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REVIEW

Neurotransmitter release: vacuolar ATPase V0 sector c-subunits in possible gene or cell therapies for Parkinson's, Alzheimer's, and psychiatric diseases

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Abstract We overview the 16-kDa proteolipid mediatophore, the transmembrane c-subunit of the V0 sector of the vacuolar proton ATPase (ATP6V0C) that was shown to mediate the secretion of acetylcholine. Acetylcholine, serotonin, and dopamine (DA) are released from cell soma and/or dendrites if ATP6V0C is expressed in cultured cells. Adeno-associated viral vector-mediated gene transfer of *ATP6V0C* into the caudate putamen enhanced the depolarization-induced overflow of endogenous DA in Parkinson-model mice. Motor impairment was ameliorated in hemiparkinsonian model mice when ATP6V0C was expressed with DA-synthesizing enzymes. The review discusses application in the future as a potential tool for gene therapy, cell transplantation therapy, and inducible pluripotent stem cell therapy in neurological diseases, from the view point of recent findings regarding vacuolar ATPase.

Keywords Secretion · Neurotransmitter · ATPase · Gene therapy · IPSC · Psychiatric diseases

Introduction

Secretion is one of major topics in the physiology [1–6]. Most cells are naturally equipped with the vesicular-soluble N-ethylmaleimide-sensitive factor attachment protein receptor (v-SNARE) that enables vesicular traffic and endo- and exocytosis as well as neurotransmitter release [7, 8]. However, another secretory mechanism has been known since 1966 [9]. In electric eels, acetylcholine (ACh) is released in a paracrine fashion from the soma of electric organ cells and acts on nicotinic ACh receptors in adjacent cells to accumulate a voltage of up to 1000 V [10–12]. Mediatophore, a 16-kDa proteolipid, was originally found in the presynaptic membrane of the *Torpedo* electric organ. This protein from the electric organ synaptosome was characterized, and its remarkable ACh translocation properties were reported [12–16].

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Acetylcholine release by mediatophore

The role of mediatophore in ACh release was revealed by projects in which reconstruction of mediatophore in a variety of cells that had no release machinery for ACh [13]. Israël, Dunat, and their colleagues reported that many cells—splenic cells, hepatocytes, erythrocytes, myeloma cells, and several neuroblastoma cell lines were unable to release ACh in response to calcium (Ca^{2+}) influx [13–16]. Cholinergic neuroblastoma or neuroblastoma-derived hybrid culture cell lines were cloned in Nirenberg's laboratory at the National Institutes of Health, USA, from 1965 to 1975 [17–20]. Neuroblastoma clones that are inactive with respect to neurotransmitter synthesis were also isolated. N18 neuroblastoma cells are representative of these cells. Other cultured cells, i.e., the C6BU-1 glioma cell line followed by the fibroblastic L cell line, PC12, Neuro 2A, and NG108-15 cells, were competent for ACh release provided that they were loaded with the transmitter [13–16, 21–27]. Interestingly, the ACh release mechanism expressed by the latter cells was Ca^{2+}-dependent, with release elicited by an influx of Ca^{2+} using the Ca^{2+} ionophore A23187 [11, 13, 16].

ACh was expected to be produced and released if the choline acetyltransferase (ChAT) gene was overexpressed in inactive N18 neuroblastoma cells. However, ACh release was not detected [22, 23]. Instead, when N18 neuroblastoma cells were transfected with mediatophore and the expressing cells were soaked with ACh, the cells began to secrete ACh [14–16]. Based on these results, it is concluded that ACh release from the neuroblastoma cell soma likely depends on mediatophore. From this view point, the ability to form synapses (ACh release detected in cultured muscle cells) in more than 25 different neuroblastoma clones [19–21] was clearly classified into two groups: the synapse formation-positive and -negative groups (Table 1 and see Table 1 of ref. [20]). The positive phenotype was derived from endogenous mediatophore, and the negative phenotype was caused by the absence of mediatophore [14, 15, 23].

Fisher et al. [28] reported that fibroblasts genetically modified to express ChAT became able to release the transmitter on addition of A23187. Some other cell lines, such as NG108-15 cells, expressed voltage-dependent Ca^{2+} channels that can be activated after depolarization, and so Ca^{2+}-dependent ACh release was elicited by electrical stimulation [24]. Of particular interest is the work of Higashida and colleagues in co-cultures of myocytes and neuroblastoma (Neuro 2A) or hybrid cell lines (NG108-15 hybrid cell line derived by Sendai virus-induced fusion of C-1300 mouse neuroblastoma clone N18TG-2 resistant to 6-thioguanie with rat glioma clone C6Bu-2, resistant to 5-bromodeoxyuridine; NBr-10A hybrid cell line derived from N18TG-2 neuroblastoma x BRL-30E rat liver cell, resistant to 6-thioguanie) [18–24], in which the release was measured as endplate potential of the ACh receptor origin [24]. The ACh release capacity of NG108-15 or NBr-10A hybrid cells seems to be derived from C6Bu-1 glioma or Buffalo rat liver BRL-30E cells equipped with mediatophore, rather than N18TG-2 neuroblastoma cells [19, 20, 23].

Mediatophore as an ortholog of ATPase

Mediatophore was later shown to be an ortholog of the mammalian c-subunit of the V0 transmembrane sector of the vacuolar proton ATPase (ATP6V0C) [10–16, 29; Fig. 1]. ATP6V0C plays a central role in H^+ transport as one part of the multi-subunit complex of ATPase [30–39]. Israël and his group initially proposed that mediatophore forms a proteinaceous pore with the six transmembrane c-subunits of ATPase [10, 11, 34]. However, the amino acid sequence and recent understanding of the c ring of the V0 domain suggest high hydrophobicity of the inner part of the ring, which seems unsuitable for a channel pore [31, 32, 36–38]. Therefore, though it is likely that ATP6V0C is permeable to much smaller ions, such as Na^+ and K^+ [37–39], Israël's hypothesis regarding the role of ATP6V0C in neurotransmitter release must be revisited. However, more importantly, to discuss the release source, we need to know whether or not some cell lines possess or lack vesicles, the V0 or V1 component of ATPase sector in the above cultured cell lines or in transfected cells with exogenous *ATP6V0C*. Such data are not available currently.

In addition, a direct interaction between ATP6V0C and the v-SNARE synaptobrevin and associated modulatory effects on ACh release have been reported [13, 33, 34]. Therefore, the role of mediatophore/ATP6V0C should be carefully reconsidered according to recent advances in knowledge regarding vacuolar ATPase.

Serotonin release from NG108-15 cells

The possibility of increasing efflux of other transmitters is of particular interest. NG108-15 neuroblastoma x glioma hybrid cells or NBr-10A neuroblastoma x Buffalo rat liver cell hybrid cells release ACh upon high potassium (K^+) depolarizing stimulation and evoke endplate potentials in muscle cells via electrical stimulation of differentiated NG108-15 or NBr-10A cells due to ACh release [20, 22–24; Table 1].

Falk-Vairant et al. demonstrated the presence of endogenous ATP6V0C in NG108-15 cells via western blotting and concluded that the capacity of NG108-15 cells to release ACh from the cell soma and/or dendrites is due to ATP6V0C [15, 34]. We examined whether NG108-15 cells can release serotonin, which is another neurotransmitter. First, NG108-15 cells were soaked with [^{3}H]serotonin, and

Table 1 Capacity for acetylcholine release and synapse formation with muscle cells in neuroblastoma tumor cells with or without ATP6V0C expression

Cell line	ACh synthesis	ACh secretion	Synapse formation	ATP6V0C expression
NG108-15	++	++	++	++
NBr-10A	+++	+++	+++	+++
N18	0	0	0	0
N18 + ACh*	++	0	0	0
N18 + ACh* + V0C	++	++	+	++

ACh*, cells were soaked in solution containing ACh or transfected with ChAT

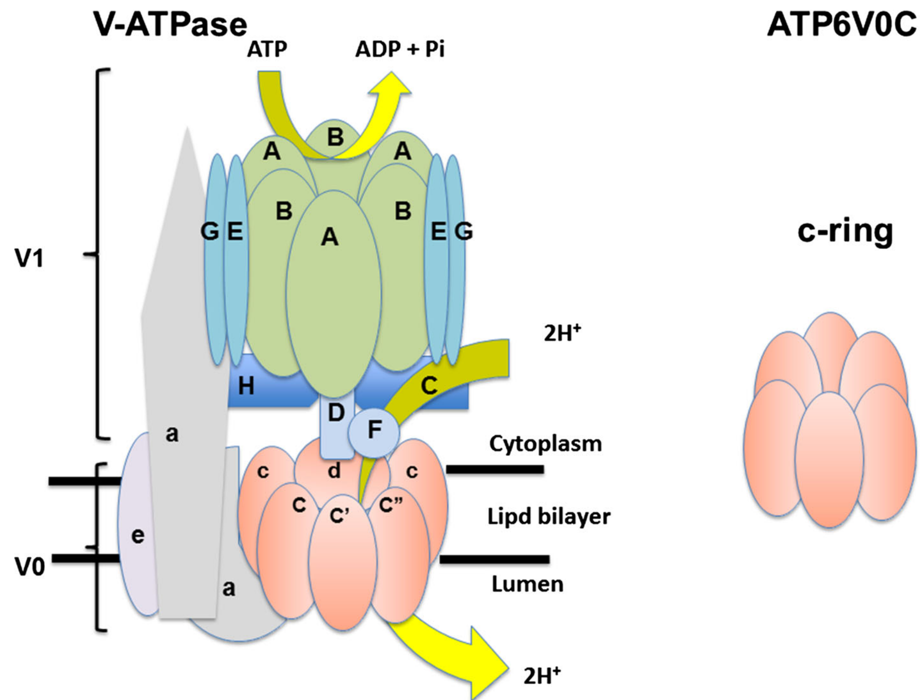

Fig. 1 Schema of major molecular components of vacuolar ATPase (V-ATPase). (*Left*) a cartoon of whole v-ATPase. ATP hydrolysis is shown at the peripheral V1 domain composed of *A*, *B*, *C*, and other components. The downhill movement of protons is shown in the V0 sector (*a*, *c*, *d* and other components). (*Right*) six c subunits in the V0 transmembrane sector of the vacuolar proton ATPase. Redrawn from Forgac and his colleagues [37, 38]

the cells were filled with [^{3}H]serotonin by uptake from the extracellular medium. After intensive washing, the cells were stimulated with depolarizing high-K$^+$ medium. [^{3}H]Serotonin was released in the presence of but not in the absence of extracellular Ca^{2+}, indicating that this behavior is Ca^{2+}-dependent [40]. NG108-15 cells possess both small clear vesicles and large dense-core vesicles [40, 41]. Therefore, it is not completely clear if ATP6V0C facilitated the exocytosis of serotonin-containing vesicles or the release of free serotonin in the cytoplasm. And an alternative question remains if release is not through ATP6V0C.

Dopamine release from N18 cells expressing ATP6V0C or NG108-15 cells

Next, we examined the possibility of DA release from N18 cells transfected with expression vectors containing the (5′ to 3′) forward or inverse (3′ to 5′) form of *ATP6V0C* [39, 42]. After transfection, the cells were preloaded for 1 h with 1 μM [^{3}H]DA in the presence of pargyline, a monoamine oxidase inhibitor, added to prevent degradation of [^{3}H]DA [40]. Stimulation with 80 mM K$^+$ in the perfusion medium resulted in a detectable increase in [^{3}H]DA release in a Ca^{2+}-dependent manner [42]. However, when identical experiments were performed in N18 cells transfected with reverse-ATP6V0C, [^{3}H]DA was not released after high-K$^+$ depolarizing stimulation. It is worth mentioning that ACh and DA share an ATP6V0C-dependent mechanism for DA release from the cell soma or dendrites of ATP6V0C-expressing neuroblastoma cells or NG108-15 cells with endogenous ATP6V0C (Table 1).

Effects of ATP6V0C overexpression on dopamine release in the intact mouse brain

The functional roles of ATP6V0C were elucidated in vivo. Although ATP6V0C is expressed in different brain regions (data not shown), ATP6V0C was overexpressed in the mouse brain using adeno-associated virus (AAV) vectors individually harboring cDNAs of rat *ATP6V0C*, reverse-*ATP6V0C*, and *ATP6V0C-GFP*. Infection with these viruses in the substantia nigra of the intact mouse brain using the microinjection technique [43] revealed ATP6V0C-GFP expression in both tyrosine hydroxylase (TH)-positive neurons and TH-negative cells, which were other non-neuronal cells, probably astrocytes (see Fig. 1 in ref. [42]).

Armed with this information, DA overflow from the synaptic cleft in the mouse striatum after treatment with AAV vectors [42] in the substantia nigra was measured using the in vivo microdialysis method [43]. DA release was significantly higher in mice infected with *AAV-ATP6V0C* under both resting (5 mM extracellular K$^+$ concentration) and depolarizing (50 mM) conditions (see Fig. 2 in ref. [42]). The result indicates that the facilitated DA release is likely from dopaminergic nerve terminals, not from the soma of striatal neurons. In contrast, viral

Table 2 The number of rotations was significantly decreased in mice

Mice transfected with	Rotations/3 min	% Recovery
Three enzymes	14.9 ± 1.2**	53
Three enzymes plus reverse ATP6V0C	13.9 ± 4.3*	56
Three enzymes plus ATP6V0C	4.0 ± 1.0***	87
PBS	31.7 ± 2.0	0

$N = 4$, one-way ANOVA followed by Bonferroni's post hoc test, $F_{3,12} = 55.44$, $P < 0.001$. * $P < 0.01$, ** $P < 0.002$, and *** $P < 0.001$ in comparison with PBS

infection with the reverse direction of *ATP6V0C* had little or no effect on DA release, with levels comparable to the control (sham-operated with phosphate-buffered saline (PBS) injection) mice.

Behavior in hemiparkinsonian mice with or without ATP6V0C

First, we generated hemiparkinsonian mice. A 2-μL aliquot of 6-hydroxydopamine (6-OHDA) dissolved in 0.02 % ascorbic acid in saline was injected into the substantia nigra over a period of 2 min at a rate of 1 μL/min (total injected amount: 28 μg of 6-OHDA) [42]. The needle was left in place for an additional 3 min and then withdrawn slowly. The control animals received PBS in the substantia nigra according to the same procedure.

The motor performance of mice with 6-OHDA-induced lesions in the unilateral substantia nigra was examined based on the latency to fall from an accelerated rotating rod (rotarod test). If the fall latency increased, we judged the change as an effect of recovery from motor impairment. Four AAV vectors individually containing three DA synthetic enzymes (tyrosine hydroxylase (*TH*), or aromatic L-amino acid decarboxylase (*AADC*), or GTP cyclohydrolase I (*GCH*)) and either one of the forward or reverse form of *ATP6V0C* were infected into the lesion side of the caudoputamen. 6-OHDA-lesioned mice treated with three DA-synthesizing enzymes and ATP6V0C showed significantly improved performance on the rotarod (134.2 ± 14.6 s) in comparison with mice treated with three DA-related genes plus reverse-*ATP6V0C* (78.1 ± 17.0 s, $P < 0.01$, $n = 5$ each, ANOVA (see Fig. 2 in ref. [42]). Recovery was much greater in these mice than in those treated with only these three enzymes alone (75.6 ± 17.2 s) or PBS-treated control mice (24.4 ± 12.2 s) ($P < 0.01$ and 0.001, respectively, in comparison with the value obtained for mice treated with all three enzymes plus *ATP6V0C*). The observed recoveries of mice treated with the three enzymes plus ATP6V0C or its reverse form were approximately 73 and 36 % of that observed in the wild-type mice (174.5 ± 12.4 s), respectively [42].

Amphetamine-induced rotations in hemiparkinsonian mice with or without ATP6V0C overexpression

d-Amphetamine-induced rotation is a strong predictor of nigral TH cell loss [44, 45]. Three weeks after lesion generation, the mice exhibited ipsilateral turning induced by the intraperitoneal injection of 3 μg/kg of *d*-amphetamine [42]. The number of rotations was significantly decreased in mice transfected with the three enzymes, the three enzymes plus reverse-*ATP6V0C*, and the three enzymes plus *ATP6V0C* in comparison to control mice injected with PBS (Table 2). In contrast, in sham-operated mice, no rotation was induced by *d*-amphetamine [42].

Overview of gene or cell transplantation therapy for neurodegenerative diseases

In both rodent and non-human primate models of Parkinson's disease (PD), viral vector-mediated gene delivery of one enzyme (*AADC*) or three DA-synthesizing enzymes (*TH*, *AADC*, and *GCH*) into the striatum has been shown to ameliorate motor symptoms, with efficient signal transduction of putaminal neurons [42, 46–54]. Many clinical trials (phase I and phase II) of gene therapy for PD were performed using AAV vectors [55]. In these protocols, gene transfer of *AADC* into the human putamen is usually combined with oral administration of the precursor L-3,4-dihydroxyphenylalanine (L-DOPA) [48, 49]. Most of the transduced cells are medium spiny neurons (MSNs), the principal projection neurons that account for 90–95 % of all neurons in the striatum. After gene transfer, the MSNs synthesize DA in addition to their inherent inhibitory neurotransmitter, γ-aminobutyric acid (GABA). Gene delivery of DA-synthesizing enzymes would be a useful means of supplying DA continuously in the putamen (Fig. 2).

The nerve terminals of dopaminergic neurons of the substantia nigra are selectively degraded and mostly lost in the putamen in the motor phase of chronic PD patients

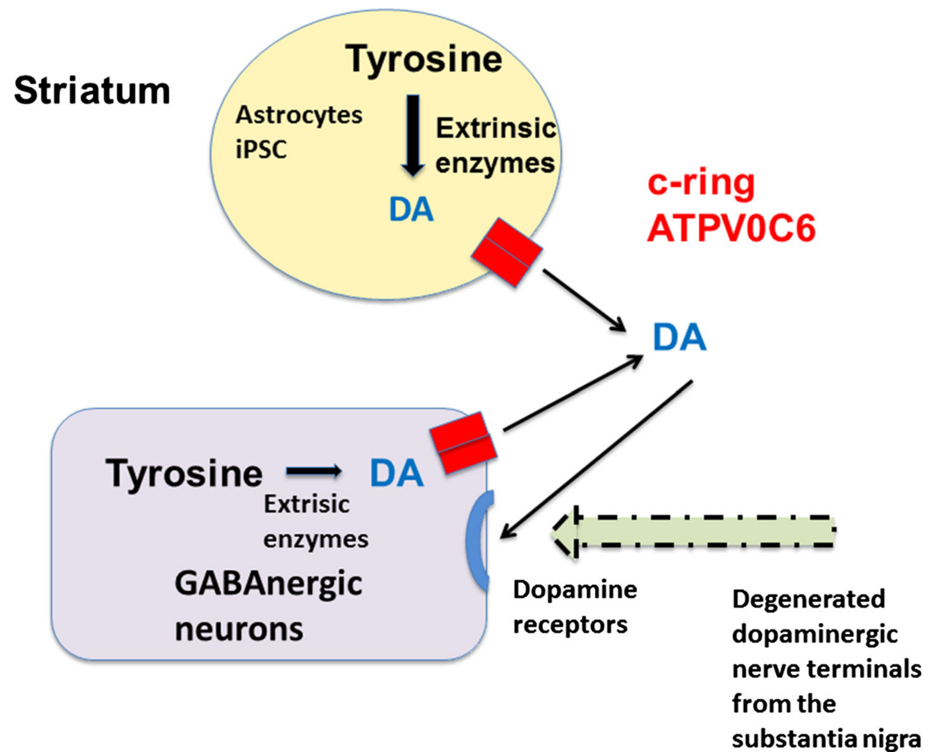

Fig. 2 Scheme showing autocrine or paracrine secretion of dopamine from cells expressing dopamine-synthetic enzyme and ATPVC6. Dopamine (DA) is synthetized by AAV-TH/-AADC/-GCH in astrocytes, neurons, and iPSC (*yellow*) GABAergic neurons (*brown*). DA release from the cell soma through the stalk or pore of ATPV0C6. DA released from adjacent cells (paracrine) or GABAergic neurons (autocrine) binds to DA receptors at the synaptic or non-synaptic domains on GABAergic neurons. GABAergic neurons relay signals via DA receptors activated by DA from dopaminergic neurons in the substantia nigra in intact tissue. In the PD brain, DA released in an autocrine or paracrine fashion activates DA receptors

[55]. This observation suggests that even DA synthesized by extrinsic enzymes in the putamen could not be released from the nerve endings of dopaminergic neurons. Rather, cell-somatal release is likely in transduced neurons (Fig. 2), in which the fusion of exocytotic vesicles and the vesicular secretory apparatus are not identical to those observed in the intact nigral nerve terminals [55, 56].

However, the following cautions should be considered. It is possible that injection of the genes encoding ATP6V0C and DA-synthesizing enzymes into the striatum would have augmented the exocytotic DA release from the remaining dopaminergic nerve terminals. Because Jin et al. [42] did not explicitly indicate how many dopaminergic neurons were actually destroyed by their unilateral 6-OHDA injection procedure, although functionally dopaminergic responses were substantially destroyed. In addition, somatic or dendritic release of DA through ATP6V0C in non-dopaminergic neurons or glia is not clearly evident.

It is possible to postulate another mechanism whereby a complex of proteolipid channels involved in SNARE fusion may effectively secrete DA from DA-accumulating cells in the striatum, as described in yeast and *Drosophila* [7, 57, 58]. ATP6V0C binds to syntaxin in SNARE complexes [59] or interacts directly with the v-SNARE synaptobrevin [33], and ATP6V0C may cooperate with SNARE proteins and vesicles for release during the late stage of fusion.

ATP6V0C as a useful gene in the future

Baseline striatal dopaminergic neurotransmission in the normal striatum is maintained by tonic synaptic and non-synaptic DA release, which are largely independent of changes in neuronal impulse flow in the nigrostriatal pathway. As shown in the previous studies [46, 47, 53], neurons and other types of cells were transfected with AAV, although the majority of transfected cells were neurons. We previously detected efficient baseline and L-DOPA-induced DA release in the *AAV-TH/-AADC/-GCH*-injected putamen via microdialysis in primates [47], indicating release of the DA synthesized by these extrinsic enzymes. The observation described above, including *ATP6V0C*, suggests that DA is released from the cell soma of the transfected cells via a non-synaptic mechanism or from non-neuronal elements, such as astro- or microglial cells. DA released from nearby cells binds to the DA receptors on GABAergic neurons and functions in a paracrine or autocrine manner, respectively (Fig. 2).

The concept suggests that *ATP6V0C* may be useful in future gene or cell transplantation therapy [60, 61]. Gene and cell therapies for other diseases related to ACh and serotonin, such as Alzheimer's disease, depression, schizophrenia, and syndromic autism, may utilize ATP6V0C in the future [62]. This approach is also applicable for iPSC therapy for similar categories of disease [63–65] because better recovery would be expected if iPSC

contains genes for neurotransmitter-synthesizing enzymes and *ATP6V0C*, because most iPS cells derived from skin fibroblasts are likely silent [62].

Conclusion

We discussed previous results for the efflux of cytosolic and/or vesicular neurotransmitters due to mediatophore/ATP6V0C from the current view involving various possible mechanisms mainly based on recent structural studies [36, 38] and a potential deal of progress in gene therapy and cell transplantation therapy.

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Conflict of interest S. M. owns equity in a gene therapy company (Gene Therapy Research Institution) that commercializes the use of AAV vectors for gene therapy applications. To the extent that the work in this manuscript increases the value of these commercial holdings, S. M. has a conflict of interest.

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