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journal or publication title	Neurochemistry International
volume	61
number	6
page range	907-912
year	2012-11-01
URL	http://hdl.handle.net/2297/32817

doi: 10.1016/j.neuint.2011.12.021

Dopamine release via the vacuolar ATPase V0 sector c-subunit, confirmed in N18 neuroblastoma cells, results in behavioral recovery in hemiparkinsonian mice

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Abstract

A 16-kDa proteolipid, mediatophore, in *Torpedo* electric organs mediates Ca^{2+}-dependent acetylcholine release. Mediatophore is identical to the pore-forming stalk c-subunit of the V0 sector of vacuolar proton ATPase (ATP6V0C). The function of ATP6V0C in the mammalian central nervous system is not clear. Here, we report transfection of adeno-associated viral vectors harboring rat ATP6V0C into the mouse substantia nigra, in which high potassium stimulation increased overflow of endogenous dopamine (DA) measured in the striatum by *in vivo* microdialysis. Next, in the striatum of 6-hydroxydopamine-lesioned mice, a model of Parkinson's disease (PD), human tyrosine hydroxylase, aromatic L-amino-acid decarboxylase and guanosine triphosphate cyclohydrolase 1, together with or without ATP6V0C, were expressed in the caudoputamen for rescue. Motor performance on the accelerating rotarod test and amphetamine-induced ipsilateral rotation were improved in the rescued mice coexpressing ATP6V0C. [^{3}H]DA, taken up into cultured N18 neuronal tumor cells transformed to express ATP6V0C, was released by potassium stimulation. These results indicated that ATP6V0C mediates DA release from nerve terminals in the striatum of DA neurons of normal mice and from gene-transferred striatal cells of parkinonian mice. The results suggested that ATP6V0C may be useful as a rescue molecule in addition to DA-synthetic enzymes in the gene therapy of PD.

1. Introduction

In both rodent and non-human primate models of Parkinson's disease (PD), viral vector-mediated gene delivery of one or three dopamine (DA)-synthesizing enzyme(s) into the striatum has been shown to ameliorate motor symptoms with efficient transduction of putaminal neurons (Shen et al., 2000; Muramatsu et al., 2002; Sun et al., 2004: Li et al., 2006; Bankiewicz et al., 2006; Svendsen, 2007; Fiandaca et al., 2008). In addition to tyrosine hydroxylase (TH) and aromatic L-amino-acid decarboxylase (AADC), GTP cyclohydrolase I (GCH) is necessary for efficient DA production (Shen et al., 2000; Muramatsu et al., 2002; Sun et al., 2004; Iancu et al., 2005). Using adeno-associated virus (AAV) vectors, two phase 1 clinical investigations of gene therapy for PD are underway based on these animal studies (Christine et al., 2009; Muramatsu et al., 2010b). In these protocols, gene transfer of AADC into the human putamen is combined with oral administration of L-3,4-dihydroxyphenylalanine (L-dopa). Expression of AADC would convert L-dopa to DA in the putamen. However, the mechanism of DA release from transduced cells remains to be elucidated.

Nerve terminals from dopaminergic neurons of the substantia nigra (SN) are selectively degraded and mostly lost in the putamen of PD (Nagatsu and Ichinose, 1999). This indicates that DA synthesized by extrinsic enzymes could be released from the soma of transduced neurons

or astrocytes, in which the fusion of exocytotic vesicles and vesicular secretory apparatus are not completely identical to those in the nerve terminals (Montana et al., 2006). Therefore, we postulated another mechanism in which a complex of proteolipid channels in SNARE fusion as described in yeast and *Drosophila* (Peters et al., 2001; Almers, 2001; Hiesinger et al., 2005) is effective in DA secretion from gene therapeutically DA-expressing cells in the striatum.

To assess the validity of this mechanism, we used mediatophore, a 16-kDa proteolipid originally found in the presynaptic membrane of the *Torpedo* electric organ (Israel et al., 1986 and 1991). Mediatophore was later shown to be an ortholog of mammalian C subunit in the V0 transmembrane sector of vacuolar proton ATPase (ATP6V0C; Nezu et al., 1992; Dunant & Israel, 2000; Hinton et al., 2009; Ediger et al., 2009; Di Giovanni et al., 2010; El Far and Seagar, 2011). ATP6V0C forms a proteinaceous pore (Zimmerberg, 2001; Dunant & Israel, 2000; Morel *et al.*, 2001;Drory et al., 2004; Inoue and Forgac, 2005; Drory and Nelson, 2006; Zhang et al., 2006) and mediates the Ca^{2+}-dependent and fast release of acetylcholine (ACh; Falk-Vairant *et al.*, 1996a and b; Dunant & Israel, 2000; Malo & Israel, 2003; Dunant *et al.*, 2009). In addition, the direct interaction between ATP6V0C and vesicular-soluble N-ethylmaleimide-sensitive factor attachment protein receptors (v-SNARE) synaptobrevin and its effects on modulation of ACh release

have been reported (Di Giovanni *et al.*, 2010; El Far and Seagar, 2011). However, the roles of ATP6V0C in the central nervous system on DA secretion have not yet been reported.

Here, we examined the effects of ATP6V0C on DA release in the mouse striatum after transfection with AAV harboring ATP6V0C and DA-synthesizing enzymes (Muramatsu et al., 2002). We examined the effects of ATP6V0C on motor behavior in a unilateral 6-hydroxydopamine (6-OHDA)-lesioned mouse model of PD. Furthermore, we examined DA release more directly using cultured neuronal cells. NG108-15 neuroblastoma × glioma hybrid (Nirenberg *et al.*, 1983a and b; Higashida *et al.*, 1986) and C6 glioma cells possess ATP6V0C, while no such protein was detected in N18 neuroblastoma clonal cells (Amano *et al.*, 1972; Falk-Vairant *et al.*, 1996a and b). It has been reported that ACh is not synthesized in N18 cells (Amano *et al.*, 1972) and that ACh is not released from transformed N18 cells to express choline acetyltransferase (Zhong *et al.*, 1995a and b) but released from N18 cells forced to express ATP6V0C (Falk-Vairant *et al.*, 1996a and b). Therefore, we examined whether [^{3}H]DA is released from NG108-15 hybrid cells with endogenous ATP6V0C or N18 neuroblastoma cells transfected with ATP6V0C.

2. Experimental procedure

2.1. Animals

Wild-type adult male mice of the ICR strain were kept in the animal center under standard conditions (24°C; 12-h light/dark cycle, lights on at 08:00) and received food and water *ad libitum*. The experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University.

2.2. 6-Hydroxydopamine-lesioned mice

Mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed in a stereotaxic apparatus. A small hole was made in the skull using a dental drill, and a 25-gauge stainless steel injection needle was inserted into the right striatum. A 2-μL aliquot of 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). The needle was left in place for an additional 3 min and then withdrawn slowly. Control animals received PBS into the substantia nigra according to the same procedure.

To validate that 6-OHDA effectively induced DA depletion, *d*-amphetamine-induced rotations were quantified at 4 weeks after microinjection of 6-OHDA. To measure rotational behavior, each mouse was placed in the center of an open field (a circle with a radius of 30 cm), and the number of complete turns was recorded during each 5-min period for 60 min. Mice were allowed 15 min to habituate to the open field before administration of *d*-amphetamine (3 mg/kg, i.p.). Only those animals exhibiting ≥ 7 ipsilateral rotations/min in a 60-min period at 4 weeks after 6-OHDA injection were included in further analysis.

2.3. Production and use of AVV

For generation of AAV vectors, we used vector plasmids containing an expression cassette, in which a human cytomegalovirus immediate-early promoter (CMV promoter) was followed by the first intron of the human growth hormone gene, the cDNA of TH, AADC, GCH, forward or reverse form ATP6V0C (Nezu et al., 1992), ATP6V0C-GFP, woodchuck hepatitis virus posttranscriptional regulatory element and simian virus 40 polyadenylation signal sequence between the inverted terminal repeats (ITR) of the AAV-2 genome. We used two helper plasmids, pAAV-RC and pHelper, harboring the AAV *rep* and *cap* genes, and the *E2A*, *E4*, and *VA1* genes of the adenovirus genome, respectively

(Agilent Technologies, Santa Clara, CA). HEK293 cells were cotransfected with the vector plasmid, pAAV-RC and pHelper using the calcium phosphate coprecipitation method. AAV particles were then harvested and purified by two sequential continuous iodoxale ultracentrifugations. The vector titer was determined by quantitative PCR of DNase-I-treated vector stocks. We routinely obtained 10^{12} – 10^{13} vector genome copies (vg).

2.4. Infection with AAV

AAV vector expressing either forward or reverse form of ATP6V0C was injected stereotaxically into the bilateral caudoputamen (CPU) of intact mice. Phosphate buffer solution (PBS) was injected in the CPU of control mice. In other experiments, mixtures of three separate AAV vectors expressing TH, AADC, and GCH, or in addition of two types of ATP6V0C, were injected stereotaxically into the unilateral CPU of hemiparkisonian mice six weeks after the 6-OHDA treatment.

2.5. In vivo *microdialysis*

Wild-type ICR mice (11 – 13 weeks old; n = 6 – 8) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and a guide cannula (AG-4; Eicom Corp., Kyoto, Japan) was implanted into the CPU

(+2.0 mm anterior, –0.5 mm lateral from the bregma, –2.0 mm ventral from the skull) according to the mouse brain atlas. On recovery from surgery, a dialysis probe (A-I-4-01; membrane length 1 mm; Eicom Corp.) was inserted through the guide cannula, and perfused with artificial cerebrospinal fluid (aCSF; 147 mmol/L NaCl, 4 mmol/L KCl, and 2.3 mmol/L $CaCl_2$) at a flow rate of 1.0 μL/min. The outflow fractions were collected every 10 min. After collection of baseline fractions, high potassium-containing aCSF (50 mM; isomolar replacement of NaCl with KCl) was perfused for 20 min through the dialysis probe. DA levels in the dialysates were analyzed using an HPLC system (HTEC-500; Eicom Corp.) equipped with an electrochemical detector, as described previously (Jin et al., 2007). The probe recoveries of DA were approximately 25%.

2.6. Immunostaining of brain sections

Brains were perfused with 4% paraformaldehyde, soaked in 30% sucrose, and dissected into coronal sections (30 μm). TH monoclonal antibody (1:800 or 1:8000; DiaSorin, Stillwater, MN) was used. Appropriate fluorescence-tagged (Invitrogen, Carlsbad, CA) or biotinylated (Vector Laboratories, Burlingame, CA) secondary antibodies were used for visualization. Immunoreactivity was assessed under microscopy (Olympus, Tokyo, Japan).

2.7. Motor behavior test with the rotating rod

The degrees of motor impairment were evaluated by measuring latency until falling from an accelerating rotating rod (diameter, 3 cm), the speed of which was slowly increased from 3 to 30 rpm within 5 min (Rotarod Apparatus Model MK-670; Muromachi Kikai, Tokyo, Japan). For 3 days before the 6-OH-injection to produce the nigra-caudate lesion, all mice were habituated to the rotarod test to minimize novelty stress and to achieve similar performance in the task. The time until falling off the rotating rod was measured.

2.8. Amphetamine-induced rotation test

d-amphetamine-induced rotation was used to estimate the extent of DA release in the striatum on three successive days three weeks after vector injection. *d*-amphetamine (3 mg/kg) was administrated intraperitoneally to the mice that had been treated with AAV vectors expressing TH, AADC, GCH, forward or reverse form ATP6V0C in the lesion side of the striatum. The criterion for ipsilateral abnormal rotation was more than 10 full turns per minute over 30 minutes on each examination day, as described previously (Imamura et al., 2003).

2.8. Experiments on release of [^{3}H]DA

N18 neuroblastoma and NG108-15 hybrid cells (Amano *et al.*, 1972; Nirenberg *et al.*, 1983a; Suzuki et al., 1983) were grown for 6 days in the presence of 1 mM dibutyryl cyclic AMP on 60-mm polyornithine-coated dishes (Higashida *et al.*, 1986). The experiment was performed in the presence of 10 μM pargyline, as described previously (Furuya *et al.*, 1985). Cultures were incubated with PBS for 30 min at 37°C. After rinsing, they were incubated with 1 μM [^{3}H] DA (1.11 TTBq/mmol; PerkinElmer, Wellesley, MA) for 1 h. They were then washed 3 times with PBS and incubated for successive 4-min periods in changes of PBS. High K^+ solution was prepared by iso-osmolar substitution of KCl (to 80 mM) for NaCl. $CaCl_2$ (1.8 mM) was omitted and 0.1 mM EGTA was added in some incubations. After the end of each 4-min period, the incubation medium was removed and fresh medium was added to the cultures. The incubation medium was passed through a 3 × 1 cm Sephadex G-10 column and eluted with 6 mL of H_2O followed by 3.1 mL of 0.5 M formic acid. The radioactivity in each fraction was estimated by liquid scintillation spectrometry.

2.9. Statistical analysis

All results are expressed as means ± SEM. One-tailed *t* test and one-way ANOVA combined with the Bonferroni test were used to analyze data with unequal variance between groups using the Stata data analysis and statistical software (StataCorp, College Station, TX). In all analyses, $P < 0.05$ was taken to indicate statistical significance.

3. Results

3.1. Effects of ATP6V0C on DA release in intact mice

To identify functional roles of ATP6V0C in the central nervous system, we expressed ATP6V0C in the mouse brain using the constructed AAV vectorsharboring cDNAs of rat ATP6V0C, its reverse form, and ATP6V0C-GFP. Infection of these viruses in the substantia nigra of the intact mouse brain revealed ATP6V0C-GFP expression in both TH-positive neurons and TH-negative cells, probably astrocytes (Fig. 1a – c).

Based on this information, DA overflow from the synaptic cleft in the striatum of mice treated with AAV vectors in the substantia nigra was measured by the *in vivo* microdialysis method. DA release was significantly higher in the mice infected with AAV-ATP6V0C under both resting (at 5 mM of extracellular potassium concentration) and depolarizing (50 mM) conditions (Fig. 2; $n = 6 – 8$, one-way ANOVA, $F_{5,49} = 19.96$ $^{*}P < 0.05$ or $^{**}P < 0.001$). Viral infection with reverse direction of ATP6V0C had no or little effect on DA release, comparable to the control (sham-operated with PBS injection) mice. We used reverse ATP6V0C as an additional control, instead of controls with vector alone, in the subsequent experiments.

3.2. Behaviors in hemiparkinsonian mice with or without ATP6V0C

Next, we examined the effects of ATP6V0C on recovery from motor impairment elicited in a unilateral 6-OHDA-lesioned mouse model of PD, in which AAV vectors of two types of ATP6V0C in addition to DA synthetic enzymes, TH, AADC, and GCH were infected on the striatum. Motor performance of 6-OHDA-treated mice was examined based on the latency to fall from the rotarod. 6-OHDA mice treated with 3 DA-synthesizing enzymes and ATP6V0C showed significantly improved performance on the rotarod compared with control lesioned mice treated with PBS alone (Fig. 3). The mean recovery by enzymes, enzymes with reversed ATP6V0C, and enzymes with ATP6V0C was to 43, 45 and 77% levels from 19% of the ability of the intact mice, respectively (*, **, ***P < 0.05, 0.01 and 0.001, respectively; $F_{4,20} = 40.42$, $P < 0.001$; $n = 5$).

As *d*-amphetamine-induced rotation shows the highest predictability of nigral TH cell loss (Iancu *et al.*, 2005; Fleckenstein *et al.*, 2007), we next examined the effects of amphetamine. Three weeks after lesion generation, mice exhibited ipsilateral turning induced by intraperitoneal injection of 3 μg/kg of *d*-amphetamine (Fig. 4). The number of rotations was significantly decreased in mice transfected with 3 enzymes (14.9 ± 1.2 rotations/3 min), 3 enzymes plus reverse ATP6V0C (13.9 ± 4.3), and 3 enzymes plus ATP6V0C (4.0 ± 1.0), compared with control

mice injected with PBS (31.7 ± 2.0) (one-way ANOVA, $F_{3,12} = 55.44$, $P < 0.001$. *$P < 0.01$, **$P < 0.002$, and ***$P < 0.001$, respectively ($n = 4$)). In sham-operated mice, no rotation was induced by amphetamine (n = 4).

3.3. [^{3}H]DA release from N18 cells expressing ATP6V0C or NG108-15 cells

Finally, we tested the hypothesis of [^{3}H]DA release through ATP6V0C *in vitro*. We cultured N18 neuroblastoma cells, which have no endogenous ATP6V0C (Falk-Vairant *et al.*, 1996a and b) and no ACh release capacity (Zhong *et al.*, 1995a and b; Falk-Variant *et al.*, 1996a and b). The cells were preloaded for 1 h in 1 μM [^{3}H]DA in the presence of pargyline (Furuya *et al.*, 1985). The release of [^{3}H]DA and its metabolites were measured. As expected, an increase in K^+ concentration in the perfusion medium to 80 mM (at 20 and 24 min) resulted in no detectable increase in release, compared from concentrations in the other period (one-way ANOVA, $F_{7,16} = 2.89$, $P = 0.037$; Fig. 5a). However, when identical experiments were performed in N18 cells transfected with ATP6V0C, [^{3}H]DA was released due to high-potassium stimulation in a Ca^{2+}-dependent manner ($F_{8,27} = 18.61$, $P < 0.001$ (Fig. 5b)).

NG108-15 neuroblastoma × glioma hybrid cultured cells with endogenous ATP6V0C (Falk-Vairant *et al.*, 1996a and b) were preloaded

with [^{3}H]DA. An increase in the K^{+} concentration resulted in a 1.8 ± 0.5-fold (n = 3) increase in release of radioactivity. Repeating the K^{+} pulse in the absence of Ca^{2+} abolished the K^{+}-evoked release.

4. Discussion

The results of the present study demonstrated for the first time that DA could be released from nigrostriatal cells overexpressing ATP6V0C of mice and from cultured neuronal cells endogenously or exogenously expressing ATP6V0C in a Ca^{2+}-dependent manner. The precise mechanism underlying the Ca^{2+}-dependent DA release is not yet clear. However, as ATP6V0C binds to syntaxin in SNARE complexes (Shiff et al., 1996) or shows direct interaction with v-SNARE synaptobrevin (Di Giovanni et al., 2010; El Far and Seagar, 2011), ATP6V0C may cooperate with SNARE proteins and vesicles for release at the late stage of fusion. Our finding of DA release through proteolipid fusion pores suggested that the vesicles may be recycled without undergoing full fusion with the plasma membrane, i.e., the “kiss-and-run” process (Ales et al., 1999; Fesce and Meldolesi, 1999; Valtorta et al., 2001; Rizo and Rosenmund, 2008).

PD is a common neurodegenerative disorder in the elderly (Samili et al., 2004). 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 (Santini et al., *2*009). Replacement of DA in the striatum is important for functional recovery regardless of damage sustained by the nigrostriatal DA pathway. With recombinant AAV vectors, gene therapy is becoming a feasible therapeutic option for PD (Lees et al., 2009;

Muramatsu et al., 2010a).

Efficient and long-term expression of genes for DA-synthesizing enzymes in the striatum restored local DA production and allowed behavioral recovery in animal models of PD (Muramatsu et al., 2002). 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 in previous studies (Shen et al., 2000; Muramatsu et al., 2002; Bankiewicz et al., 2006), the majority of transduced cells were neurons. As most of the striatal neurons express DA receptors that are internalized in response to alterations in dopaminergic tone (Dumartin et al., 1998; Muriel et al., 1999), DA produced in striatal neurons may bind to cytoplasmic DA receptors. However, we previously detected efficient baseline and L-dopa-induced DA release in the AAV-TH/-AADC/-GCH-injected putamen by microdialysis (Muramatsu et al., 2002), suggesting that the DA synthesized by these extrinsic enzymes was released by a non-synaptic mechanism or from non-neuronal elements such as astroglial cells. To our knowledge, these results represent the first insight into the possible involvement of ATP6V0C in these phenomena.

We demonstrated that ATP6V0C expression in the striatum increased endogenous DA release due to depolarization in the intact brain (Fig. 2). However, we did not measure DA release in the 6-OHDA-

lesioned brain with the same paradigm. Therefore, it will be interesting if we can show an evidence of recovery at the DA release level using in vivo microdialysis for ameliorating behavioral impairments with 3 enzymes and/or ATP6V0C. Such experiments currently underwent.

The results of the present study indicated that DA is similarly released from N18 neuroblastoma cells expressing ATP6V0C and NG108-15 cells with endogenous ATP6V0C and dense core vesicles (Furuya et al., 1985), although it is not yet clear what types of neurotransmitters are packed into such organelles. NG108-15 cells synthesize and uptake serotonin from the extracellular medium when loaded with [^{3}H]serotonin, and released [^{3}H]serotonin in a Ca^{2+}-dependent manner (Suzuki et al., 1983; Furuya et al., 1985). Therefore, it is possible that serotonin and DA share the ATP6V0C-dependent mechanism for release from NG108-15 cells.

In conclusion, we observed better improvement in movement impairment in mice expressing 4 proteins (TH, AADC, GCH, and ATP6V0C) than 3 (TH, AADC, and GCH). This concept of additional effects with ATP6V0C suggested that ATP6V0C may be useful in future gene therapy for PD, in addition to TH, AADC, GCH, and the DA transporter (Sun *et al.,* 2004).

ACKNOWLEDGMENTS

We thank Naomi Takino, Mika Kodera, and Hiroko Nishida for their help with producing AAV-vectors. This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and from the Ministry of Health, Labour and Welfare of Japan.

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Figure legends

Fig. 1. A sample with ATP6V0C-GFP expression in cells in the substantia nigra of mice at two weeks after transfection with AAV. **a**, Tyrosine hydroxylase (red) in dopaminergic neurons. **b**, ATP6V0C (green) expressed in cells. **c**, Merge of the two, showing expression in a dopaminergic neuron and non-dopaminergic cells (arrowheads). Bars 20 μm.

Fig. 2. Extracellular striatal DA levels measured by *in vivo* microdialysis in wild-type mice at two weeks after transfection with PBS or reverse ATP6V0C (rev) and ATP6V0C (V0C) genes. Dialysates were collected by perfusion with artificial CSF containing 5 mM and 50 mM K. $n = 6 – 8$, $*P < 0.01$, $**P < 0.001$.

Fig. 3. Rotarod performance tested in the 6-OHDA mouse model of PD by treatment with AAV-TH/-AADC/-GCH (Enz) with or without ATP6V0C or reverse ATP6V0C. Mice with relatively complete lesions were used for gene transfer; these mice received the 3 or 4 gene vectors, or PBS alone. The mice were tested starting 3 weeks after gene transfer. $n = 5$ lesion-positive mice. $*, **, ***P < 0.05$, 0.01, and 0.001, respectively.

Fig. 4. Amphetamine-induced rotations/3 minutes in mice prepared similarly to those described in Fig. 3. Each bar indicates the net mean turns (mean ± SEM) for each test, in the ipsilateral direction over 90 min after injection of *d*-amphetamine. $n = 3$. *$P < 0.01$, **$P < 0.002$, or ***$P < 0.001$.

Fig. 5. Release of [^{3}H]DA. Three and four N18 neuroblastoma cells transfected with (b) or without (a) ATP6V0C were preloaded with 1 μM [^{3}H]DA for 1 h. The cells were incubated for successive 4-min periods in the indicated solutions (normal PBS containing 1.8 mM Ca^{2+} with or without calcimycin, PBS excluding Ca^{2+} and including 0.1 mM EGTA, or 80 mM K-PBS). Bars represent SEM. N.s., not significant bewtten two bins during high potassium stimulation and other others in (a). *, **$P < 0.01$ and 0.001 in (b).

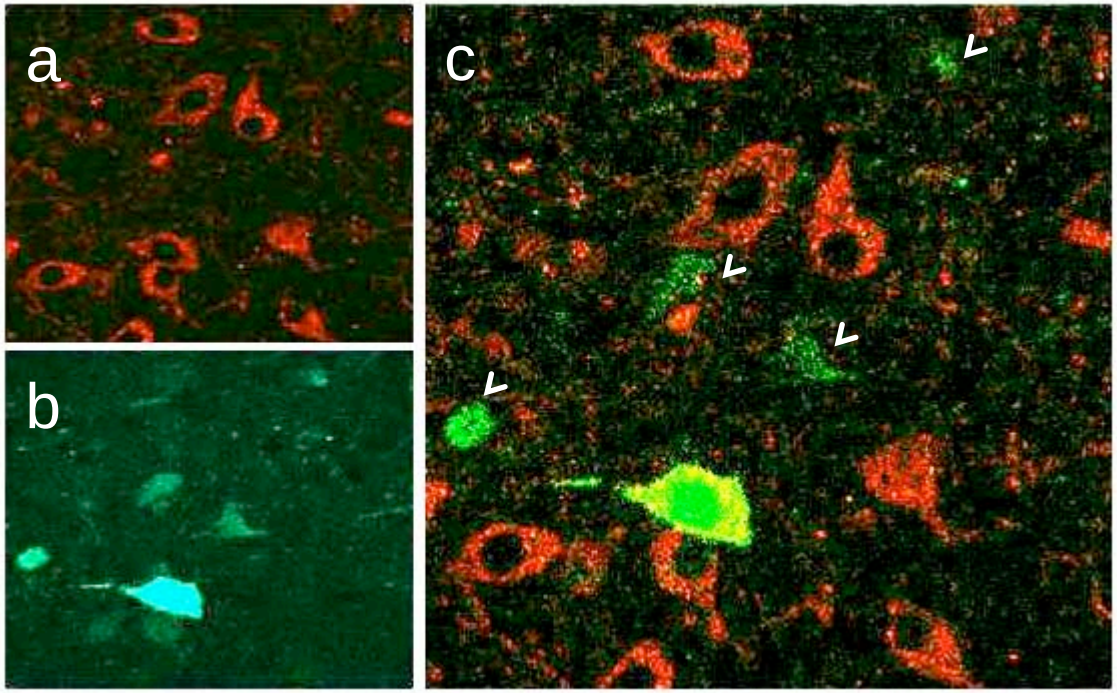
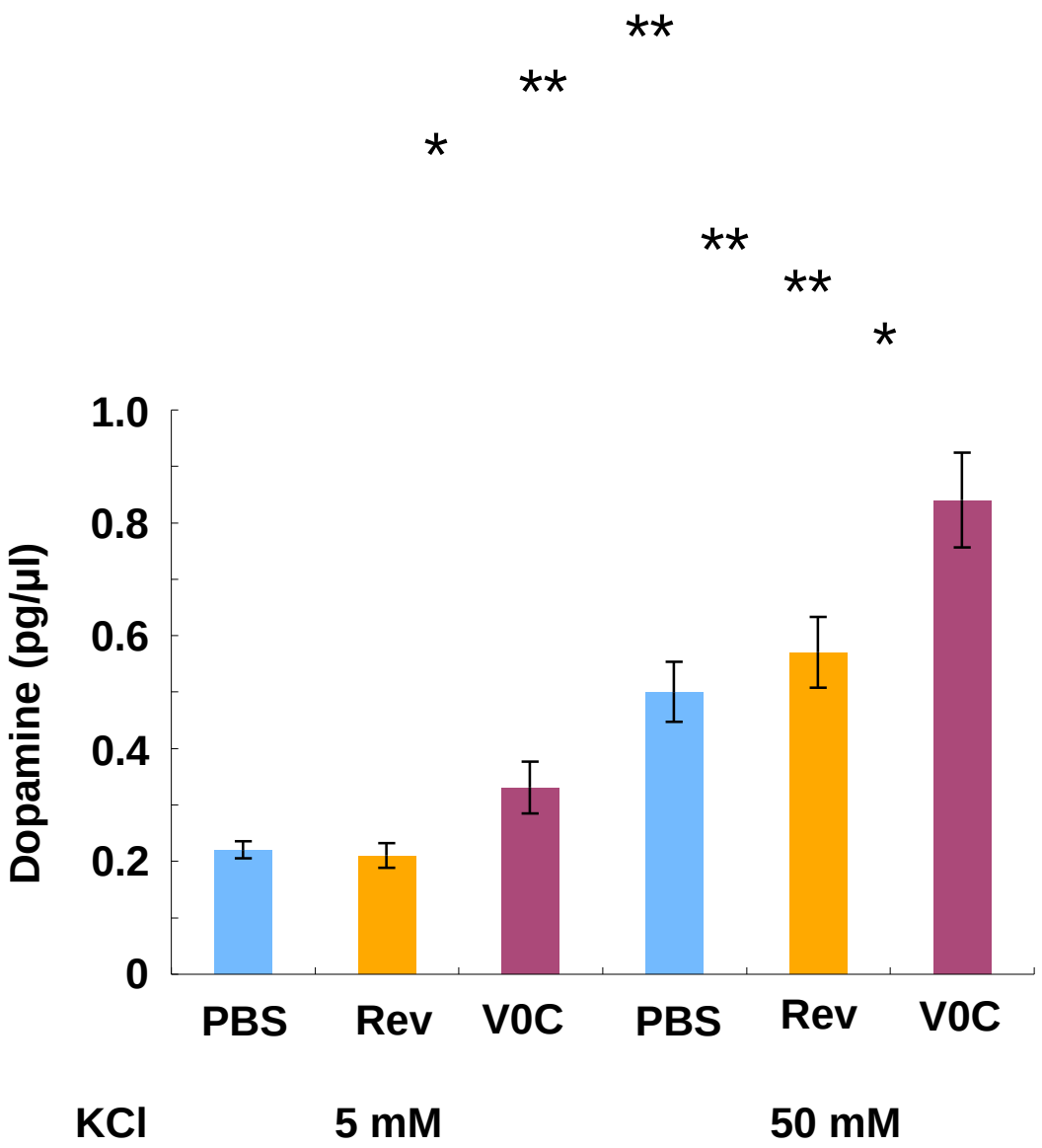


Fig. 1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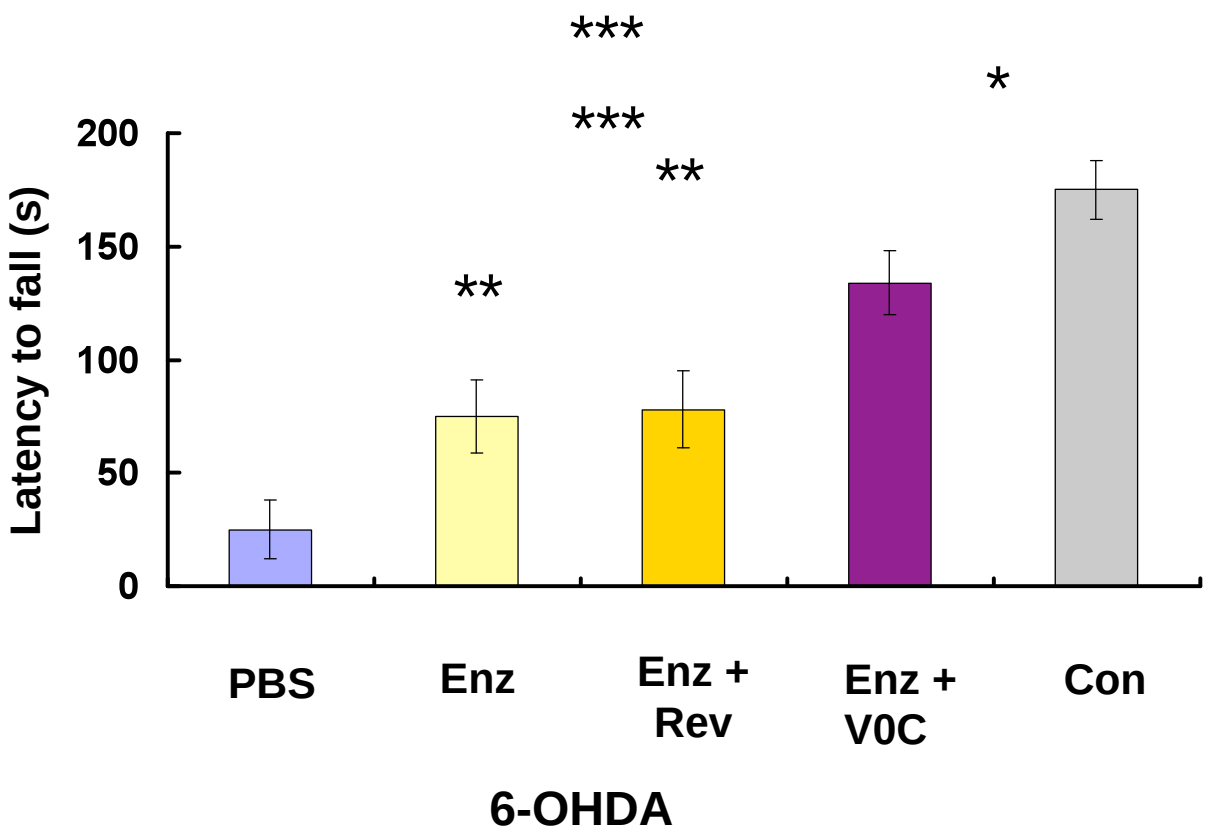
1.0
0.8
0.6
0.4
0.2
0
Dopamine (pg/µl)
PBS
Rev
V0C
PBS
Rev
V0C
KCl
5 mM
50 mM

Fig. 2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200
150
100
50
0
Latency to fall (s)
PBS
Enz
Enz + Rev
Enz + V0C
Con
6-OHDA

Fig. 3

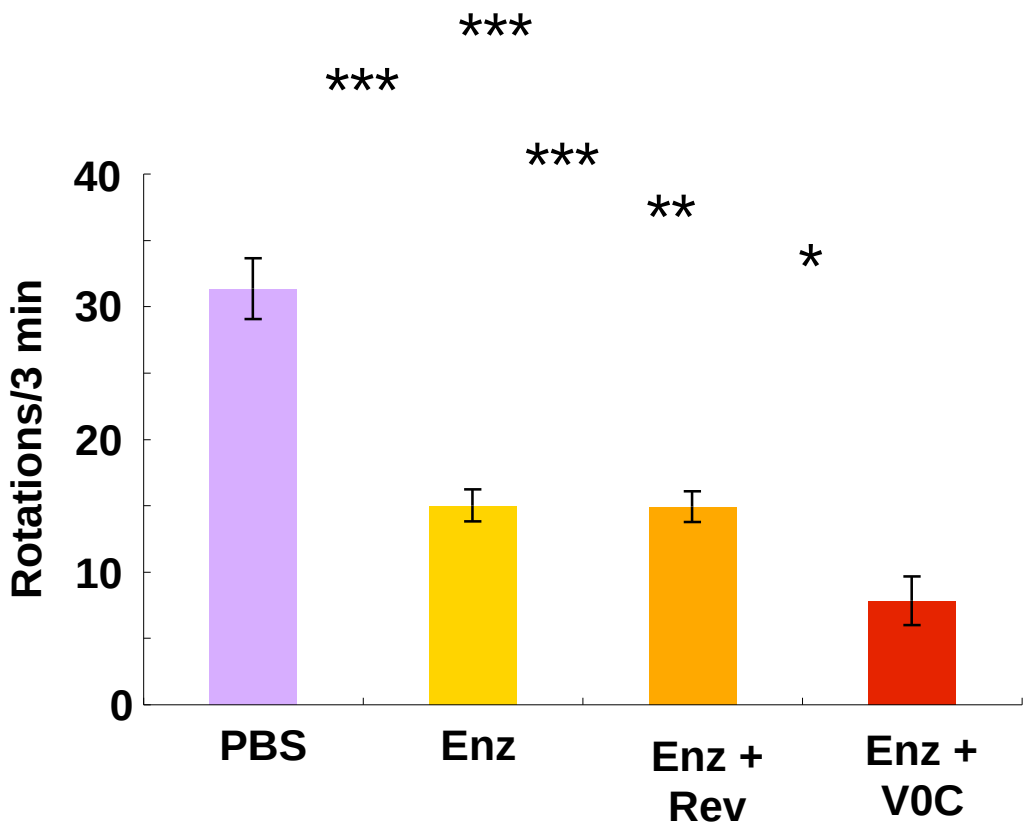

Fig. 4

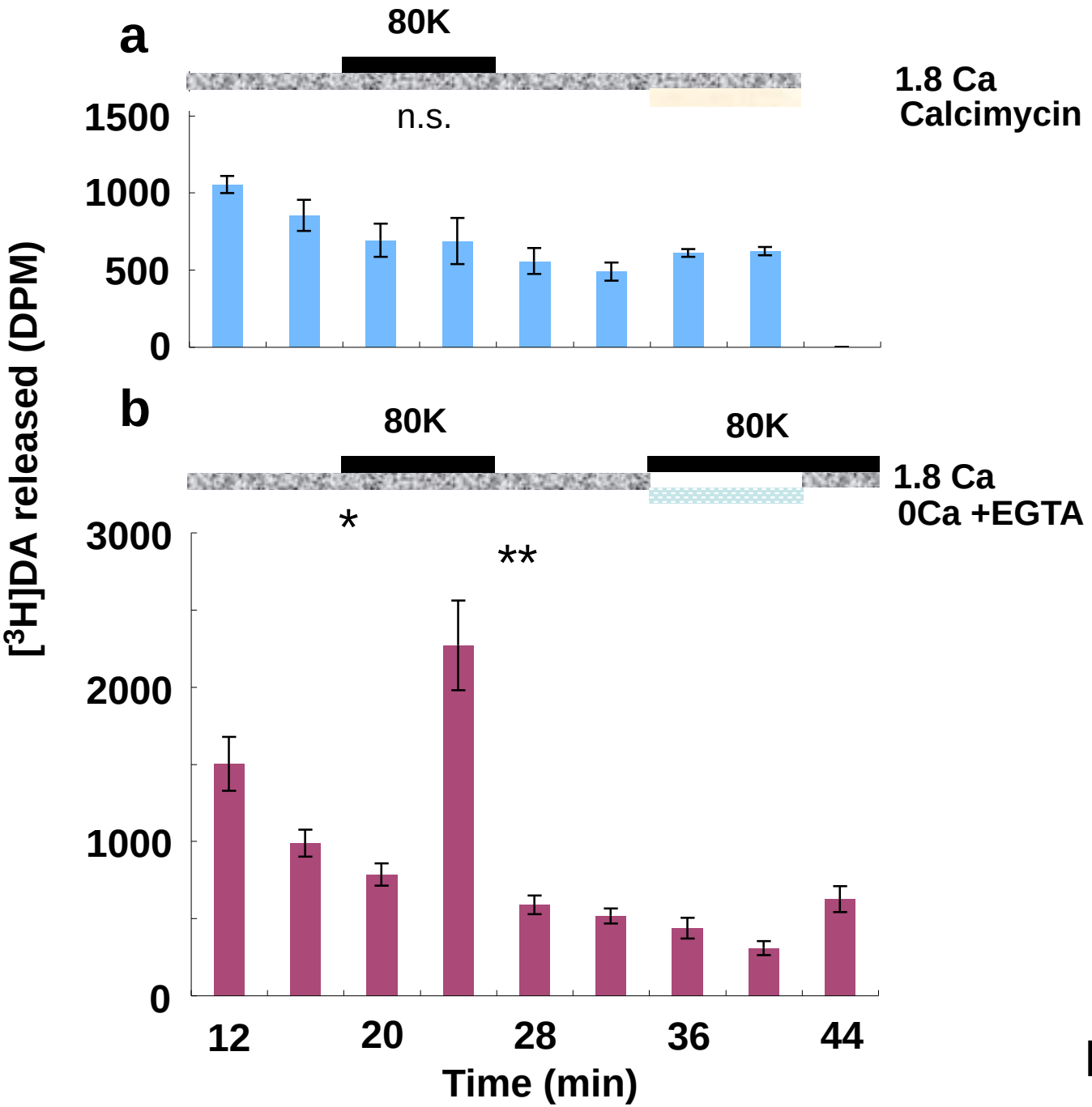
a
80K
1.8 Ca
Calcimycin
n.s.
1500
1000
500
0
b
80K
80K
1.8 Ca
0Ca +EGTA
*
**
3000
2000
1000
0
[3H]DA released (DPM)
12
20
28
36
44
Time (min)

Fig. 5