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

Somatic neurotransmitter release by vacuolar ATPase V0 sector c-subunits: potential use in gene or cell therapies for Parkinson's, Alzheimer's and psychiatric diseases

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Abstract

Gene therapy or cell transplantation therapy has made great progress in recent years. We overview mediatophore as a potential medical usage for gene and transplantation therapies in the nervous system. The 16-kDa proteolipid mediatophore was shown to mediate the secretion of acetylcholine in a Ca^{2+}-dependent manner. Mediatophore is known to be identical to the transmembrane c-subunit of the V0 sector of the vacuolar proton ATPase (ATP6V0C). Recent development of structural understandings reveals that ATP6V0C is not a simple pore-forming stalk enable to permeate transmitters. Therefore, it is time to review this topic revisited from the recent knowledge on ATPase. 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 rat *ATP6V0C* into the mouse caudate putamen enhanced the depolarization-induced overflow of endogenous DA in the 6-hydroxydopamine-lesioned substantia nigra. Motor impairment was ameliorated more efficiently in hemiparkinsonian model mice when ATP6V0C was expressed with DA synthesizing enzymes in comparison to the gene delivery of three DA synthesizing enzymes without *ATP6V0C*. These results suggest that ATP6V0C mediates neurotransmitter release from the cell soma and/or dendrites in the brain in an autocrine or paracrine fashion and may thus apply as a useful tool for gene therapy, cell transplantation therapy and inducible pluripotent stem cell therapy for Parkinson's disease, Alzheimer's disease and psychiatric disorders in the future.

Introduction

Secretion is one of major topics in the physiological science field [1-6]. Most cells are naturally equipped with the vesicular-soluble N-ethylmaleimide-sensitive factor attachment protein receptor (v-SNARE) that ensures the vesicular traffic and endo-exocytosis as well as neurotransmitter release [7,8]. However, another attractive secretion mechanism has been known since 1966 [9]. In the 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 1,000 V [10-12]. Mediatophore, a 15-kDa proteolipid, has been originally found in the presynaptic membrane of the *Torpedo* electric organ. French physiologists characterized this protein from the electric organ synaptosome and showed its remarkable ACh translocation properties (see review [13-16]).

Acetylcholine release by mediatophore

The much clear answer for the role of mediatophore in ACh release was rendered by projects in which reconstruction of mediatophore in a variety of cells that had no release machinery for ACh [13]. Israel, Dunat and their colleagues found that many cells were unable to release ACh in response to a calcium influx: this was the case for splenic cells, hepatocytes, erythrocytes, myeloma cells and several neuroblastoma cells [13-15]. In Marshall Nirenberg's laboratory at the National Institutes of Health, USA from 1965-1975, cholinergic neuroblastoma or neuroblastoma-derived hybrid culture cell

lines were cloned [17-20]. Neuroblastoma clones that are inactive with respect to neurotransmitter synthesis were also isolated. N18TG-2 neuroblastoma cells were typical such cell type. Contrarily, other culture cells were very competent for ACh release provided they were loaded with the transmitter: the C6BU-1 glioma cell line followed by the fibroblastic L cell line, PC12, Neuro 2A and NG108-15 cells [13-15,21-25]. Interestingly, the ACh release mechanism that is expressed by the latter cells was calcium dependent, when release was elicited by an influx of calcium with the calcium ionophore A23187 [11,13,16]. Fisher et al. [13] noticed that fibroblasts genetically modified to express choline acetyltransferase (ChAT) became able to release the transmitter by A23187 [26,27]. Some of other cell lines such as NG108-15 cells expressed voltage-dependent calcium channels that can be activated after depolarization [24,25], so that Ca^{2+} dependent ACh release was elicited by electrical stimulation [26]. Of particular interest is the series of work of Higashida and his colleagues who studied in co-culture with myocytes and neuroblastoma (Neuro 2A) or hybrid cell lines (NG108-15, N18TG-2 x C6Bu-1; NBr-10A, N18TG-2 x BRL-30E liver) [19-23], in which the detected release was measured as endplate potential of the ACh receptor origin [24]. The N18TG2 cells that were transfected with ChAT was *insufficient* to induce transmission at the synapse in co-cultures, and were unable to elicit miniature endplate potentials, as similarly as those experiments after loading them with ACh using the chemiluminescent ACh detection [16,21,22,27]. In Table 1 opposite cell lines are listed: One has a very efficient ACh release mechanism (the C6BU-1 glioma cells); another has no capability (the N18TG-2 neuroblastoma cells). The ACh release capacity

of NG108-15 or NBt-10A hybrid cells cells seems to be derived from C6Bu-1 glioma or Buffalo rat liver BRL-30E cells which equipped with mediatophore, rather than N18-TG-2 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) [10,11,13-15,28,29; Figure 1]. ATP6V0C plays a central role in H^+ transport as one part of the multi-subunit complex of ATPase [30-44]. Israel and his group initially proposed that medatophore forms a proteinaceous pore by the six transmemebrane c subunits of ATPase [10,11]. However, Israel's hypothesis about ATP6V0C in neurotransmitter release must be delineate by considering that V-ATPase is widely expressed in most types of cells and that, if ATP6V0C is indeed non-selectively permeable to cationic neurotransmitters [37,42-44]]. It is highly likely that ATP6V0C is also permeable to much smaller ions, like Na^+ and K^+ [44] Thus ionic currents through ATP6V0C might be recordable using an electrophysiological technique, when ATP6V0C is overexpressed in the plasma membrane. Of course such reports are not available, because nobody has been tested along such idea [44]. If ATP6V0C is not permeable to small ions and/or the currents through ATP6V0C are not clearly recorded, ATP6V0C might be classified as a transporter or an enzyme, and ATP6V0C is unlikely to be permeable to so diverse types of neurotransmitter. The amino acid sequence and recently reported structures of the c ring of the V0 domain [44] suggest very high hydrophobicity of the inner part of the

ring, which seems unsuitable for an ion channel pore. In addition, a direct interaction between ATP6V0C and the vesicular SNARE synaptobrevin and associated modulatory effects on ACh release have been reported [13,35,36]. Therefore, now it is time to carefully re-consider the role of mediatophore/ATP6V0C and thus, revisited it from the recent advances in knowledges of vacuolar ATPase.

Serotonin release from NG108-15 cells

The possibility to increase efflux other transmitters is of particularly interest. NG108-15 neuroblastoma x glioma hybrid cells or NBr-10A neuroblastoma x Buffalo rat liver cell hybrid cells release ACh upon high K^+ depolarizing stimulation and evoke endplate potentials in muscle cells via electrical stimulation of differentiated NG108-15 or NBr10-A 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 NG10-15 cells to release ACh from the cell soma and/or dendrites is due to ATP6V0C [13-15]. Then, we examined whether NG108-15 cells can release serotonin, which is another neurotransmitter. First, NG108-15 cells were soaked with [^{3}H]serotonin, and the cells were filled with [^{3}H]serotonin by uptake from the extracellular medium. After intensive washing, the cells were stimulated with a depolarizing high potassium medium. [^{3}H]Serotonin was released in the presence of extracellular Ca^{2+} but not in the absence of Ca^{2+}, showing that this behavior is Ca^{2+}-dependent [45,46]. NG108-15 cells are known to have both small clear vesicles and large dense core vesicles. Indeed, Furuya et

al. (1985) [45] clearly demonstrated them and suggested serotonin in dense core vesicles. Thus the evidence for serotonin in the dense core release through ATP6V0C is very weak; instead, it is likely that ATP6V0C just facilitate the exocytosis of serotonin-containing vesicles or the release of free serotonine in the cytoplasm (cytosolic serotonin).

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

Next, we examined the possibility of DA release in N18 cells transfected with vectors containing (5' to 3') forward form of *ATP6V0C* or the reverse-form of *ATP6V0C* that is newly produced in the expression vector with 3'- to 5' sequence of ATP6V0C [47]. After transfection, the transfected cells were preloaded for 1 h with 1 μM [^{3}H]DA in the presence of pargyline, a monoamine oxydase inhibitor that would have been added for preventing degradation of [^{3}H]DA [45]. Stimulation with 80 mM K^+ in the perfusion medium resulted in a detectable increase in [^{3}H]DA release in a Ca^{2+}-dependent fashion [45]. However, when identical experiments were performed in N18 cells transfected with reverse ATP6V0C, [^{3}H]DA was not released after high-potassium depolarizing stimulation. It is worth mentioning that ACh, serotonin 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 over-expressed in the mouse brain using adeno-associated virus (AAV) vectors individually harboring cDNAs of rat *ATP6V0C* [38], reverse form *ATP6V0C*, and *ATP6V0C-GFP* [47] Infection with these viruses in the substantia nigra of the intact mouse brain using the microinjection technique [47] revealed ATP6V0C-GFP expression in both tyrosine hydroxylase (TH)-positive neurons and TH-negative cells, which were other non-neuronal cells, probably astrocytes (see Figure. 1 of ref. 47).

Armed with this information, DA overflow from the synaptic cleft in the mouse striatum after treatment with AAV vectors [47] in the substantia nigra was measured using the *in vivo* microdialysis method [48]. DA release was significantly higher in the mice infected with *AAV-ATP6V0C* under both resting (5 mM extracellular potassium concentration) and depolarizing (50 mM) conditions (see Figure 2 of ref. 47). In contrast, viral infection with the reverse direction of *ATP6V0C* had no or little effect on DA release, with levels comparable to the control (sham-operated with 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 speed of 1 μL/min (total injected amount: 28 μg of

6-OHDA) [47]. 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 6-OHDA-lesioned mice in the unilateral substantia nigra was examined based on the latency to fall from the accelerated rotating rod. If the fall latency increased, we judged the changed 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 forward and reverse forms of *ATP6V0C* were infected stereotaxically into the lesion side of the caudoputamen. 6-OHDA-lesioned mice treated with three DA-synthesizing enzymes and ATP6V0C displayed significantly improved performance on the rotarod (134.2 ± 14.6 s) in comparison to mice treated with three DA-related genes plus reverse *ATP6V0C* (78.1 ± 17.0 s, $P < 0.01$, n = 5, ANOVA; see Figure 2 of ref. 47). Recovery was much greater in these mice than in mice treated with 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 to the value obtained for mice treated with all three enzymes plus *ATP6V0C*). The observed recovery of mice with the three enzymes plus ATP6V0C or its reverse form was approximately 73% or 36% of that observed in the wild-type mice (174.5 ± 12.4 s), respectively [47].

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

d-Amphetamine-induced rotation is a strong predictor of nigral TH cell loss [49,50]. Three weeks after lesion generation, the mice exhibited ipsilateral turning induced by the intraperitoneal injection of 3 μg/kg of *d*-amphetamine [47]. 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 amphetamine [47].

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 [47,51-64]. Many clinical trials (phase-one and phase-two) of gene therapy for PD were performed using AAV vectors [56]. 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) [53,54]. 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 way to supply DA continuously in the putamen (Figure 2).

The nerve terminals of dopaminergic neurons of the substantia nigra (SN) are selectively degraded and mostly lost in the putamen of the motor phase of chronic PD patients [63]. 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 (Figure 2), in which the fusion of exocytotic vesicles and the vesicular secretory apparatus is not identical to that observed in the intact nigral nerve terminals [63,64].

Alternatively, if the neurons do not possess the vesicular monoamine transporter, DA would not be incorporated into vesicles and not released through vesicular exocytosis. On the other hand, the vesicle fusion and its apparatus must be more or less similar among different types of neurons [63-67]. In any case, however, the evidence for somatic or dendritic release of DA through ATP6V0C in non-dopaminergic neurons or glia is likely to be very weak at this moment. For example, Jin et al. [47] did not explicitly indicate how many dopaminergic neurons were actually destroyed by their unilateral 6-OHDA injection procedure, though functionally dopaminergic responses were substantially destroyed. Thus it is still possible that injection of the genes of ATP6V0C and DA-synthesizing enzymes into the striatum would have just augmented the exocytotic DA release from the remaining dopaminergic nerve terminals. Indeed, it has been shown in many studies, including Jin et al. [47], that the enzyme introduction alone, i.e. even without ATP6V0C, may significantly improve the symptom.

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* [7,65,66]. ATP6V0C binds to syntaxin in SNARE complexes [67] or interacts directly with the v-SNARE synaptobrevin [35,36], and ATP6V0C may cooperate with SNARE proteins and vesicles for release during the late stage of fusion []. ~~DA release through proteolipid fusion pores suggests that the vesicles may be recycled without undergoing full fusion with the plasma membrane (i.e., the "kiss-and-run" process) [51-54].~~

ATP6V0C as a useful gene in the future

PD is a common neurodegenerative disorder in the elderly [62]. The characteristic motor symptoms, which include resting tremor, muscular rigidity, and bradykinesia, are caused by a severe decrease in the DA content of the striatum secondary to progressive loss of nigrostriatal DA neurons [68]. The refilling of DA in the striatum using extrinsic methods is important for functional restoration, regardless of the damage sustained by the nigrostriatal DA pathway. With recombinant AAV vectors, gene therapy is becoming a feasible therapeutic option for PD.

Baseline striatal dopaminergic neurotransmission in the normal striatum is maintained by tonic synaptic and non-synaptic DA release, which is largely independent of changes in neuronal impulse flow in the nigrostriatal pathway. As shown in previous studies [51,52,58], 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 [52], indicating that the DA synthesized by these extrinsic enzymes was released. This observation 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 micro-glial cells. DA released from nearby cells binds to the DA receptors on GABAergic neurons and functions in paracrine or autocrine mode, respectively (Figure 2). ~~Can cytoplasmic DA bind to the receptors, even when the DA-binding sites of the receptors are never exposed to the cytoplasm once they have been internalized? The papers [56, 57] just indicated down- or up-regulation of DA receptors in the presence or absence of preceding DA receptor activation, respectively.~~

Of course, ~~we pay less consideration to the binding of internalized DA receptors in response to alterations in dopaminergic tone [56,57].~~

Conclusion

The mediatophore is of course not sufficient to explain all the release properties of a natural synapse [16]. It can only give an partial explanation for the efflux of cytosolic and/or vesicular ACh, and probably for the most elementary events related to sub-quanta, as mentioned by Israel et al. [11,28]. They tried to analyze what would be the effect of transfecting them with a plasmid encoding for ChAT, and then for vesicular ACh transporter (VAChT) that allows them to store the transmitter in vesicles [13]. Several groups have found that ChAT and VAChT genes are adjacent and co-regulated

forming a cholinergic genomic locus (see for example [37]). Though we do not now that N18TG-2 cells equip with the VAChT and synaptic vesicles, we cannot exclude the possibility that the transfection with ATP6V0C may induce vesicle formation and transporter expression. In such case, it is possible that cytosolic as well as vesicular ACh is released [69].

We described a stronger improvement in movement impairment in mice expressing four proteins (TH, AADC, GCH, and ATP6V0C) than in mice expressing three proteins (TH, AADC, and GCH), largely due to the facilitated release of DA. We discussed these results not from an old view of the pore formed by ATP6V0C, but from the current view with various possible mechanisms for transmitter release mainly based on the recent structural understandings [44]. Such concept suggests that *ATP6V0C* may be useful in future gene or cell transplantation therapy [70,71]. As ACh and serotonin can be released in the same fashion, 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. This approach is applicable for iPSC therapy for similar categories of diseases [72-74] because better recovery would be expected if iPSC contains transmitter synthesizing genes and *ATP6V0C*, because most of iPS cells drived from the skin fibroblasts are likely silent [27].

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Conflicts of interests: 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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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	++	++	++	++
NBr10A	+++	+++	+++	+++
N18	0	0	0	0
N18+ACh*	++	0	0	0
N18+ACh*+V0C	++	++	+	++

ACh*, Cells were soaked in solution containing ACh (Ref). The same results was obtained by choline acetyltransferase transfection.

Table 2.

The number of rotations was significantly decreased in mice.

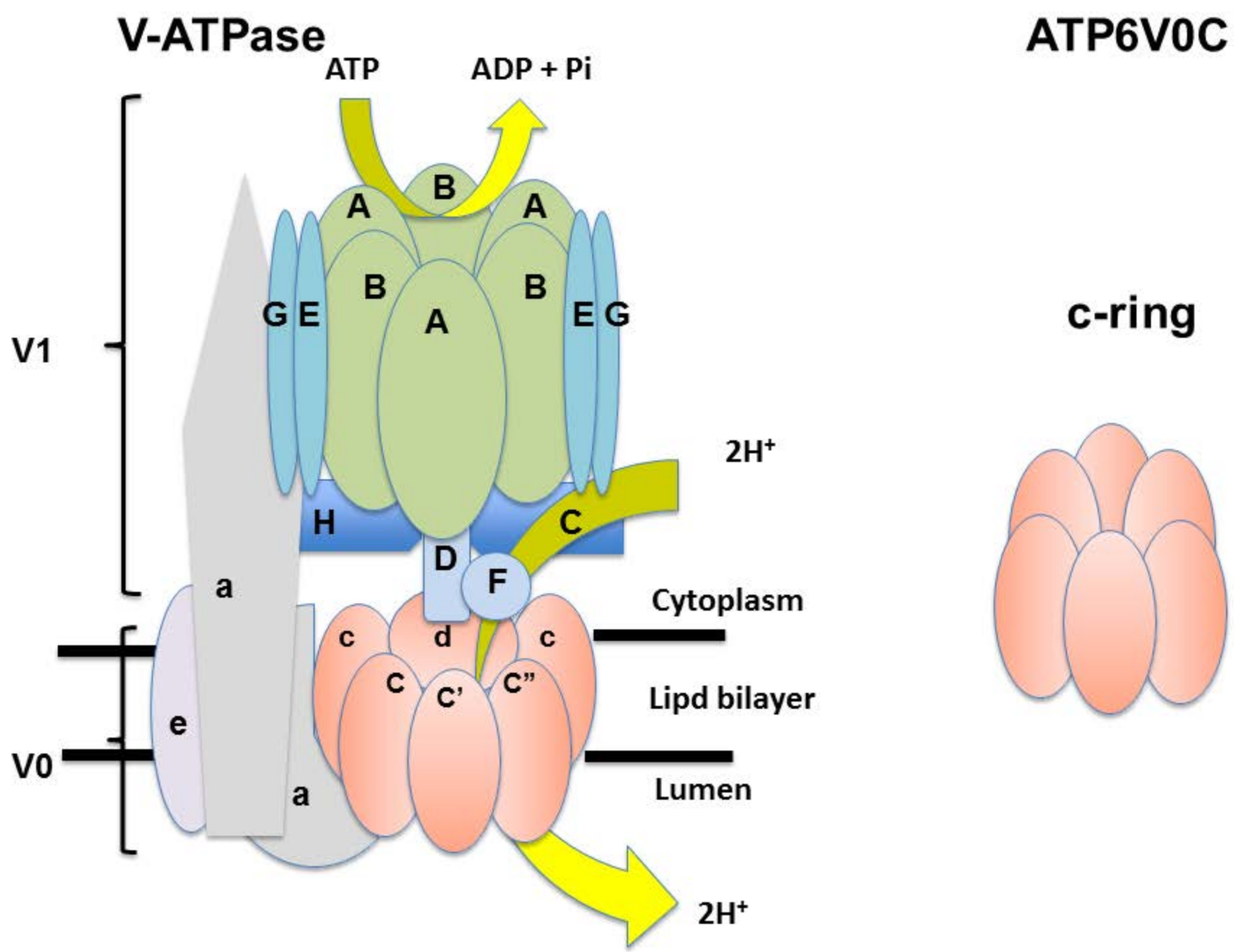
Mice transfected with	Rotations/3 min (% Recovery)	
3 enzymes	14.9 ± 1.2**	(53)
3 enzymes + reverse ATP6V0C	13.9 ± 4.3*	(56)
3 enzymes plus ATP6V0C	4.0 ± 1.0***	(87)
PBS	31.7 ± 2.0	(0)

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 to PBS.

Figure legends

Figure 1. Schema of molecular components of V-ATPase. (A) 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 (α, c, d and other components.) (B) c subunits in the V0 transmembrane sector of the vacuolar proton ATPase. Six c subunits form the stalk or pore. Redrawn from Nishi and Forgac (62).

Figure 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) or glutamatergic neurons (brown). DA release from the cell soma through the stalk or pore of ATPV0C6. DA released from adjacent cells (paracrine) or glutamatergic neurons (autocrine) binds to DA receptors at the synaptic or non-synaptic domains on glutamatergic neurons. Glutamatergic 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.


V-ATPase
ATP
ADP + Pi
V1
V0
A
B
G
E
H
C
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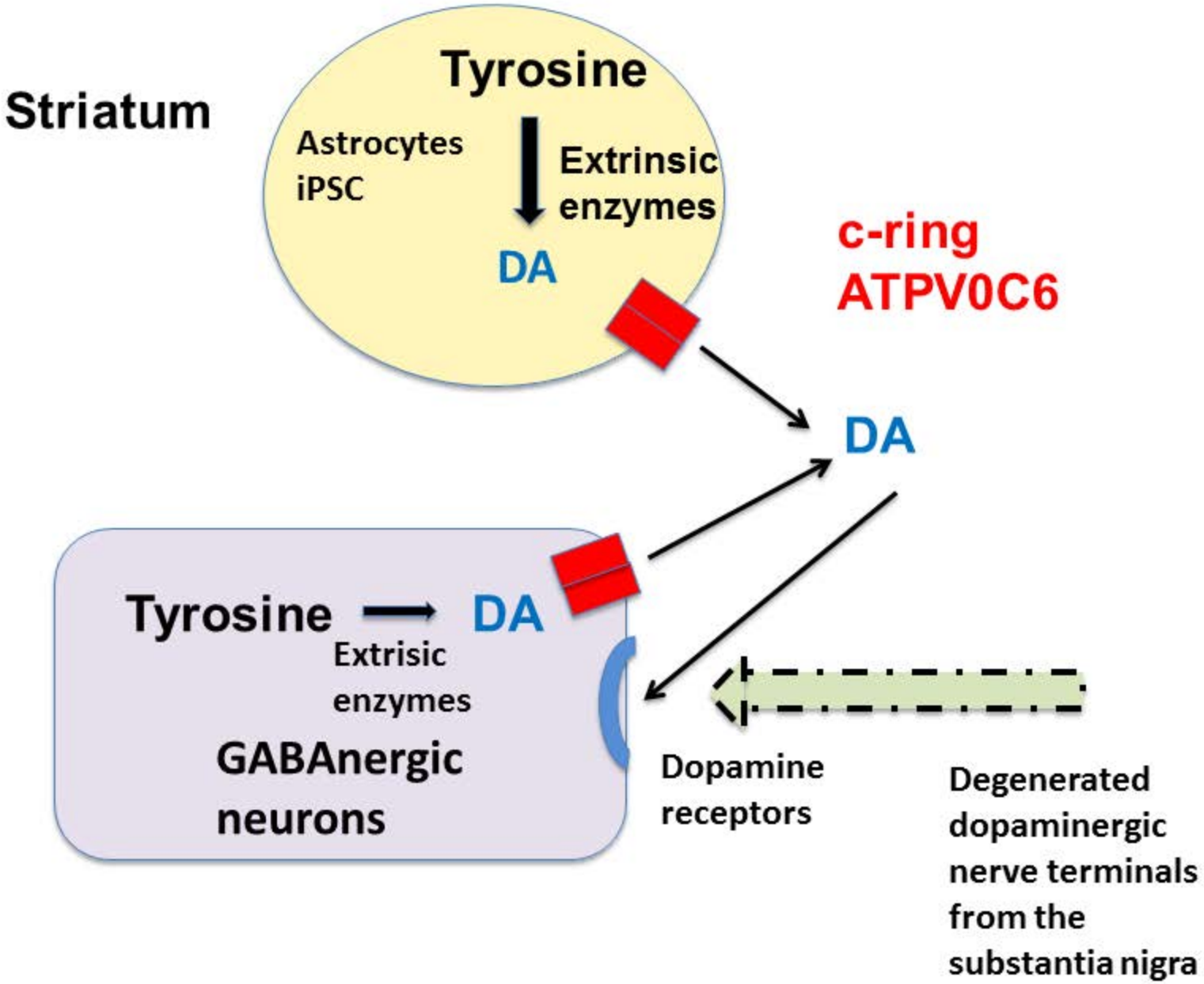
C"
e
2H+
Cytoplasm
Lipd bilayer
Lumen
ATP6V0C
c-ring

Figure 1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neurons
Dopamine
receptors
Degenerated
dopaminergic
nerve terminals
from the
substantia nigra

Figure 2