Current Topics

Functional Changes Induced by Long-Term Stimulation of Nicotinic Acetylcholine Receptors

Na\(^+\)–Ca\(^{2+}\) Exchanger Expression and Its Modulation

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Here we reviewed our recent work on the chronic effects of nicotine on the Na\(^+\)–Ca\(^{2+}\) exchanger (NCX) gene and protein expressions in various organs of rats treated with nicotine in the drinking water for 4—12 weeks. Microarray analysis and reverse transcriptase-polymerase chain reaction (RT-PCR) did not detect significant changes in NCX mRNA expression in cerebral cortex, hippocampus, heart, and skeletal muscle. However, NCX1 protein was up-regulated by nicotine in cerebral cortex and hippocampus, but was down-regulated in the heart. NCX2 protein was up-regulated by nicotine in hippocampus. We suggest that although mRNA change was insignificant, NCX protein expression was altered by chronic nicotine administration in brain and heart in rats. We also reviewed our work on modulators of NCX gene expression and function in cardiac myocytes.

Key words nicotine; Na\(^+\)–Ca\(^{2+}\) exchanger; gene expression; microarray analysis; cardiac myocyte; brain

1. INTRODUCTION

Nicotinic receptor was first cloned by Shosaku Numa and his colleagues.3 Nicotinic receptor is a pentameric ion channel which allows the permeation of cations including Na\(^+\), K\(^+\) and Ca\(^{2+}\). An endogenous ligand, acetylcholine, or an exogenous ligand, nicotine, binds to and activates the nicotinic ion channel and depolarizes the membrane. The depolarization triggers an action potential in neurons and muscles by activating the adjacent Na\(^+\) channels and subsequently Ca\(^{2+}\) channels, leading to Ca\(^{2+}\) entry into the cell. Ca\(^{2+}\) entry induces muscle contraction, neurotransmitter release and activation of various signal transduction pathways. Ca\(^{2+}\) that entered the cells should be extruded; otherwise the cells are overloaded with Ca\(^{2+}\).

Ca\(^{2+}\) extrusion systems from the cell seem to be limited to a rather small number of transporters, including the Na\(^+\)–Ca\(^{2+}\) exchanger (NCX) and the sarcoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) or Ca\(^{2+}\) pump, in contrast to a wide variety of molecules mediating Ca\(^{2+}\) entry, including voltage-gated Ca\(^{2+}\) channels of various types (L, N, P/Q, R, T) and non-selective cation channels, either ligand-gated or voltage-gated. In general, Ca\(^{2+}\) concentration is about 1000 times higher outside than inside the cells. Therefore, it is not possible for Ca\(^{2+}\) to exit from the cells on its own, and a driving force is necessary for Ca\(^{2+}\) to be extruded from the cell. NCX is a membrane transporter which carries one Ca\(^{2+}\) efflux in exchange for 3 Na\(^+\) influx. NCX easily reverses its direction and brings Ca\(^{2+}\) into the cells, if the Na\(^+\) concentration gradient decreases and/or the membrane potential becomes less negative. The following equation is used for the calculation of equilibrium potential of NCX:

\[ E_{NCX} = 3E_{Na} - 2E_{Ca} \]

where \( E_{NCX} \) is the equilibrium potential for NCX, \( E_{Na} \) is the equilibrium potential for Na\(^+\) and \( E_{Ca} \) is the equilibrium potential for Ca\(^{2+}\).

NCX is comprised of 9 transmembrane segments with a long internal loop between the 5th and 6th transmembrane segments.4) There are three isoforms of NCX, namely NCX1, NCX2 and NCX3.5) NCX1 is the ubiquitous type and the one most abundantly expressed in the heart. NCX2 is expressed in the brain and NCX3 in the skeletal muscle. Ni\(^{2+}\) was used as a nonselective inhibitor of NCX6—8) until the first selectively selective NCX inhibitor, KB-R7943, was developed9,10) and subsequently SEA040010 as a more selective NCX inhibitor. In the meantime, we found that various antiarrhythmic drugs listed in the Sicilian gambit, including aprindine, azimilide, bepridil, cibenzoline, amiiodarone and its derivative dronedarone, inhibited cardiac NCX current at a concentration range overlapping or slightly higher than the therapeutic concentrations.11—13) More recently, YM-24476914) and SN-615) were developed as new NCX inhibitors.

It is well known that long-time smoking exerts various hazardous effects including dependency and cancer. Recently, partial agonists of nicotinic receptors have been developed and beginning to be used for the therapy of smoking habit and nicotine dependency.16) The mechanism of carcinogenicity of nicotine has begun to be elucidated.17) Our study was instigated by Katsuura et al.18) who showed in primary cultured neuronal cells from mouse cerebral cortex that chronic nicotine treatment induces up-regulation of nicotinic receptor and Ca\(^{2+}\) channel expression. If the Ca\(^{2+}\) channel expression is up-regulated and Ca\(^{2+}\) entry is enhanced, then the Ca\(^{2+}\) exit system should also be accelerated, otherwise, Ca\(^{2+}\) overload ensues. Therefore, we investigated whether chronic administration of nicotine enhanced NCX expression in various organs of rats. In this review, we present our results on the mRNA and protein expression of NCX and the recent reports relating to the NCX expression.
and the effects of nicotinic stimulation.

2. CHRONIC NICOTINE EFFECT ON THE BODY WEIGHT

The weight loss effect of nicotine had already been well known. During the course of our study, we also observed the effect of nicotine on the body weight of rats. Chronic administration of nicotine (100 mg/l) in the drinking water for 4 or 12 weeks reduced the body weight of rats significantly. The body weights of the control male Wister rats at the beginning (8 weeks of age) were 225±1 g (n=4), and increased to 311±4 g (n=4) after 4 weeks (12 week of age). In contrast, the body weights of Wister rats at 8 weeks of age before nicotine administration were 228±1 g (n=4) and after 4 weeks of nicotine administration were 259±4 g (n=4). Therefore the weight gain during the 4 weeks was 86 g in control, while it was only 31 g in nicotine-treated rats. A similar result of significant difference in body weight was also reported previously.21,22) A possible explanation is that there were no dramatic changes induced by nicotine in the expression levels of the genes including NCX (Figs. 1A, B). Although the microarray analyses indicated no drastic changes induced by nicotine in the expression levels of the genes including NCX, there were no significant differences in the NCX mRNA expression levels in the cerebral cortex and in the hippocampus in the brain. NCX2 and NCX3 protein were also predominantly expressed in hippocampus, and NCX3 protein particularly in the lacunosomolecular region of hippocampus.

3. MICROARRAY ANALYSIS OF VARIOUS ORGANS OF RATS WITH CHRONIC NICOTINE ADMINISTRATION

We administered nicotine (100 mg/l) in the drinking water of rats for 12 weeks. The mRNA of various organs including brain, heart, lung, liver, blood, kidney, and skeletal muscles were extracted. Using these samples, microarray analyses were performed. The detailed method is described below in the ‘RNA Preparation’ and ‘Acquisition of Gene Expression Profiles.’

The samples were from two control rats and three rats with nicotine administration. The microarray analysis revealed that there were no dramatic changes induced by nicotine in the expression levels of the genes including NCX (Figs. 1A, B).

Although the microarray analyses indicated no drastic changes with nicotine, it has been reported that nicotine induced modest but significant changes in the gene expression of nicotinic receptors and Ca2+ channels.21,23) Therefore, we further investigated the rats with 4-week nicotine treatment using real-time reverse-transcription polymerase chain reaction (real-time RT-PCR) and Western blot, whether chronic nicotine administration had any effect on NCX mRNA and protein levels in various organs.

RNA Preparation The tissues were lysed with TRIzol Reagent (Invitrogen, Carlsbad, CA U.S.A.). Total RNA was extracted from the tissue lysate according to the manufacturer’s instructions. Poly(A)+RNA was purified from the total RNA using a MicroPoly(A)Purist Kit (Ambion, Austin, TX, U.S.A.) according to the manufacturer’s instructions. The purified poly(A)+RNA was divided into aliquots of 1.5 μg, precipitated with ethanol, and stored at −20 °C.

Acquisition of Gene Expression Profiles Synthetic polynucleotides of 80 mer representing 11468 species of rat transcripts (MicroDiagnostic, Tokyo, Japan) were printed on a glass slide using a custom-made arrayer.24—26) The aliquots of 1.5-μg RNA were subjected to first-strand cDNA synthesis in the presence of Cyanine-5 dioxuryridine triphosphate (dUTP) (PerkinElmer, Boston, MA, U.S.A.) for samples obtained from the rat tissues or Cyanine-3 dUTP (PerkinElmer) for a common reference RNA (MicroDiagnostic) in a reaction mixture derived from a labeling and hybridization kit (MicroDiagnostic). The red fluorescence-labeled cDNA of the samples and the green fluorescence-labeled common reference RNA were equally mixed and hybridized to a microarray printed with the 80-mer polynucleotides using the labeling and hybridization kit. The hybridized microarray was washed with the labeling and hybridization kit and subsequently scanned by using a GenePix 4000A scanner (Axon Instruments, Union City, CA, U.S.A.). Fluorescence signals were detected and processed by GenePix Pro 3.0 software (Axon Instruments). The processed raw data (median of ratios) were normalized by multiplying with the normalization factors provided by the GenePix Pro 3.0 software. The normalized data (expression ratios) were converted into log 2 values (designated log ratios).

4. BRAIN

Uniquely localized expression of NCX isoforms in rat brain were reported by Papa et al.27) using in situ hybridization and immunohistochemical analysis. According to their report, NCX1 was expressed mainly in the granular layer of the cerebral cortex and in the hippocampus of the brain. NCX2 and NCX3 protein were also predominantly expressed in hippocampus, and NCX3 protein particularly in the lacunosomolecular region of hippocampus.

We investigated the three isoforms of NCX mRNA and protein in the cerebral cortex and hippocampus of rat brain after 4 weeks of nicotine administration in the drinking water. We found that in cerebral cortex, NCX2 mRNA was more easily detected compared to NCX1 mRNA, but there was no statistical difference in the expression levels of NCX1, NCX2 or NCX3 mRNA (Fig. 2A). However, the protein level of NCX1 in the cerebral cortex was significantly increased by nicotine (Fig. 2B). In the hippocampus, NCX2 mRNA was most readily detected but nicotine did not induce any significant difference in the NCX mRNA expression levels from the control (Fig. 3A). However, protein levels of NCX1 and NCX2 increased by 1.5 and 2.7 folds, respectively, in the hippocampus of nicotine-administered rats (Fig. 3B).

5. HEART

NCX1 is abundantly expressed in the cardiac myocytes and expels Ca2+ at each beat under physiological conditions.28) In pathological conditions where [Na+]i, is increased, such as during cardiac ischemia/reperfusion, NCX1 functions in a Ca2+ entry mode and become arrhythmogenic. Recently, we showed in guinea pig cardiac myocytes that NCX could be a target of preconditioning, which prevents ischemia reperfusion injury.29)

Long QT syndrome (LQTS) is associated with sudden cardiac death resulting from torsades de pointes (TdP), which are triggered by early afterdepolarizations (EADs). The cardiac NCX has been suggested to work as a trigger for EADs. In guinea pig cardiac ventricular myocytes, we also demon-
Fig. 1A. Overview of the Entire Gene Expression Profiles

Gene expression profiles obtained from the samples were converted into log ratios and assembled into a single data matrix. Rows and columns of the data matrix represent transcripts (11468) and samples (30), respectively. Rows of the matrix are aligned according to the result of clustering analysis; dendrogram is not shown. Tissues and experimental conditions (C: control, N: nicotine-administered) are shown at the top of the data matrix. A color bar indicates higher levels of gene expression in the sample in red gradation and lower levels in blue gradation when compared with those in the common reference RNA.

Fig. 1B. Expression of NCX-Related Transcripts

Gene expression profiles obtained from the samples were converted into log ratios and assembled into a single data matrix. Rows and columns of the data matrix represent transcripts (55) and samples (30), respectively. Tissues and experimental conditions (C: control, N: nicotine-administered) are shown at the top of the data matrix. The gene symbols and accession numbers for the 55 species of the transcripts are shown on the left of the matrix. A color bar indicates higher levels of gene expression in the sample in red gradation and lower levels in blue gradation when compared with those in the common reference RNA.
strated an oscillatory NCX current which could be inhibited by KB-R7943. Recently, Milberg et al. tested a selective NCX inhibitor, SEA0400, on Langendorff-perfused rabbit hearts with the IKr-blocker sotalol and veratridine, an inhibitor of sodium channel inactivation. These drugs significantly increased the monophasic action potential duration, thereby mimicking LQTS2 and LQTS3. When the K<sup>+</sup> concentration was lowered, sotalol (56%) and veratridine (63%) induced TdP. Perfusion with SEA0400 suppressed EADs in sotalol and veratridine-treated hearts. SEA0400 significantly shortened the action potential duration and reduced the dispersion of repolarization in both groups. These effects lead to the reduction of TdP incidence in the sotalol and the veratridine group. They further demonstrated using a computer model of rabbit ventricular myocytes that the blockade of NCX activity accelerated the repolarization of cellular action potential and prevented EADs. They suggested a therapeutic benefit of selective NCX inhibition in LQTS.

In heart failure, NCX1 mRNA and protein expressions are up-regulated in animal models and human heart. Xu et al. studied the promoter region of NCX1 gene responsible for NCX up-regulation in response to α-adrenergic stimulation in adult and neonatal cardiac myocytes from rat, cat and mouse. Maeda et al. in our laboratory found that fluvastatin, an hydroxymethylglutaryl-coenzyme A (HMG)-CoA reductase inhibitor used to treat high cholesterol, reduced NCX1 expression levels in H9c2 cells, which were originally derived from rat neonatal ventricle. The reduction in NCX1 expression was mediated by a small GTPase, RhoB, which required geranylgeranyl pyrophosphate for its activation to stabilize NCX1 mRNA. Maeda et al. also found that lysophosphatidylcholine, an activator of Rho signaling, enhanced NCX1 expression. We do not yet know the significance of those pathways in physiological or pathological circumstances.

Nicotine is known to increase the risk of sudden cardiac death. Satoh investigated the effects of nicotine on the spontaneous action potentials and ionic currents of rabbit sinoatrial (SA) nodal cells using current-clamp and whole-cell voltage-clamp modes. Nicotine (30 μM to 1 mM) produced a negative chronotropic effect in a concentration-dependent manner. Nicotine at 300 μM significantly decreased the maximum rate of depolarization by about 10%. Atropine (1 μM) and hexamethonium (1 mM) did not modify the nicotine-induced effects. Nicotine at 300 μM inhibited the L-type Ca<sup>2+</sup> current at 10 mV by about 20%. He concluded that nicotine depresses the action potentials and induces negative chronotropic effect due to inhibitions of the ionic currents in the SA nodal cells. Satoh further demonstrated in guinea pig cardiomyocytes that a high concentration of nicotine...

Fig. 2. NCX Expression in Cerebral Cortex
(A) mRNA ratio between NCX1, NCX2 or NCX3 and GAPDH from control and nicotine treated rat brains. (B) NCX proteins. Upper part indicates Western blot results of NCX1 and actin. The lower graph compares protein ratio between NCX and actin.

Fig. 3. Hippocampus
(A) mRNA ratio between the RT-PCR products of each NCX isoform and GAPDH form control and nicotine treated rat brains. (B) Protein ratio between NCX1 or NCX2 and actin from control and nicotine treated rat brain.
(30 μM to 1 mM) inhibited the Ca$^{2+}$ current, the inwardly rectifying K$^+$ current (IK1) and the delayed rectifier K$^+$ current (IKr), but not slowly activated delayed rectifier K$^+$ current (IKs). These effects of nicotine were not modified by atropine, hexamethonium, or nicotinic receptor antagonists (d-tubocurarine and benzoquinonium). He suggested that nicotine inhibits the ionic currents with relatively high sensitivity to IKr and IKr, resulting in the modulation of cardiac functions.

Wang et al.\textsuperscript{37} reported in canine ventricular myocytes and Xenopus oocytes with over-expressed Kv4.3 and Kv4.2 channels that nicotine potently inhibited Kv4 current with IC$_{50}$ of 40 nmol/l, and the current was abolished by 100 μM nicotine. The IC$_{50}$ for the blockade of native transient outward K$^+$ current (Ito) was 270 nm/l. Nicotine reduced single-channel conductance, open probability and open time, but increased the closed time of Kv4.3. The effects of nicotine were not altered by antagonists of various neurotransmitter receptors, indicating direct effects on Ito channels. Thus, nicotine is a potent inhibitor of cardiac Ito type K$^+$ channels that probably blocks both closed and open channels. This action may contribute to the ability of nicotine to affect cardiac electrophysiology and induce arrhythmias.

Apoptosis develops in several heart diseases, but therapeutic options are limited. Suzuki et al.\textsuperscript{38} hypothesized that nicotine, which inhibits apoptosis in several types of cells, inhibits cardiac apoptosis induced by lipopolysaccharide (LPS). Over-the-counter nicotine products can sustain circulating LPS (30 μM to 1 mM) inhibited the Ca$^{2+}$ current, the inwardly rectifying K$^+$ current (IK1) and the delayed rectifier K$^+$ current (IKr), but not slowly activated delayed rectifier K$^+$ current (IKs). These effects of nicotine were not modified by atropine, hexamethonium, or nicotinic receptor antagonists (d-tubocurarine and benzoquinonium). He suggested that nicotine inhibits the ionic currents with relatively high sensitivity to IKr and IKr, resulting in the modulation of cardiac functions.

We demonstrated in rat heart that NCX1 mRNA was abundant, but no NCX2 or NCX3 mRNA were detected (Fig. 4A). NCX1 mRNA level was not affected by nicotine treatment for 4 weeks (Fig. 4A). The protein level of NCX1 in rat cardiac ventricle was significantly reduced, to about 60% of control (Fig. 4B). This decrease is opposite to the effect of nicotine on NCX1 in the brain, and the cause of this discrepancy is currently unknown.

6. SKELETAL MUSCLE

HMG-CoA reductase inhibitors, or “statins,” induce adverse effects on skeletal muscles including rhabdomyolysis. Recently, Sakamoto et al.\textsuperscript{39,40} in our laboratory investigated the effect of fluvastatin, an HMG-CoA reductase inhibitor, on isolated rat skeletal myofibers, and found that statin induced the formation of vacuoles and blebs in myofibers. They further found that the inactivation of Rab small GTPases by fluvastatin is the most likely cause of vacuolation in skeletal myofibers. Nicotine is known to inhibit tendon healing.\textsuperscript{41} Recently an effect of smoking on skeletal muscle has been reported.\textsuperscript{42} According to the report, there was structural and metabolic damage in peripheral muscles of smokers. They investigated the vastus lateralis muscle in 14 smokers and 20 healthy control subjects. In smokers, type I muscle fiber cross-sectional area was decreased, and a similar trend was found in type Iia fibers. Lactate dehydrogenase levels and the percentage of fibers with low oxidative and high glycolytic capacity were increased in smokers. Neuronal nitric oxide synthase (nNOS) and endothelial NOS (eNOS) levels were increased in smokers.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Cardiac Ventricle}
\end{figure}

(A) mRNA ratios between each NCX isoform and GAPDH from control and nicotine treated rat hearts. (B) Protein ratio between NCX1 and actin from control and nicotine treated rat hearts.
were lower in smokers. They concluded that smokers presented with some alterations of skeletal muscle, such as oxidative fiber atrophy, increased glycolytic capacity, and reduced expression of the constitutive NO synthases (eNOS and nNOS).

We found in adult rat skeletal muscle (gastrocnemius) a high level of NCX3 mRNA which was not affected by nicotine administration for 4 weeks (Fig. 5A). The protein levels of NCX3 showed a tendency to decrease but the difference was not statistically significant (Fig. 5B).

7. BLOOD VESSELS

Insulin resistance and smoking are significant risk factors for cardiac and cerebral vascular diseases. Because vascular smooth muscle cells play a key role in the development and progression of atherosclerosis, Wada et al.44) investigated the effect of nicotine on insulin-induced mitogenic signaling in aortic vascular smooth muscle cells isolated from Sprague-Dawley rats. They found that acute exposure to nicotine enhances insulin-induced mitogenesis predominantly by affecting the phosphorylation of p44/42-MAPK and that chronic exposure further augments the insulin signal via up-regulation of alpha7-nAChR, which may be crucial for the development and progression of atherosclerosis in large vessels.

In the above-mentioned study43) with vascular smooth muscle, Src kinase and MAP kinase were activated by nicotine. In our laboratory, Hinata et al.,44) found that acute administration of H2O2 enhanced NCX current in guinea pig ventricular myocytes, and this effect was mediated by two different signaling pathways. An initial common pathway was the H2O2 conversion to -OH, which activated pertussis toxin-sensitive Gi/o protein and subsequently MAP kinase (MEK). Then a low concentration of H2O2 activated Na+/H+ exchanger via phosphoinositide 3-kinase, increased intracellular Na+ concentration and increased the Ca2+ entry mode of NCX in cardiac myocytes. On the other hand, a high concentration of H2O2 (above 1 mM) activated Src and enhanced NCX. Direct tyrosine phosphorylation by Src was not found in NCX and the enhancing mechanism of NCX by Src is still unclear.44)

Recent studies show that nAChRs are expressed in a variety of non-neuronal tissues including the lung, and stimulation of those non-neuronal nAChRs can induce cell proliferation and angiogenesis.45,46) Analysis of the molecular mechanisms underlying nicotine-mediated cell proliferation showed the involvement of Src kinase.45)

Src kinase also has a pivotal role in neuronal nAChR signaling.46) Tyrosine phosphorylation and kinases regulate muscle and neuronal nAChRs. Thus the synaptic distribution of nAChRs at the neuromuscular junction is regulated by Src-family kinases (SFKs). SFKs activate heteromeric neuronal nAChRs in adrenal chromaffin cells, thereby enhancing catecholamine secretion. In contrast, the activity of homomeric neuronal nAChRs, as found in the hippocampus, is negatively regulated by tyrosine phosphorylation and SFKs. It appears that tyrosine kinases provide the means to regulate all nAChRs; but the functional consequences, even those caused by the same kinase family, are specific for each receptor subtype and location.

8. ADRENAL CHROMAFFIN CELLS

Adrenal chromaffin cells of adrenal medulla express nAChRs with α3 and β4 subunits that have Na+, K+ and Ca2+ permeability.47) Homomeric α7 nicotinic receptors are expressed in a species-dependent manner and do not contribute to catecholamine secretion. nAChR expression increases, when cells are chronically exposed to either selective antagonists or agonists such as nicotine, a protocol mimicking the condition of chronic heavy smokers. In this case, up-regulation of nAChRs occurs even though most of the extra nAChRs remain inside the cells, creating a mismatch between the increase in total nAChRs and increase in functional nAChRs on the cell surface.47)

NCX1 is expressed in adrenal chromaffin cells.48) Pintado et al.49) found in bovine adrenal chromaffin cells that KB-R7943 inhibited Na+-gradient-dependent 45Ca2+ uptake into chromaffin cells with an IC50 of 5.5 μM. However, they found that KB-R7943 also inhibited ACh-stimulated 45Ca2+ entry with an IC50 of 6.5 μM. In oocyte expression systems, α3β3 and α3 nAChRs were blocked by KB-R7943 with IC50 of 0.4 μM and the Hill coefficient of 0.9. They concluded that KB-R7943 is a potent blocker of nAChR and may be an open channel blocker of rat brain α3β3 nAChR. Soma et al.50) tested SEA0400, another inhibitor of NCX, on Na+–depend-
ent Ca\(^{2+}\) uptake and catecholamine release and found that SEA0400 inhibited Na\(^+-\)dependent Ca\(^{2+}\) uptake with an IC\(_{50}\) of 40 nm and ACh-stimulated catecholamine release with IC\(_{50}\) of 100 nm. Similar to KB-R7943, SEA0400 also blocked \([\text{H}]\) nicotine binding with IC\(_{50}\) of 90 \(\mu\)M, indicating that both NCX inhibitors had an effect on nAChRs.

We detected expression of NCX1 and NCX2 mRNA in PC12 cells from rat pheochromocytoma (see Fig. 1 in ref. 51) So far, we could not detect significant changes in protein expression levels of NCX in PC12 cells with chronic nicotine treatment.\(^{51}\)

9. CONCLUSION

We found that chronic nicotine treatment in rats \textit{in vivo} did not significantly change the mRNA levels of NCX in cerebral cortex, hippocampus, heart, and skeletal muscle. However, protein level of NCX1 was increased in cerebral cortex and hippocampus. In contrast, a decrease of NCX1 protein was detected in rat cardiac ventricle. At the moment, we do not know the reason for these changes. However, modest but significant changes of gene expression have been reported with nAChR and Ca\(^{2+}\) channels in various organs, and therefore we think that our results are not an exception. It is necessary to further pursue the functional aspect of the changes in gene expression and also the mechanism of gene expression change by nicotine. We hope that these studies will one day clear the smoke surrounding nicotine, as nicotine is still a drug in a pharmacological black box.

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