 10 s through the three-barrel electrode tubing. At the end of the experiment, the cat was killed by saturated KCl administered intravenously. The brain was removed and frozen-sectioned at 40 μm thickness on a Cr40 microtome (2800 Frigocut). Proper placement of the probe was confirmed upon microscopic examination. Only cats with correctly positioned electrode tubing in the DFA were considered for data analysis.

Data analysis

Changes in the SAP, HR and CCA blood flow responding to microinjections of chemicals were calculated as (response value−control value)/(control value) × 100%. Data were expressed as means ± s.e.m. and analyzed statistically by Student's *t*-test. The probability level of a significant difference was $P<0.05$.

Results

Dose-dependent responses of nicotine and choline

The mean systemic arterial pressure (MSAP), HR and CCA blood flow in the normal control cats were 140 ± 28 mm Hg, 238 ± 40 beats min^{-1} and 30 ± 5 ml min^{-1}, respectively ($n=52$). In control experiments, microinjections into the DFA of any drug vehicle used did not affect MSAP, HR and CCA blood flow.

Repeated microinjections of either 20 nmol nicotine or 20 nmol choline into the DFA at an interval of 30 min ($n=3$ for each drug) induced reproducible increases in the CCA blood flow in each animal (Figure 1).

Microinjections of nicotine (Figure 2a and c, $n=5$) or choline (Figure 2b and d, $n=5$) into the DFA caused dose-dependent increases of the CCA blood flow, but did not affect the MSAP and HR (Table 1). Nicotine at doses of 10–40 nmol elicited dose-dependent increases in the CCA blood flow, reaching a maximal increase of 114% as compared with the basal level (Figure 2a). The increase was reduced at higher

Table 1 Effect of intra-DFA microinjection of nicotine or choline on changes of MSAP, HR and both sides of CCA blood flow

nmol	*10*	*20*	*40*	*80*	*160*
Nicotine					
MSAP (%)	5.3 ± 1.7	6.6 ± 0.5	7.7 ± 2.0	6.4 ± 1.3	6.4 ± 1.7
HR (%)	4.8 ± 4.9	5.9 ± 0.8	6.8 ± 1.3	7.6 ± 1.1	6.7 ± 2.1
ICCA (%)	37 ± 9**	80 ± 11**,[a]	113 ± 14**,[b]	93 ± 12**	80 ± 14**
CCCA (%)	5 ± 2	6 ± 1	6 ± 2	7 ± 3	7 ± 3
Choline					
MSAP (%)	3.3 ± 0.6	5.6 ± 1.6	6.5 ± 2.1	7.3 ± 1.8	6.3 ± 1.8
HR (%)	4.8 ± 4.9	4.2 ± 0.8	4.8 ± 2.2	6.6 ± 3.2	6.5 ± 3.1
ICCA (%)	22 ± 5**	31 ± 9**,[c]	54 ± 14**,[d]	108 ± 11**,[e]	108 ± 15**
CCCA (%)	4 ± 1	5 ± 2	5 ± 3	6 ± 2	6 ± 3

CCA, common carotid arterial blood flow; CCCA, contralateral common carotid artery blood flow; DFA, dorsal facial area; HR, heart rate; ICCA, ipsilateral common carotid artery blood flow; MSAP, mean systemic arterial pressure.
$N=5$ for either nicotine or choline group. Values are mean ± s.e.m., **$P<0.01$ vs vehicle; [a]$P<0.01$ vs nicotine 10 nmol; [b]$P<0.01$ vs nicotine 20 nmol; [c]$P<0.05$ vs choline 10 nmol; [d]$P<0.05$ vs choline 20 nmol; [e]$P<0.01$ vs choline 40 nmol by Student's *t*-test.

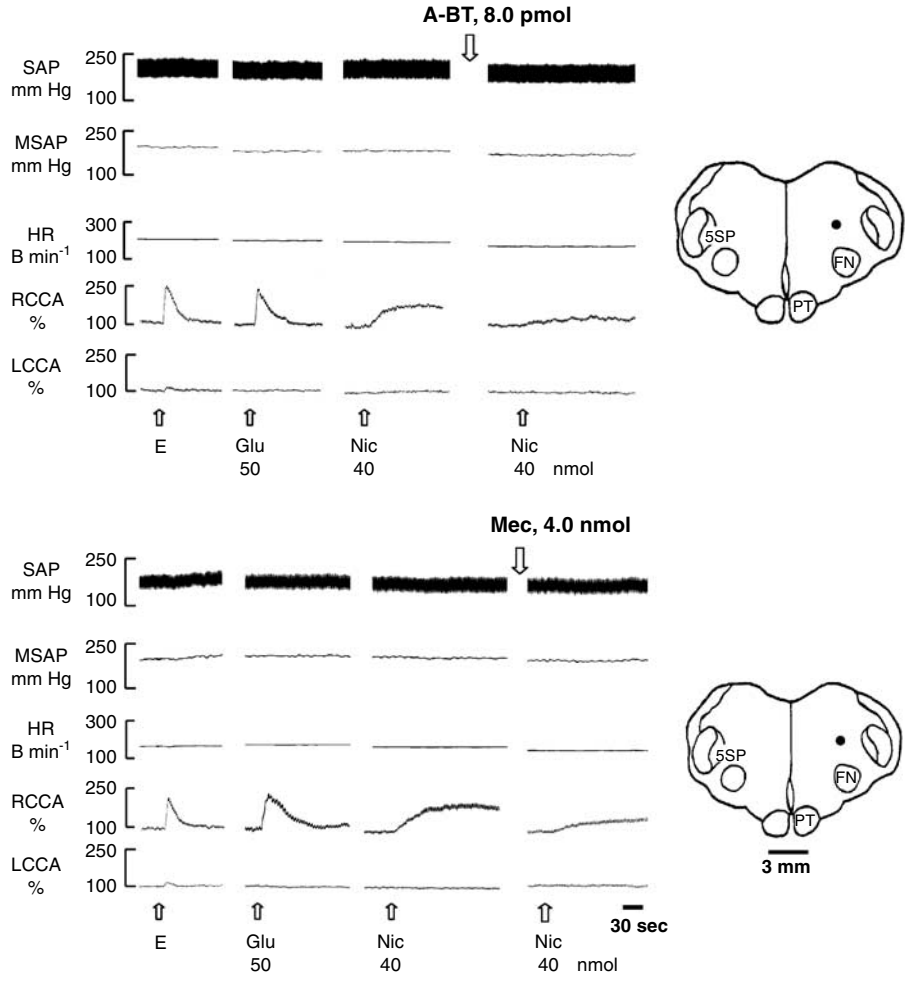

Figure 3 Typical tracings demonstrate that the increase of CCA blood flow induced by microinjection of nicotine into the DFA was markedly inhibited by pretreatment of α-bungarotoxin (α7 nAChR antagonist) or mecamylamine (α3β4 nAChR antagonist) microinjected into the DFA. Abbreviations: A-BT, α-bungarotoxin; Mec, mecamylamine.

doses of 80 and 160 nmol, probably owing to development of tachyphylaxis to nicotine (Zhang *et al*., 1998). Microinjections of choline, at doses similar to nicotine, also dose-dependently increased the CCA blood flow (Figure 2b). Choline at 10–80 nmol elicited dose-dependent increases in the CCA blood flow, reaching a maximal increase of 108% as compared with the basal level. The flow increase was not further increased by a greater dose of 160 nmol. Based on these results, 40 nmol of nicotine and 80 nmol of choline were selected for subsequent studies.

Effects of nAChR antagonists on nicotine-induced increase in the CCA blood flow

To determine whether nicotinic action was mediated by α7-, α3β4- and α4β2-nAChR subunits in the DFA, selective antagonists were used. The increased CCA blood flow induced by microinjection of 40 nmol nicotine into the DFA was markedly inhibited by pretreatment with 8.0 pmol α-bungarotoxin (A-BT, a selective α7-nAChR antagonist) and 4.0 nmol mecamylamine (Mec, a relative selective α3β4-nAChR antagonist) microinjected into the DFA (Figure 3). The nicotine-induced increases in the CCA blood flow were dose-dependently suppressed by pretreatments of A-BT (Figure 4a, $n=4$), methyllycaconitine (Met, a selective α7-nAChR antagonist) (Figure 4b, $n=4$), Mec (Figure 5a, $n=4$) and dihydro-β-erythroidine (DBE, a relative selective α4β2-nAChR antagonist) (Figure 5b, $n=4$). In control experiments, all antagonists used did not affect the basal CCA blood flow.

Effects of nAChR antagonists on choline-induced increase in the CCA blood flow

To further test the involvement of α7-, α3β4- and α4β2-nAChRs in nicotinic action in the DFA, choline, a selective α7-nAChR agonist, was used to elicit increases in the CCA blood flow. The increased CCA blood flow was dose-dependently suppressed by prior microinjections in the DFA of A-BT (Figure 6a, $n=4$) and Mec (Figure 6b, $n=4$). A-BT and Mec alone did not affect the basal CCA blood flow. DBE affected neither the basal nor the choline-

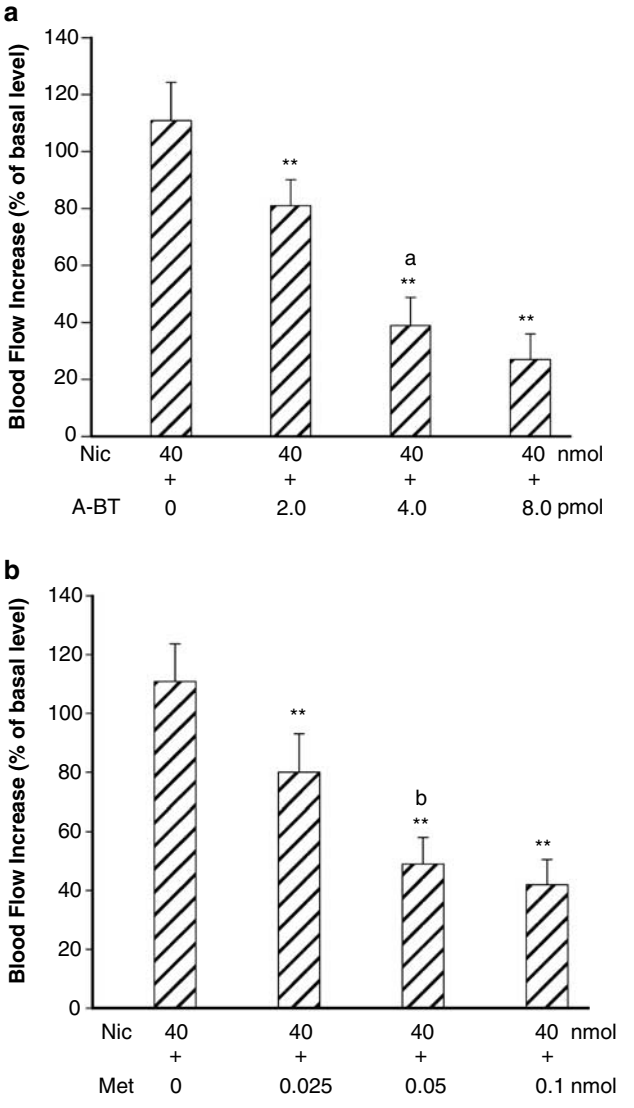

Figure 4 Interactions of nicotine with selective α7-nAChR antagonists (α-bungarotoxin) ($n=4$) (**a**) and methyllycaconitine ($n=4$) (**b**), in the DFA. (**a**) Microinjection of nicotine in the DFA caused an increase of CCA blood flow. The flow increase was dose-dependently reduced by α-bungarotoxin. (**b**) Microinjection of the same dose of nicotine in other group produced an increase of CCA blood flow. This flow increase was dose-dependently reduced by methyllycaconitine. Abbreviations: Met, methyllycaconitine. Data are expressed as means$\pm$s.e.m. and analyzed by Student's *t*-test. $^{**}P<0.01$ vs Nic 40 nmol; $^{a}P<0.01$ vs A-BT 2.0 pmol; $^{b}P<0.05$ vs Met 0.025 nmol.

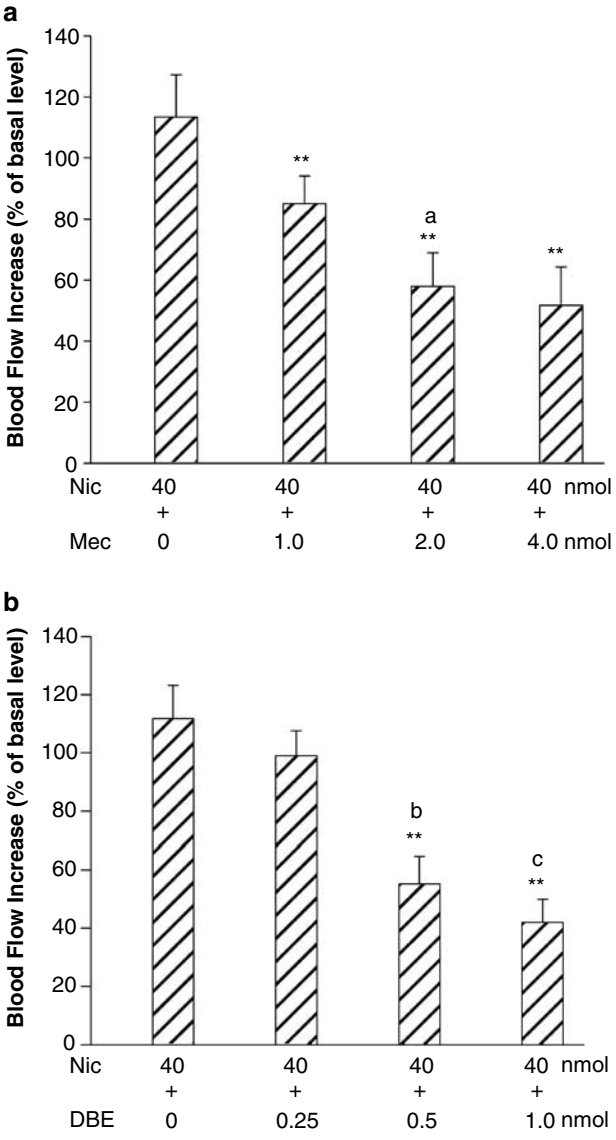

Figure 5 Interactions of nicotine with mecamylamine, $\alpha3\beta4$ nAChR antagonist ($n=4$) (**a**), and dihydro-β-erythroidine, $\alpha4\beta2$ nAChR antagonist ($n=4$) (**b**), in the DFA. (**a**) Microinjection of nicotine induced an increase of the CCA blood flow. The flow increase was dose-dependently attenuated by pretreatment with mecamylamine. (**b**) Microinjection of the same dose of nicotine in another group produced an essentially similar increase of the CCA blood flow. This flow increase was dose-dependently reduced by pretreatment with dihydro-β-erythroidine. Data are expressed as means$\pm$s.e.m. and are analyzed by Student's *t*-test. $^{**}P<0.01$ vs Nic 40 nmol; Abbreviations: DBE, dihydro-β-erythroidine. $^{a}P<0.05$ vs Mec 1.0 nmol; $^{b}P<0.01$ vs DBE 0.25 nmol; $^{c}P<0.05$ vs DBE 0.5 nmol.

induced increase in the CCA blood flow ($n=3$, data not shown).

Effects of muscarine and methacholine

Whether muscarinic receptors were involved in the DFA was assessed by microinjections in the DFA of muscarinic agonists, muscarine (5 nmol in one animal and 10 nmol in two animals) and methacholine (10 nmol in one animal and 20 nmol in two animals). Neither agonists nor antagonist affected the basal CCA blood flow or the MSAP.

Verification of injection site

Figure 7 shows a photograph of the coronal section of the medulla indicating the injected site in the DFA. This site is located 6 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.

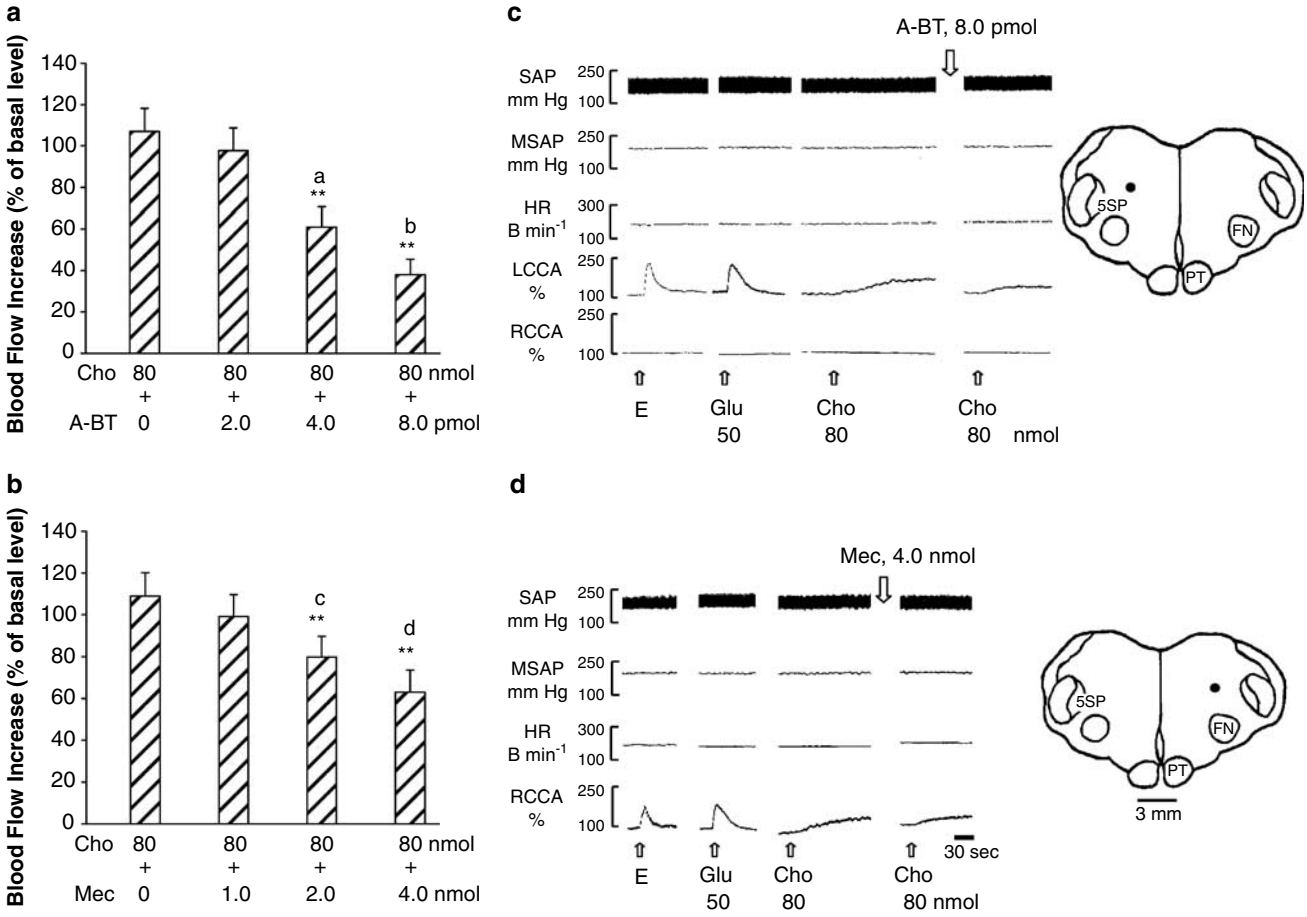

Figure 6 Interactions of choline with α-bungarotoxin, α7 nAChR antagonist ($n=4$) (**a**, **c**), and mecamylamine, α3β4 nAChR antagonist ($n=4$) (**b**, **d**), in the DFA. (**a**) Microinjection of choline resulted in an increase of the CCA blood flow. The flow increase was dose-dependently reduced by α-bungarotoxin. (**b**) Microinjections of the same dose of choline in other group (**b**) produced an essentially similar increase of the CCA blood flow. This flow increase was reduced by mecamylamine. (**a**, **b**) Statistical analysis; (**c**, **d**) original experimental tracings and microinjection sites. The dots on the drawing medulla sections indicate the injected loci. Data are expressed as means ± s.e.m. and analyzed by Student's *t*-test. $^{**}P<0.01$ vs Cho 80 nmol; $^{a}P<0.01$ vs A-BT 2.0 pmol; $^{b}P<0.01$ vs A-BT 4.0 pmol; $^{c}P<0.05$ vs Mec 1.0 nmol; $^{d}P<0.05$ vs Mec 2.0 nmol.

Discussion

In the present experiment, we assessed the effects of various nicotinic and muscarinic agonists and antagonists on the DFA in regulation of the CCA blood flow. The results suggest for the first time that three functional subunits of nAChRs, namely, α7, α4β2 and α3β4 subunits, on the neurons in the DFA participate in regulation of the CCA blood flow. On the other hand, muscarinic receptors, if any, on the DFA neurons do not appear to be involved in regulation of the CCA blood flow.

The presence of nAChRs on the DFA neurons (or synaptic terminals) was suggested from the present finding that microinjections into the DFA of various doses of nicotine, a non-selective nAChR agonist that stimulates various subunits of nAChRs (Chavez-Noriega *et al*., 1997; Si and Lee, 2002; Amtage *et al*., 2004), resulted in a dose-dependent increase in the CCA blood flow (Figure 2a and d). This increase was dose-dependently inhibited by different nicotinic receptor antagonists, A-BT, Met, Mec and DBE (Figures 4 and 5).

The presence of α7-nAChR on the DFA neuron was suggested based on the following findings. First, choline, a precursor of acetylcholine and a metabolic product of acetylcholine, acts as a relatively selective agonist for α7-containing nAChR (Alkondon *et al*., 1997; Si and Lee, 2002). Microinjections of various doses of choline in the DFA resulted in a dose-dependent increase of CCA blood flow (Figure 2b and c). Second, A-BT (CaChelin and Rust, 1995; Lopez *et al*., 1998; Ferreira *et al*., 2000, 2001; Si and Lee, 2002) and Met (Alkondon *et al*., 1997; Si and Lee, 2002), selective antagonists for nAChRs containing α7 subunit, dose-dependently suppressed the increase in the CCA blood flow induced by nicotine (Figure 4) and choline (Figure 6a and c).

The presence of α3β4- and α4β2-nAChRs on the neurons of the DFA was evident from our findings that pretreatments in the DFA of various doses of either Mec (Figure 5a) or DBE (Figure 5b) attenuated the nicotine-induced increase in the CCA blood flow in a dose-dependent manner. Mec and DBE are partial antagonists for nAChRs containing α3β4 and α4β2 subunits, respectively (Alkondon *et al*., 1997; Webster *et al*.,

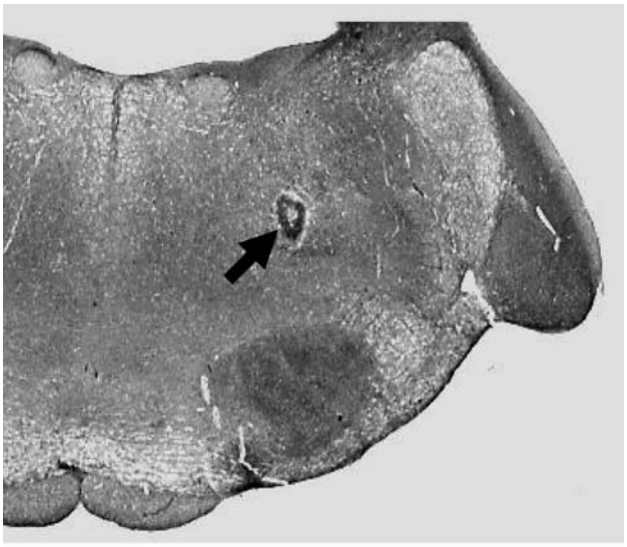

Figure 7 A photograph of a coronal medulla section, 6 mm rostral to the obex, 3.5 mm lateral to the midline and 3.5 mm ventral to the dorsal surface of the medulla, indicates microinjection site (indicated by an arrow) in the DFA.

1999). They, however, have been considered as unselective nAChR antagonists that may act at nAChRs subunits other than $\alpha3\beta4$ and $\alpha4\beta2$ in human (Chavez-Noriega *et al*., 1997; Amtage *et al*., 2004; Si and Lee, 2002). Therefore, the present findings do not preclude the presence of nAChRs subunits other than $\alpha3\beta4$ and $\alpha4\beta2$.

Choline, a selective $\alpha7$ nAChR agonist (Alkondon *et al*., 1997; Si and Lee, 2002), appears to act as a partial agonist at $\alpha3\beta4$-nAChRs on PC12 cells (Alkondon *et al*., 1997). In concert with the finding, the present experiment demonstrated that Mec, a partial $\alpha3\beta4$-nAChR antagonist (Alkondon *et al*., 1997; Webster *et al*., 1999), attenuated the choline-induced increase in the CCA blood flow (Figure 6b), indicating that Mec also partially blocked $\alpha7$-nAChR. The order of preferential targets for Mec in human appears to be $\alpha4\beta4 \sim \alpha2\beta4 > \alpha2\beta2 \sim \alpha4\beta2 \sim \alpha7$ (Chavez-Noriega *et al*., 1997; Amtage *et al*., 2004). Choline, however, did not act as an agonist on $\alpha4\beta2$-nAChR, as DBE did not affect the choline-induced increase in the CCA blood flow (data not shown), supporting the finding that choline did not activate $\alpha4\beta2$-nAChRs on hippocampal neurons (Alkondon *et al*., 1997).

The DFA gives rise to axons that contribute to the parasympathetic preganglionic fibers of the seventh and ninth cranial nerves (Chyi *et al*., 1995, 2005). Through these nerves, glutamate stimulation of the DFA increases the CCA blood flow (Kuo *et al*., 1987, 1992, 1995; Chyi *et al*., 1995, 2005) via AMPA and NMDA receptors on the neurons in the DFA (Gong *et al*., 2002). The release of serotonin (5-HT) in the DFA, acting through 5-HT2 receptor, suppresses the release of glutamate in the DFA (Li *et al*., 1996). The present experiment for the first time demonstrated that activation of $\alpha7$-, $\alpha4\beta2$- and $\alpha3\beta4$-nAChRs in the DFA increased the CCA blood flow. It is not yet known whether these nAChRs are modulated by glutamatergic and serotonergic receptors in the DFA. Nevertheless, nicotinic activation to the DFA may likely mediate through the seventh and ninth parasympathetic nerves to increase the CCA blood flow as glutamatergic and serotonergic activations.

Electrical or glutamate stimulation of the DFA induces not only the increase in the CCA blood flow but also the increase in the cerebral blood flow (Kuo *et al*., 1995). Glutamate stimulation of the parasympathetic cerebrovasodilator center in rats (Nakai *et al*., 1993), an area likely equivalent to the DFA in cats (Kuo *et al*., 1987, 1992, 1995, 1999; Chyi *et al*., 1995, 2005; Li *et al*., 1996; Gong *et al*., 2002), increases cortical blood flow. Whether the cerebral or cortical blood flow is also regulated by nicotinic action in the DFA deserves further investigation.

Muscarinic receptors may not be present on the DFA neurons to regulate CCA blood flow. This was evident from the present finding that microinjections of muscarinic receptor agonists (muscarine and methacholine) and antagonist (atropine) did not affect the basal or the nicotine-increased CCA blood flow. Muscarinic receptors, if any, on the DFA neurons are not likely involved in regulation of the CCA blood flow.

In summary, we demonstrate for the first time that the neurons in the DFA contain at least three functional subunits of nAChRs, namely, $\alpha7$, $\alpha4\beta2$ and $\alpha3\beta4$-nAChRs. Activation of these receptors results in increased CCA blood flow. Nevertheless, the present findings do not preclude the presence of nAChRs subunits other than $\alpha7$, $\alpha3\beta4$ and $\alpha4\beta2$. On the other hand, muscarinic receptors, if any, in the DFA are not likely involved in regulation of the CCA blood flow. Based on our previous (Li *et al*., 1996; Kuo *et al*., 1999; Gong *et al*., 2002) and the present findings, glutamate, serotonin and nAChRs are present in the DFA and may play roles in regulation of the CCA and possibly the cerebral blood flows (Kuo *et al*., 1995).

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Conflict of interest

The authors state no conflict of interest.

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