ART & TECHNOLOGY PUBLISHING PTE. LTD.

Biomed Communication

2023 Volume 3, Issue 1

ISSN: 2529-7635

The Physiological Effects of the Non-Neuronal Cardiac Cholinergic System on the Heart and Extra-Cardiac Organs

Yoshihiko Kanikuma*

Department of Bioregulatory Science, Graduate School of Medicine, Nippon Medical School, Bunkyo, Tokyo 113-0031, Japan

Corresponding author: Yoshihiko Kanikuma, k12417853@nms.ac.jp

Abstract

It has been independently reported that cardiomyocytes possess a system to synthesize acetylcholine (Ach), which is called non-neuronal ACh, and therefore, this system in the heart is named the non-neuronal cardiac cholinergic system (NNCCS). This system is involved in many cardiac physiological aspects including cardiac homeostasis, for example, negative regulation of oxygen consumption, enhancement of glucose preference as a cardiac energy substrate, acceleration of angiogenesis, and upregulation of gap junction function, etc., in other words, enhancement of resilience against damages by ischemia or hypoxia in the heart. The history of the establishment of the NNCCS concept, significant findings of NNCCS functions, the pathogenic roles in the impairment of NNCCS, the link between NNCCS and extra-cardiac organs through the vagal nerve, and its influences on extra-cardiac organ functions are provided in this review.

Keywords

Acetylcholine (ACh)
Non-neuronal cardiac cholinergic system (NNCCS)
Heart
Oxygen consumption
Energy metabolism
Angiogenesis
Gap junction
Anti-ischemia/hypoxia
Blood brain barrier
Anti-inflammation

1. Introduction

The concept of non-neuronal acetylcholine (ACh) is distinct from ACh, which is a transmitter at the inter-synaptic or neuromuscular junction and is derived from its intracellular synthetic potential by non-neuronal cells that are not originally part of the nervous system. In other words, non-neuronal ACh is synthesized by cells outside the nervous system and thus exists independently of ACh derived from the original parasympathetic nervous system. Such a non-neuronally derived ACh-producing system is termed the non-neuronal cholinergic system (NNCS). Many such cell groups

have been reported, including immunocompetent cells (T and B cells), tracheal epithelial cells, and vascular endothelial cells [1-3]. This system was first reported to retain in cardiac myocytes [4], which had not been previously reported, and this intracardiac production of ACh has important effects on cardiac myocytes and physiological functions of the heart, hence the system is referred to as the non-neuronal cardiac cholinergic system (NNCCS) as there was no unified name, but the expression NCS in the heart is sometimes used for the same meaning. This review focuses on the NNCCS and discusses the physiological functions and significance of the NCS-derived ACh in the heart, as well as its apparent and significant effects on non-cardiac organs. Although the findings will probably come as a great surprise to readers who are new to the concept, about half of these findings have been independently by other research organizations as well, and a global consensus seems to be well established.

2. ACh production in cardiomyocytes

The synthesis or production of ACh is carried out by a very simple system involving choline acetyltransferase (ChAT), an ACh synthase, to synthesize ACh from choline and acetyl CoA. Therefore, if ACh is produced in cardiomyocytes, they must have at least CHT1 and ChAT for choline uptake, and a transporter, vascular Ach transporter (VAChT), to store the ACh synthesized in the cell. In addition, the presence of acetylcholinesterase (AChE), which is involved in ACh degradation, regulates ACh levels.

The expression of the aforementioned components in cardiomyocytes was confirmed in a previous study using immunohistochemical and cytochemical staining [4]. In addition, HPLC measurement of ACh in cell extracts from primary cultures of rat cardiomyocytes, in which admixture of other cellular components was removed as far as possible and cardiomyocytes were contracting synchronously on one side of the culture dish, showed that intracellular ACh was only detected in the presence of the AChE inhibitor physostigmine [4].

When measured by HPLC using samples only at the ventricular level (left and right ventricular muscles) with completely ablated atria, where the cardiac vagal nerve (a nerve in the parasympathetic nervous system that is preferentially distributed in the heart) is more abundant, ACh was well detected in the ventricular-derived samples [4].

It is well known that the distribution of vagal nerve endings in the ventricular muscles of rodents and humans is much smaller and sparser than in the sympathetic nervous system [5]. Nevertheless, ACh is found in the ventricle myocardium, suggesting that ACh is produced at the cardiomyocyte level, i.e. cardiomyocytes are capable of producing ACh, which was reported as NNCCS in 2009 [4].

Other research groups later reported similar findings, albeit with different methods: Rana *et al.* compared ACh content at the atrial and ventricular levels in rat hearts and reported that ACh was also detected at the ventricular level and that the ACh-producing capacity of young and old rat hearts was reduced with aging [6]; Rocha-Resende *et al.* showed that ACh content in ventricular myocytes of adult rat hearts was increased in the presence of AChE inhibitors and that the β-receptor agonist isoproterenol suppressed cardiomyocyte hypertrophy, and reported ACh production capacity in adult rat cardiomyocytes [7].

As aforementioned, the successive reports of ACh production in the heart or cardiomyocytes concentrated in a short period of time from 2009 to 2012, have almost reached a consensus on non-neuronal ACh or NNCCS in the heart. A conceptual diagram of the NNCCS is shown in **Figure 1**, and the functions represented in it are described.

3. Physiological roles or functions of the NNCCS

3.1. Physiological functions within the heart

It is now clear that ACh is produced in cardiac

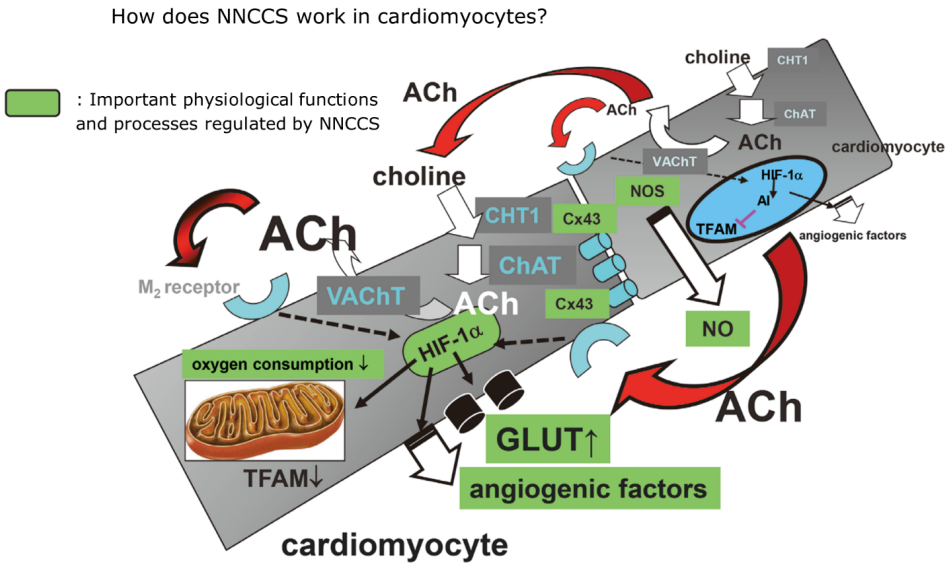

Figure 1. NNCCS is involved in various functions in the heart. ACh produced within cardiomyocytes is thought to act on itself and neighboring cardiomyocytes in an autocrine/paracrine fashion. However, the point of action of ACh within the cell is unclear. Abbreviations: AI, apoptosis inhibitor; TFAM: mitochondrial transcription factor A; NOS: nitric oxide synthase.

myocytes, but the meaning of ACh production in cardiomyocytes and the heart has yet to be discussed. Although there have been many reports demonstrating the existence of NNCCS, unfortunately, the analyses and reports on its function have been limited to several research organizations [7,8].

In other words, the NNCCS intrinsic to the heart is an essential system for the maintenance of homeostasis in pathological conditions (pathophysiology) of the heart, which also means that it is cardioprotective. Among the various pathological states of heart disease, it has been reported that NNCCS plays a particularly important role in cardioprotection against cardiac hypertrophy, myocardial ischemia, and infarction [9]. Another way of describing this is the reduction of physiological or pathological stress load on the heart [7]. Thus, dysfunction of NNCCS may result in accelerated cardiac remodeling and cardiac hypersympathetic nervous system responses [8,10,11].

Among the autonomic nervous systems that control the heart, it is known that the predominance of the parasympathetic nervous system over the sympathetic nervous system reduces the load on the heart of an individual, resulting in a relatively better cardiac life span or prognosis of cardiovascular disease [12]. Based on such reports, it is easy to imagine the cardioprotective effect of NNCCS, even if it is derived from NNCCS and independent of the parasympathetic nervous system, since it is produced in the same way as ACh. However, the detailed molecular mechanism as to why this might be the case was very poorly reported at the time. Therefore, several relevant reports were made and described in the following subsections.

3.1.1. ACh has the effect of increasing the level of the hypoxia-inducible factor-1α (HIF-1α) protein in cardiomyocytes via activation of the non-hypoxia-inducible pathway [13]

Cells exposed to a hypoxic environment will upregulate the expression of a set of genes regulated by hypoxia-inducible factor-1α (HIF-1α) in order to adapt and survive in the given environment. Due to the hypoxic environment, they usually adapt by altering their own intracellular metabolism to compensate for ATP from the glycolytic system. On the other hand, treatment with ACh, even under normoxic conditions, elicits the same response as if the cells were exposed to a hypoxic environment. This is called the normoxic induction pathway. In other words, since cardiomyocytes have a basic level of ACh production on a steady or constant

basis (i.e., as long as the NNCCS function is working normally), this system may have an intrinsic capacity to adapt to hypoxic environments to some extent.

3.1.2. ACh has the effect of negatively regulating oxygen consumption in cardiomyocytes, resulting in a slow decrease in intracellular ATP levels [13]

Muscarinic receptors expressed in cardiomyocytes are of the M_2 type and are distributed throughout the heart. Meanwhile, vagal innervation in the cardiac ventricles is very sparse compared to sympathetic nerve endings, which are rather relatively abundant in the atria [5]. In contrast, sympathetic nerve endings are distributed throughout the cardiac ventricles. The distribution of ligands and their receptors is highly unbalanced in the cholinergic innervation of the cardiac ventricles. Therefore, the NNCCS can be seen as filling this gap.

Knockdown of non-neuronal ACh production in cultured cardiomyocytes *in vitro* or cell line HL-1 cells derived from mouse atrial muscle has been shown to increase oxygen consumption and decrease the amount of intracellular ATP under these circumstances as a result of greater consumption [10]. Thus, it is suggested that non-neuronal ACh-producing systems, including NNCCS, have the ability to maintain higher intracellular ATP. Based on these results, it is expected that cells with impaired function of NNCCS would undergo more apoptosis and generate more intracellular reactive oxygen species when cultured under specific conditions such as hypoxia or serum-free conditions, resulting in higher cytotoxicity and fewer viable cells [10].

3.1.3. NNCCS has the effect of promoting sugar utilization in the cell

In the aforementioned subsections, it has been suggested that NNCCS negatively regulates oxygen consumption and that HIF-1α is involved. In this regard, it was hypothesized that the enhanced function of NNCCS enhances the expression of glycolytic and glucose transporter genes, which are part of the HIF-1α regulatory gene cluster, and that this may lead to increased intracellular utilization of glucose and consequently to a partial suppression in oxygen consumption. A previous report has shown that forced expression of intracellular ChAT enhances sugar uptake and glucose transporter expression in cells [14].

3.1.4. NNCCS has an angiogenesis-promoting effect.

Another possible mechanism from a cardioprotective perspective is to increase the expression of vascular endothelial growth factor (VEGF), another regulatory gene cluster of the transcription factor HIF-1α, which is deeply involved in angiogenesis. A previous report on oral administration of donepezil, an AChE inhibitor, to mice as a method of increasing ACh levels in mouse tissues ameliorates the inhibition-induced skeletal muscle atrophy observed in a model of lower limb ischemia induced by unilateral femoral artery ligation, partly due to its ability to promote angiogenesis in the ischemic lower limb [9]. The *in vitro* angiogenesis-promoting effects of ACh and donepezil alone have also been observed in human-derived vascular endothelial cells on Matrigel, as measured by the promotion of tube formation structures. The direct angiogenesis-promoting effect of ACh has therefore been demonstrated, and NNCCS-derived ACh may share this mechanism [9].

NNCCS-derived ACh has been reported to regulate by a positive feedback mechanism via muscarinic receptors This is due to a positive feedback mechanism mediated by muscarinic receptor agonists. This is based on the results that treatment of cardiomyocytes with the muscarinic receptor agonists pilocarpine and ACh increases the transcription and translation levels of the *ChAT* gene and the production of ACh [4].

All of these mechanisms have been clarified *in vivo* using NNCCS-enhanced mice, the world's first NNCCS-associated *in vivo* mice models, and the results are reported in the next section [15].

3.2. What are the functions of NNCCS found in NNCCS-enhanced mice?

As NNCCS-enhanced mice, transgenic mice with enhanced NNCCS function were generated by expressing the mouse *ChAT* gene under the control of the α-myosin heavy chain (MHC) promoter [8]. Although the α-MHC promoter induces cardiac-specific expression, protein expression analysis in the model showed relatively higher expression of ChAT in ventricular muscle than in atrial muscle, confirming that the mice are NNCCS hyperfunctional. The representative and noteworthy phenotypes of these mice are as follows [15]:

(1) Although ChAT tg mice had approximately 10–20 times higher than that of wild-type (WT) mice, there was no difference in blood pressure, heart rate, or other hemodynamic parameters. In addition, echocardiographic examination of cardiac function showed a tendency towards slightly higher single stroke volume and end-diastolic volume, but otherwise, the two groups were almost identical and no reduction in contractile capacity was observed. However, ventricular myocardial HIF-1α protein levels were significantly higher than in the WT, even under normoxia. Considering that HIF-1α protein levels are naturally very low under normoxia, it is likely that cardiac HIF-1α protein levels are elevated in NNCCS-enhanced mice due to activation of the hypoxia-induced pathway.

(2) ChAT tg cardiac glucose transporter Glut4 protein expression was significantly elevated compared to WT, and immunohistological studies showed that Glut4 signaling in cardiac ventricular muscle was markedly increased in ChAT tg. This phenotype strongly suggests that increased NNCCS function enhances glucose utilization in the heart. Furthermore, ChAT tg already had increased intracardiac angiogenesis, and endothelial cell marker von Willebrand Factor (vWF) signaling produced within the vascular endothelial cells was more common in ChAT tg than in the pre-pathological normal state (i.e. more angiogenesis occurred and increased vascular endothelial cell density was observed in pathological assessment).

(3) Comparison of myocardium remnant after myocardial infarction by ligation of the anterior descending branch of the left coronary artery in mice with open thoracotomy revealed that more myocardium remained in the ChAT tg myocardium. Furthermore, vWF signaling was stronger and more extensive in the post-infarction ChAT tg myocardium than in the pre-infarction myocardium, suggesting a greater promotion of angiogenesis in the ChAT tg myocardium. Moreover, post-infarction compensatory cardiac hypertrophy was rather suppressed in the ChAT tg myocardium. These cardioprotective effects were more pronounced in ChAT tg, resulting in a significantly higher survival rate of mice within 14 days after myocardial infarction (93% in ChAT tg compared to 43% in WT). This indicated that hearts with enhanced NNCCS function had enhanced ischemic tolerance and were more cardioprotective than WT. Furthermore, in the Langendorff perfusion heart experiments, when isolated hearts were perfused and then ischemia-reperfused (30 min ischemia followed by 60 min reperfusion), the infarct size in ChAT tg was smaller, the time to cardiac arrest after severe ischemia due to perfusate interruption was longer in ChAT tg, and the time to heartbeat recovery after reperfusion was shorter. These findings suggested that mice with enhanced NNCCS function have a markedly increased tolerance to ischemia.

(4) Neonatal cardiomyocytes from ChAT tg and WT were collected and cultured and compared their mitochondrial function when they were exposed to 1% oxygen for 24 hours and when they were returned to normal oxygen. Mitochondrial function was significantly enhanced in WT but was more suppressed in ChAT tg. This suggested that ChAT tg-derived cardiomyocytes had a lower oxygen demand, i.e. lower oxygen consumption.

These phenotypes of NNCCS-enhanced mice indicate that NNCCS is essential for cardiac function and has a protective effect on cardiac function by negatively regulating oxygen consumption (**Figure 2**). In addition, a recent analysis of cardiac-specific ChAT miRNA transgenic mice in which the NNCCS was knocked down reported a phenotype almost the exact opposite of that of ChAT tg [11]. In this model, the steady-state production of cardiac ACh by NNCCS was reduced by approximately 50%, and a gradual but significant reduction in cardiac function, i.e. a state of heart failure, was observed. These results indicated that the innate NNCCS function is essential for maintaining cardiac physiology at this level, whereas increasing its function is more inhibitory to disease development.

4. Physiological functions of NNCCS on non-cardiac organs

As it is technically very difficult to assess NNCCS function directly in WT, it was assumed that it is somehow affected in non-cardiac organs, thus, ChAT tg was used, and attempted further analysis due to the impression of ChAT tg being very docile in comparison with WT. The behavior of these mice was analyzed using a more objective measure of this impression [16].

4.1. ChAT tg exhibits antidepressant, anxiolytic, and antistress responses

Since ChAT tg originally expresses cardiac-specific transgene ChAT, it has already been confirmed that the distribution and number of ChAT-expressing positive neurons in the brain are comparable to those of WT. Therefore, the possibility of effects due to unexpected ectopic expression of the transgene in the brain was considered to be very low. Despite such conditions, ChAT tg exhibited the following phenotypes [16]:

(1) ChAT tg was slightly, but significantly, less active during the nocturnal period compared to WT. This suggests that the mice do not engage in much nocturnal exploratory behavior. Therefore, when

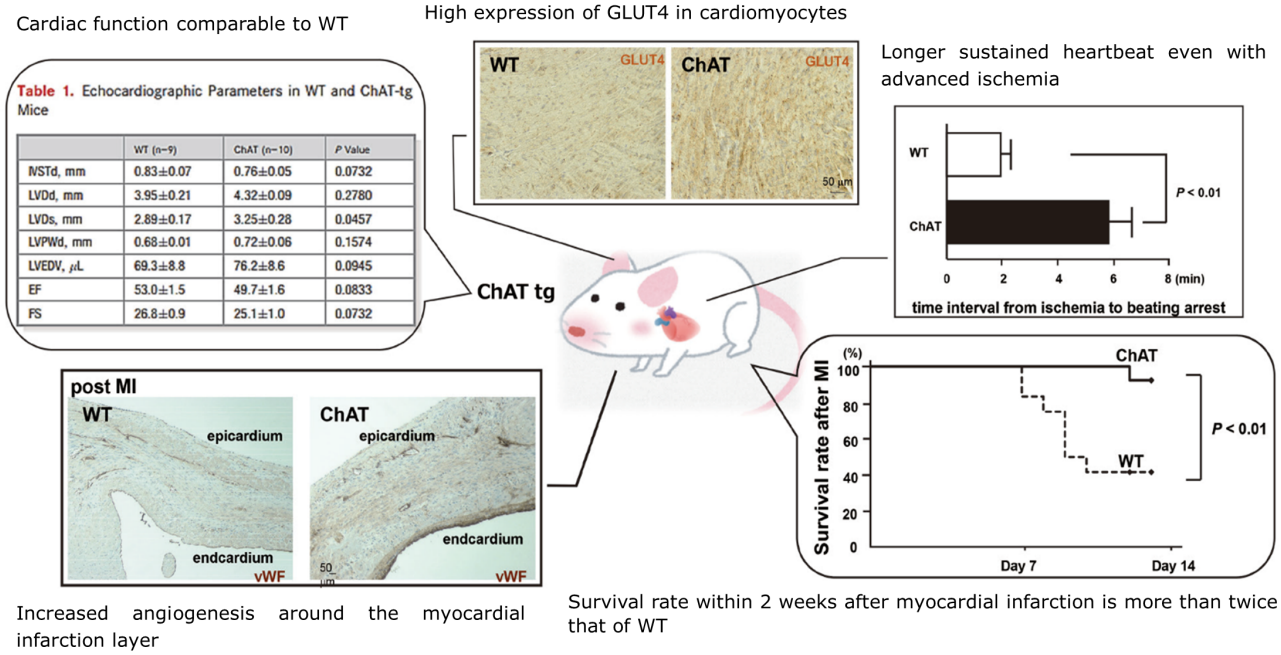

Figure 2. NNCCS-enhanced mice (ChAT tg) are characterized by a marked acquisition of cardiac ischemia tolerance. ChAT tg is hemodynamically similar to WT mice and has a similar cardiac function, but has enhanced cardiac GLUT-4 expression ChAT tg exhibits characteristic phenotypes such as more accelerated angiogenesis after myocardial infarction, longer sustained heartbeat during severe ischemia in the perfused heart and higher post-infarction survival. Abbreviations: IVSTd, interventricular septal thickness at end-diastole; LVDd, left ventricular diameter at end-diastole; LVDs, left ventricular diameter at end-systole; LVPWd, left ventricular posterior wall thickness at end-diastole; LVEDV, left ventricular end-diastolic volume; EF, ejection fraction; FS, fractional shortening.

the open field test was performed, ChAT tg showed a tendency to spend more time in the open field. Furthermore, when the elevated cruciform maze test was performed, ChAT tg spent significantly more time in the open arm than WT. These results indicate that ChAT tg showed more anxiolytic tendencies. Furthermore, when the tail suspension test and the forced swimming test were conducted as depression response tests, the ChAT tg showed a significantly shorter immobility time than the WT, indicating a greater antidepressant response. Moreover, after 60 min of restraint stress, the peak increase in blood corticosterone concentration was significantly reduced in the ChAT tg (approximately 50% of that in the WT). Taken together, these results suggested that the higher functions of ChAT tg are less prone to anxiety, stress, and depression-like symptoms than those of WT and that such central nervous system (CNS) responses are affected by increased NNCCS function.

(2) ChAT tg cerebral nerve activity was significantly suppressed compared to WT as assessed by transcranial flavoprotein fluorescence imaging. In seizure induction studies with the muscarinic receptor agonists pilocarpine or pentylenetetrazole, ChAT tg had significantly shorter seizure durations than WT, and a significantly lower mortality rate due to convulsive seizures compared to both induction agents (pilocarpine: ChAT tg 18.2% vs. WT 92.9%; pentylenetetrazole: ChAT tg 57.1% vs. WT 92.9%).

(3) To qualitatively evaluate neuronal activation in the medullary solitary bundle nucleus and dorsal nucleus of the vagal nerve, the number of c-fos positive cells was significantly increased in the medulla oblongata of ChATtg. Vagal electrical activity was also measured in the left cervical region and showed a significant increase in the frequency of nerve firing in ChAT tg. The norepinephrine content in the hypothalamus of ChAT tg was significantly increased compared to the same area in WT.

Overall, these findings suggest that vagal ascending (or afferent) pathway activity is enhanced in ChAT tg compared to WT. The involvement of nitric oxide (NO) as a molecule that enhances the activity of the vagal ascending tract was postulated. This is because cardiomyocytes originally produce NO via muscarinic receptors through ACh, and the amount of NO produced in the ChAT tg heart is significantly higher than in WT, and pretreatment with the NO synthase inhibitor L-NAME significantly reduced the frequency of vagal activity, or nerve firing, which was increased in the ChAT tg. In order words, the unique CNS phenotype of ChAT tg is triggered by information from the cardiac primary via the vagal ascending tract (NO as a candidate). Therefore, when the phenotype of ChAT tg in which unilateral vagotomy at the neck was compared with that of non-truncated ChAT tg, surprisingly, all previously observed antidepressant, anxiolytic, and antistress responses were abolished and suppressed to a level almost equivalent to that of WT. Similarly, ChAT tg without vagotomy but pretreated with the NO synthase inhibitor L-NAME not only reduced vagal ascending tract activity but also suppressed the ChAT tg-specific CNS phenotypes to near WT levels.

These results suggested that increased NNCCS function stimulates vagal nerve centers via the vagal nerve ascending tract, possibly activating cholinergic neurons to higher centers, thereby modifying the higher functions of the mouse and transforming it into a more antidepression, anxiolytic, and anti-stress state. In other words, the analysis of ChAT tg revealed for the first time that the NNCCS may regulate central higher functions via the vagal nerve, i.e., that it may control not the brain-mind but the mind-brain functional connectivity [16].

4.2. NNCCS-enhanced mice show a phenotype similar to that of autologous vagal nerve-stimulated mice and suppress not only systemic inflammation but also inflammation in the brain by stiffening the blood-brain barrier (BBB)

The NNCCS-enhanced mouse ChAT tg has been shown

to modulate higher-order functions of the CNS and lipopolysaccharide (LPS) loading was investigated as a model of systemic inflammation and the blood-brain barrier (BBB) function in the brain as a model regulator of localized inflammation. Hence, a model of BBB dysfunction using ChAT tg mice was investigated [17].

(1) The survival rate of ChAT tg mice was 50.0%, whereas that of WT mice was 14.3%, indicating that the anti-septic (i.e., anti-inflammatory) effect was significantly higher in ChAT tg mice. Furthermore, when the levels of inflammatory cytokines in various organs were evaluated after low-dose LPS administration, the cytokine levels in the blood of ChAT tg were significantly lower than those of WT. Cytokine expression levels were also significantly reduced in the primary culture system of liver-derived Kupffer cells from ChAT tg. Among several nicotinic receptors to which ACh binds, the α7 nicotinic receptor is an ion channel-type receptor consisting of only five α-subunits, which is known to exhibit an anti-inflammatory response [18,19]. The α7 nicotinic receptor is expressed in a variety of cells, and ACh may stimulate this receptor via the hepatic vagal centrifugal pathway to regulate inflammation.

(2) The expression of claudin-5, a BBB component protein that constitutes the tight junction, was significantly higher in the ChAT tg brain than in the WT, and immunohistological evaluation showed that the number of claudin-5-positive cells was significantly higher in the ChAT tg brain cortex and hippocampus. When ChAT tg brains with higher expression of claudin-5 were subjected to cryoinjury, the resulting BBB rupture was highly significantly suppressed compared to WT brains. The degree of leakage of Evans blue dye, a marker of BBB permeability, into the brain parenchyma, was significantly lower in ChAT tg than in WT, as expected when the dye was administered systemically at the time of brain cryoinjury. The expression of inflammatory cytokines at the site of injury was also significantly lower in ChAT tg than in WT. As a result, astrocyte responsiveness induced by brain injury was suppressed in ChAT tg compared to WT, and cell hypertrophy and proliferation were also reduced to a lesser extent in ChAT tg than in WT. The claudin-5 protein expression was also continuously upregulated in primary cultures of ChAT tg brain-derived vascular endothelial cells, and Evans blue dye leakage was significantly suppressed in ChAT tg-derived cells in the *in vitro* BBB reconstruction model. Thus, the BBB function in ChAT tg brains is upregulated by the expression of claudin-5 protein, indicating a specific phenotype in the brain, a non-cardiac organ, even though the model only enhances the NNCCS function. This suggested that the NNCCS system in the heart may modulate BBB function, possibly via the vagal nerve. Evidence for this was found in unilateral cervical vagotomy, where the elevated ChAT tg brain claudin-5 protein expression level was attenuated approximately 5 days after transfection. It remains clear that BBB function is affected by NNCCS [17].

(3) When a Parkinson's disease-like model was also created using the dopamine neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the survival rate of ChAT tg was 83.3%, which was significantly higher than that of WT (43.8%). Similarly, the disruption of BBB function by MPTP was significantly reduced in ChAT tg brains. MPTP-induced reduction and loss of tyrosin hydroxylase-positive cells in the substantia nigra and striatum were also significantly reduced in ChAT tg brains. As a result, the number of residual neurons in both regions was significantly increased in ChAT tg. These results suggest that the drug-induced Parkinson's model itself was suppressed in ChAT tg brains, partly due to more robust BBB function and the suppression of the inflammatory response in the brain by enhanced NNCCS function, which was also observed in the cryoinjury model [17].

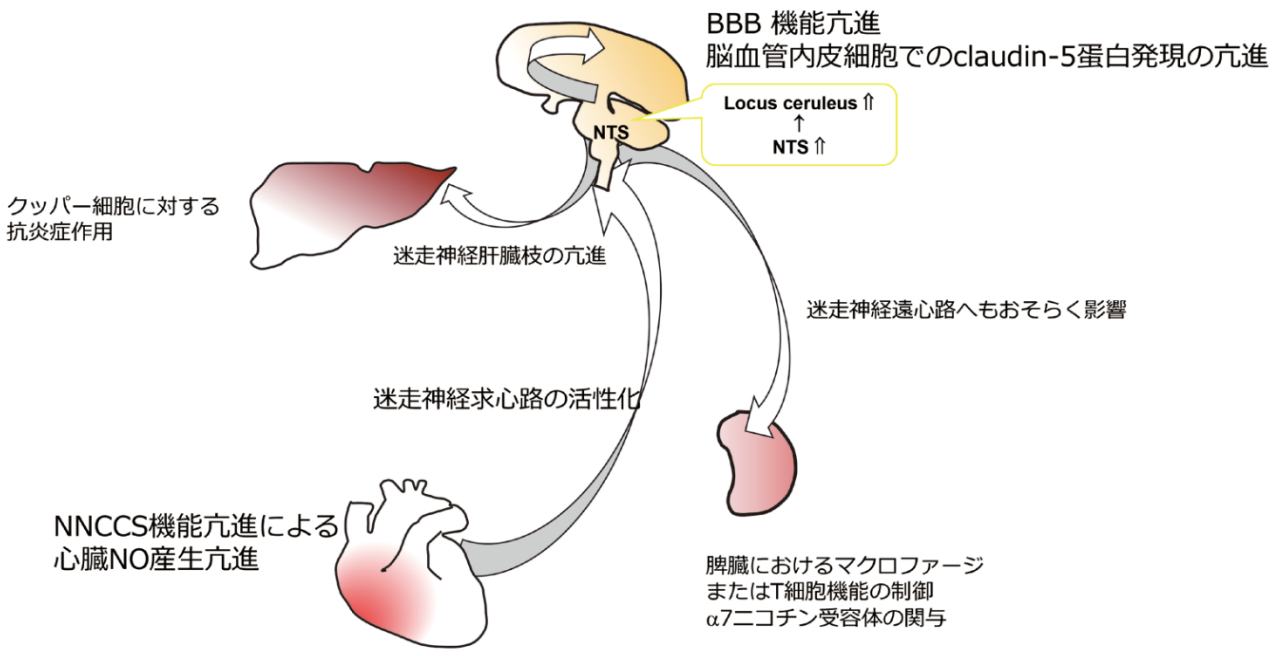

Figure 3. Crosstalk between NNCCS function and vagally innervated organs. NNCCS function is also modulated by cross-talk with the brain, spleen, and liver via the vagal nerve, where information on increased NNCCS function enters the nucleus of the solitary tract (NTS), a parasympathetic center, and then projects to the locus coeruleus (LCS). The information is then projected to the nucleus tractus solitarii (NTS), the center of the parasympathetic nervous system, and further propagated to the whole brain. On the other hand, the information is transmitted from the parasympathetic center to the innervating organs (liver, spleen, and other intra-abdominal organs) via the vagal nerve centrifugal tract.

In summary, it was clearly shown for the first time that NNCCS activation in the mouse model of NNCCS hyperfunction (ChAT tg) transmits information to the brain via the vagal afferent pathway and this may be linked to enhanced BBB function and suppression of inflammation in the brain, possibly leading to the CNS phenotypes of antidepressant, anxiolytic, anti-stress and anticonvulsant effects on other organs (i.e., the liver) in the vagally innervated area via the centrifugal pathway from the afferent pathway (**Figure 3**).

5. Possible involvement of NNCCS in humans

At present, knowledge of the NNCCS is not widely known, it is not technically possible to measure or assess this system directly in humans, and there are no reports on the extent of its involvement in humans.

However, several large clinical epidemiological studies have already been reported by several research organizations that strongly suggested the involvement of the NNCCS in human disease [20,21]. One report by Nordström *et al.* compared patients with Alzheimer's disease who were taking the AChE inhibitor donepezil, with those who were not, and found that both myocardial infarction deaths and total deaths were significantly lower in those taking donepezil. The number of deaths from myocardial infarction and all-cause mortality were significantly lower in those taking donepezil, and were even lower in those taking higher doses, suggesting that the use of donepezil is at least beneficial in reducing death from cardiovascular events [20]. Furthermore, Wu et al. reported a significant reduction in the incidence of acute coronary syndromes in Alzheimer's patients taking donepezil, with a further significant reduction at higher doses [21].

A previous report in 2009 showed that donepezil enhances the NNCCS in cultured cardiomyocytes, i.e. it not only has the pharmacological effect of AChE inhibition but also enhances ACh production [4]. Since NNCCS-enhanced mice have acquired extremely

potent ischemia tolerance, it was hypothesized that a similar treatment in humans, if available, would probably lead to a higher survival rate in myocardial infarction or chronic heart failure, and the results of clinical epidemiological studies supporting this were one of the possible mechanisms. One possible mechanism was that donepezil may have contributed to the increased NNCCS.

Furthermore, it is known that obesity and glucose intolerance in obese mice (db/db mice), a model of diabetes, becomes more pronounced as the mice grow older. NNCCS function was recently reported to be reduced during the development of diabetic cardiomyopathy, which may be associated with the onset of diabetic cardiomyopathy, i.e., impaired cardiac function and heart failure [22]. NNCCS-enhanced db/db mice (generated by mating db/+ heterozygous mice with ChAT tg and then with db/+ heterozygous mice) did not show any cardiac dysfunction due to diabetic cardiomyopathy, as compared with db/db mice of the same age. This indicates that suppression of NNCCS hypofunction and preservation of its function can inhibit the progression of diabetic cardiomyopathy in animal models [22]. To confirm this phenomenon in humans, NNCCS function in the heart tissue of diabetic patients was evaluated and reported that it was reduced in the same way as in db/db mice, thus suggesting that NNCCS is also present in humans and that its reduction may result in reduced cardiac function [22].

6. Conclusion

This review began with the history of the discovery of the NNCCS and mentioned that studies on the NNCCS have been published almost simultaneously and independently of each other. The physiological functions elucidated in the cardiovascular system thereby revealed, in a nutshell, an essential system that protects the heart through a wide variety of actions, including energy metabolism, gap junctions, angiogenesis, and inflammatory regulation. These actions are considered to be part of the consensus on the NNCCS. Furthermore, it has recently been suggested that NNCCS may affect the BBB function of the CNS and even higher functions when NNCCS function is enhanced. It is generally accepted that cardiac function is controlled by the CNS, as indicated by the dual sympathetic and parasympathetic control of the heart by the CNS, as indicated by the term “brain-cardiac coupling”. However, the ratio of ascending and descending pathways contained in the vagal nerve is said to be approximately 80% to 20%, with the ascending (afferent) nature reported to be overwhelmingly dominant. This suggests the possibility that CNS can be controlled by information from the heart, or in other words, the “cardiac-brain connection”, which may be a better description of the original physiological function of the CNS.

For example, it is well known that exercise reduces stress, and this may be due to an increase in NNCCS function. The vagal nerve, as its name suggests, innervates almost all internal organs, including abdominal and thoracic organs, and if an increase in NNCCS function can induce an anti-inflammatory effect in the innervating organs, such an active intervention in the NNCCS would present an extremely meaningful method for research.

Acknowledgments

We would like to take this opportunity to thank Dr. Toshio Takahashi of the Suntory Foundation for Life Sciences for the opportunity to write this review.

Disclosure statement

The author declares no conflict of interest.

References

[1] Fujii T, Kawashima K, 2001, The Non-Neuronal Cholinergic System: An Independent Non-Neuronal Cholinergic System in Lymphocytes. Jpn J Pharmacol, 85(1): 11–15.

[2] Kummer W, Lips KS, Pfeil U, 2008, The Epithelial Cholinergic System of the Airways. Histochem Cell Biol, 130(2): 219–234.

[3] Wilson C, Lee MD, McCarron JG, Acetylcholine Released by Endothelial Cells Facilitates Flow-Mediated Dilatation. J Physiol, 594: 7267–7307.

[4] Kakinuma Y, Akiyama T, Sato T, 2009. Cholinoceptive and Cholinergic Properties of Cardiomyocytes Involving an Amplification Mechanism for Vagal Efferent Effects in Sparsely Innervated Ventricular Myocardium. FEBS J, 276(18): 5111–5125.

[5] Hoover DB, Ganote CE, Ferguson SM, et al., 2004, Localization of Cholinergic Innervation in Guinea Pig Heart by Immunohistochemistry for High-Affinity Choline Transporters. Cardiovasc Res, 62(1): 112–121.

[6] Rana OR, Schauerte P, Kluttig R, et al., 2010, Acetylcholine as an Age-Dependent Non-Neuronal Source in the Heart. Auton Neurosci, 156: 82–89.

[7] Rocha-Resende C, Roy A, Resende R, et al., 2012, Non-Neuronal Cholinergic Machinery Present in Cardiomyocytes Offsets Hypertrophic Signals. J Mol Cell Cardiol, 53: 206–216.

[8] Roy A, Fields WC, Rocha-Resende C, et al., 2013, Cardiomyocyte-Secreted Acetylcholine is Required for Maintenance of Homeostasis in the Heart. FASEB J, 27: 5072–5082.

[9] Kakinuma Y, Furihata M, Akiyama T, et al., 2010, Donepezil, and Acetylcholinesterase Inhibitor Against Alzheimer's Dementia, Promotes Angiogenesis in an Ischemic Hindlimb Model. J Mol Cell Cardiol, 48(4): 680–693.

[10] Kakinuma Y, Akiyama T, Okazaki K, et al., 2012, A Non-Neuronal Cardiac Cholinergic System Plays a Protective Role in Myocardium Salvage during Ischemic Insults. PLoS One, 7(11): e50761.

[11] Oikawa S, Kai Y, Mano A, et al., 2021, Non-Neuronal Cardiac Acetylcholine System Playing Indispensable Roles in Cardiac Homeostasis Confers Resiliency to the Heart. J Physiol Sci, 71(1): 2.

[12] Palatini P, Benetos A, Julius S, 2006, Impact of Increased Heart Rate on Clinical Outcomes in Hypertension: Implications for Antihypertensive Drug Therapy. Drugs, 66(22): 133–144.

[13] Kakinuma Y, Ando M, Kuwabara M, et al., 2005, Acetylcholine from Vagal Stimulation Protects Cardiomyocytes Against Ischemia and Hypoxia Involving Additive Non-Hypoxic Induction of HIF-1α. FEBS Lett 579(10): 2111–2118.

[14] Oikawa S, Iketani M, Kakinuma Y, 2014, A Non-Neuronal Cholinergic System Regulates Cellular ATP Levels to Maintain Cell Viability. Cell Physiol Biochem, 34(3): 781–789.

[15] Kakinuma Y, Tsuda M, Okazaki K, et al., 2013, Heart-Specific Overexpression of Choline Acetyltransferase Gene Protects Murine Heart Against Ischemia Through Hypoxia-Inducible Factor-1α-Related Defense Mechanisms. J Am Heart Assoc, 2(1): e004887.

[16] Oikawa S, Kai Y, Tsuda M, et al., 2016, Non-Neuronal Cardiac Cholinergic System Influences CNS via the Vagus Nerve to Acquire a Stress-Refractory Propensity. Clin Sci (Lond), 130(21): 1913–1928.

[17] Oikawa S, Kai Y, Mano A, et al., 2019, Potentiating a Non-Neuronal Cardiac Cholinergic System Reinforces the Functional Integrity of the Blood Brain Barrier Associated with Systemic Anti-Inflammatory Responses. Brain Behav Immun, 81: 122–137.

[18] Echeverria V, Yarkov A, Aliev G, 2016, Positive Modulators of the α7 Nicotinic Receptor Against Neuroinflammation and Cognitive Impairment in Alzheiner's Disease. Prog Neurobiol, 144: 142–157.

[19] Wang H, Yu M, Ochani M, et al., 2003, Nicotinic Acetylcholine Receptor Alpha7 Subunit is an Essential Regulator of Inflammation. Nature, 421: 384–388.

[20] Nordström P, Religa D, Wimo A, et al., 2013, The Use of Cholinesterase Inhibitors and the Risk of Myocardial Infarction and Death: a Nationwide Cohort Study in Subjects with Alzheimer's Disease. Eur Heart J, 34(33): 2585–2591.

[21] Wu PH, Lin YT, Hsu PC, et al., 2015, Impact of Acetylcholinesterase Inhibitors on the Occurrence of Acute Coronary Syndrome in Patients with Dementia. Sci Rep, 5: 15451.

[22] Saw EL, Pearson JT, Schwenke DO, et al., Activation of the Cardiac Non-Neuronal Cholinergic System Prevents the Development of Diabetes-Associated Cardiovascular Complications. Cardiovasc Diabetol, 20: 50.