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Minagi Ozawa April 15th, 2015

Synaptic Vesicle Glycoprotein 2C (SV2C) in Parkinson's Disease-Related Striatal Vesicular Dysfunction

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An abstract of
A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Neuroscience and Behavioral Biology Program

2015

Abstract

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By Minagi Ozawa

Vesicular packaging of dopamine has two important roles in the neuron: 1) compartmentalization of dopamine to facilitate rapid neurotransmission, and 2) sequestration of dopamine and other toxicants from the cytosol. Impaired vesicular function in dopamine neurons has been shown to lead to dopaminergic degeneration, and is increasingly implicated in the pathogenesis of Parkinson's disease (PD). Identifying novel modulators of dopamine vesicle function is of utmost importance, as they represent novel pharmaceutical targets for the treatment of PD. The synaptic vesicle glycoprotein 2C (SV2C) is one such possibility; it is a vesicular protein enriched in dopaminergic brain regions, particularly the nigrostriatal pathway. Its genotype has also been shown to modulate the protective effect of cigarette smoking against PD. SV2C's function is unknown, although there is evidence to suggest that it plays a role in facilitating vesicular function with its heavily glycosylated intraluminal loop. We show here that in human PD striatum, SV2C expression is strikingly disrupted. We further shed light on to a possible function of SV2C in vesicular dopamine uptake. We show that introduction of SV2C expression in-vitro increases vesicular dopamine uptake, while genetic loss of SV2C in mice tends to decrease this. To investigate the mechanism of how SV2C may facilitate vesicular dopamine uptake, we focused on the abundant N-linked glycosylations of the large intraluminal loop of SV2C that have been proposed to adsorb intravesicular transmitters. We show that while no individual N-glycan at N480, N484, N534, or N565 is required for proper targeting of SV2C to the vesicle, each one is indispensable for SV2C to facilitate dopamine vesicular uptake. Taken together, our findings implicate SV2C's involvement in PD pathogenesis and provide convincing evidence for SV2C as a novel target for modulating dopamine vesicular function.

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2015

Table of Contents

Introduction

Parkinson's Disease (PD) is the second most common neurodegenerative disease in the United States and affects approximately 10 million people worldwide. It is characterized by various motor symptoms including tremor, rigidity, postural instability, and bradykinesia (slowness of movement), and also non-motor symptoms such as cognitive impairment and depression. Motor symptoms arise from the selective degeneration of dopaminergic cells in the substantia nigra pars compacta (SNc) and the resulting decrease in dopamine levels in the striatum (Parkinson, 1817; Olanow and Tatton, 1999). However, clinically, these symptoms are not observed until degeneration of the SNc has reached upwards of 60 to 70%. This cell death cannot be recovered and this is, in large part, the reason why disease-altering treatments currently do not exist. Treatments aiming for the prevention of cell loss will be of most use.

Genetic and epidemiological studies have provided some suggestions as to the mechanisms underlying the disease. Genes linked to familial cases of PD (only about 5% of all PD cases) have implicated mitochondrial dysfuction and dysregulation of protein folding and clearance of misfolded proteins (Abeliovich and Flint-Beal, 2006). Epidemiological studies provide insight into causes of sporadic cases of PD. These studies have found that pesticide exposure increases the risk for PD (Hatcher, 2008), while caffeine consumption and cigarette smoking decrease the risk of PD (de Lau and Breteler, 2006). Cigarette smoking is the strongest protective factor against the disease, and is consistently found to reduce the risk of PD by 50% (de Lau and Breteler, 2006). Rodent studies have also shown that exposure to cigarette smoke or nicotine protects

against dopaminergic cell death in animal models of PD (Shahi et al., 1991; Singh et al, 2010).

Research has failed to describe the mechanisms underlying this relationship, but a recent genome wide association study (GWAS) found an association between single nucleotide polymorphisms (SNPs) upstream of the synaptic vesicle glycoprotein 2C (SV2C) gene and the protection provided by cigarette smoking against PD. Individuals with a history of smoking who carry the major allele for both SNPs are less than half as likely to develop PD, while smokers who carry the minor alleles are at a 3.5 times higher risk for PD (Hill-Burns et al., 2012).

SV2C is part of the SV2 family, which includes two other closely related isoforms, SV2A and SV2B. All three isoforms are localized to synaptic vesicles and are structurally similar glycoproteins comprised of 12 transmembrane regions like VMAT2 (Janz and Sudhof, 1999). While SV2A and SV2B are ubiquitously expressed throughout the brain, SV2C expression is localized to a few regions including the basal ganglia and mesolimbic reward pathway (Fig. 1). SV2C expression has been observed in GABA and acetylcholine neurons of these regions, but it is particularly enriched in the dopaminergic cells of the SNc which project to the striatum (Janz and Sudhof, 1999; Dardou et al., 2011 and 2013).

Although the exact functions of SV2s are unknown, studies suggest that they regulate vesicular function in a few specific ways: 1) SV2 enhances transmitter loading by taking transmitters out of solution with its heavily glycosylated intraluminal loop (Reigada et al., 2003; Vautrin, 2009). 2) SV2A and SV2C bind with and regulate the trafficking of synaptotagmin, the calcium sensor for exocytosis (Schivell et al., 2005;

Yao et al., 2010). 3) SV2 facilitates neurotransmitter release by acting via presynaptic calcium (Wan et al., 2010; Nowack et al., 2012). 4) SV2 modulates the size of the readily releasable pool of vesicles (Xu and Bajjalieh, 2001). Since SV2C is preferentially expressed in dopamine neurons, collectively, these findings indicate a role for SV2C in dopamine vesicular handling.

In recent years, vesicular handling in dopamine neurons is increasingly implicated in PD. Synaptic vesicles in dopamine neurons have two critical roles. The first is the packaging of dopamine to prepare for release in neurotransmission. The second is the sequestration of dopamine out of the cytosol for neuroprotection. In the cytosol, dopamine auto-oxidizes to form cytotoxic free radicals. Normally, cytosolic dopamine levels are kept at a minimum by the continuous pumping activity of the vesicular monoamine transporter 2 (VMAT2) of the synaptic vesicle. Defects in vesicular uptake, however, lead to degeneration of dopaminergic neurons (Goldstein et al., 2012). In humans, mutations in the VMAT2 gene that critically impairs dopamine vesicular function have been linked to infantile parkinsonism (Rilstone et al., 2013), whereas, a gain-of-function VMAT2 haplotype has been shown to be neuroprotective against PD (Glatt et al., 2006). Most interestingly, a recent study has found that dopamine synaptic vesicles of PD patients have an impaired ability to sequester cytosolic dopamine (Pifl et al., 2014). Taken together, these findings indicate that dopamine vesicular dysfunction may be an abnormality that underlies and promotes the progressive degeneration of dopaminergic neurons in PD.

Considering SV2C's proposed function in dopamine vesicular handling, and that dopamine vesicle dysfunction is increasingly implicated in PD pathogenesis, SV2C

represents a major target for investigation of PD pathology and therapeutics. In this study, we investigated whether SV2C is involved in PD-related striatal vesicular dysfunction. Upon conducting immunohistochemical analysis of SV2C in post-mortem human PD striatal tissue, we find that SV2C expression is strikingly disrupted in human PD striatum as compared to an age-matched control (Fig. 4). We then tested our hypothesis that SV2C plays a role in vesicular uptake in the striatum by both an *in-vitro* and *in-vivo* approach. Expression of SV2C in HEK293 cells significantly increased vesicular uptake via VMAT2 (Fig. 6). Furthermore, genetic loss of SV2C in mice significantly decreased synaptic vesicular uptake of [^{3}H]-dopamine in the striatum (Fig. 7). Finally, the role of intraluminal glycosylation of SV2C in vesicular uptake was studied *in-vitro* by site-directed mutagenesis of SV2C's N-linked glycosylation sites (Fig. 8). Glycosylations of the large intraluminal loop of SV2's are theorized to be a key component of an intravesicular glycidic matrix that adsorbs neurotransmitters inside synaptic vesicles (Reigada et al., 2003). By taking neurotransmitters out of solution in the vesicle, SV2C may reduce the dopamine gradient and facilitate vesicular uptake of dopamine. It is also plausible that the glycans increase retention of intravesicular dopamine and by preventing efflux, SV2C glycosylation may serve to increase storage of transmitter. We observe that no single glycan is required for proper vesicular localization of SV2C, but that each one may be indispensable for SV2C to facilitate dopamine uptake into vesicles.

Materials and Methods

Animals.

Six- to 12-month old male WT C57Bl/6 mice (Charles River Laboratories) were used. All procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and previously approved by the Institutional Animal Care and Use Committee at Emory University.

Tissue Processing.

Immediately after sacrificing by rapid decapitation without anesthetization, brains were removed and hemisected at the midline. Brain halves to be used for western blotting analysis were dissected for the striatum, midbrain and cortex. These dissections were then flash frozen in liquid nitrogen or dry ice, thawed, and homogenized with a TissueTearer in homogenization buffer. Synaptosomal, vesicular, and membrane-associated fractions were achieved through differential centrifugation. Brain halves to be used for immunostaining were drop-fixed in 4% paraformaldehyde for one week and subsequently placed in 30% sucrose and 0.05% sodium azide for 3 days. Fixed brain tissue was then sectioned to 40μm with a freezing sliding microtome and preserved in cryoprotectant at -20C.

Antibodies.

Two rabbit polyclonal anti-SV2C sera were raised against a peptide in the N-terminal region (amino acids 97-114) of SV2C: one against mouse SV2C (mSV2C; sequence STNQGKDSIVSVGQPKG), and one against human SV2C (hSV2C; sequence SMNQAKDSIVSVGQPKG). Peptides were conjugated to Imject® Maleimide Activated mcKLH (Thermo Scientific) and sera were generated for our lab using Covance Custom Immunology Services. Monoclonal rabbit anti-tyrosine hydroxylase (TH) and rat anti-dopamine transporter (DAT) antibodies were purchased from Millipore. Polyclonal goat

anti-SV2A and anti-SV2B antibodies were purchased from Santa Cruz Biotechnologies. Horseradish peroxidase (HRP) conjugated secondary antibodies for western blotting were purchased from Jackson ImmunoResearch. All biotinylated secondary antibodies (bovine anti-goat, goat anti-rat, goat anti-rabbit, and goat anti-mouse) were purchased from Jackson ImmunoResearch Laboratories. AlexaFluor 594 goat anti-mouse, 594 donkey anti-goat, and 488 goat anti-rabbit (Life Technologies) were used as secondary antibodies for immunofluorescent labeling.

Western immunoblotting analysis.

Whole brain or striatal homogenate samples (20ug of total protein) were loaded on to NuPAGE 10% 1.0mm Bis-Tris Gels (Life Technologies) and subjected to SDS-PAGE at 130V for approximately 1 hour. Protein was transferred to Invitrolon PVDF 0.45µm pore size membranes (Life Technologies) at 30V for approximately 90 minutes. Membranes were blocked with 7.5% nonfat dry milk in tris-buffered saline with tween (TTBS) and incubated overnight at 4°C in rabbit anti-SV2C (1:2,500) or goat anti-SV2A/B (1:1,000) diluted in TTBS. After three 10 minute washes in TTBS, membranes were incubated for 1 hour at room temperature in goat anti-rabbit (1:5,000) or chiken anti-goat (1:5,000) HRP-conjugated secondary antibodies. Signal was detected by SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific) and imaged with a ChemiDoc XRS+ imager. Densitometric analysis was conducted using the ImageLab software (BioRad).

Immunohistochemistry.

Before each step (except after incubation in blocking solution), sections were washed three times for 10-minutes in phosphate buffered saline containing 0.2% Triton

X-100 (PBST) or tris-buffered saline with 1% Triton X-100 (TBST) except where noted. Sections were allowed to free float in net wells on a shaker.

Sections were incubated in Antigen Retrieval Citra Solution (BioGenex) for 1 hour at 70˚C for antigen retrieval. Sections were then treated with 3% hydrogen peroxide for 10 minutes to deactivate endogenous peroxidase activity. Nonspecific antibody binding was then blocked by preincubation in 3% normal horse serum or 10% normal goat serum for 1 to 4 hours at room temperature. Sections were incubated overnight at 4°C in polyclonal rabbit anti-SV2C serum (1:2500), rabbit anti-TH (1:1,000), rabbit anti-VMAT2 serum (1:50,000), or goat anti-SV2A/B (1:50). Incubation in goat anti-rabbit, goat anti-mouse, goat anti-rat, or bovine anti-goat biotinylated secondary antibody (1:800) was conducted at room temperature for 1 hour. Antigen signal was enhanced by avidin-biotin complex formation using the Vectastain ABC Elite kit (Vector Laboratories).

Visualization was performed with SIGMAFAST 3,3-diaminobenzidine (DAB) detection (Sigma-Aldrich). Sections were mounted on to Superfrost Plus Microscope Slides (Fisher Scientific), dehydrated in a graded ethanol series and xylene, and coverslipped with Fisherfinest Premium Cover Glass (Fisher Scientific) using permount. Light microscopic images were acquired with NeuroLucida (MBF Bioscience).

Immunofluorescence.

Fluorescent staining to visualize co-localization was conducted by a similar procedure to immunohistochemical DAB stainings but without treatment in 3% hydrogen peroxide and ABC. Mouse anti-TH (1:1000) primary antibodies were each combined to make cocktails with the rabbit anti-SV2C (1:2500) primary antibody. Sections were

incubated in these cocktails for simultaneous detection of each of the two proteins. AlexaFluor 594 goat anti-mouse (1:800) and 488 goat anti-rabbit (1:800) secondary antibodies were combined into a cocktail for a 1-hour incubation for TH/SV2C co-labeling. Incubation in secondary antibody and all subsequent steps occurred while protected from light.

After the final wash following secondary antibody incubation, sections were mounted on to superfrost slides and coverslipped using VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Immunofluorescence was also imaged using NeuroLucida.

Human tissue.

Paraffin-embedded 8um human sporadic PD (n=2) and control (n=4) striatal sections were obtained from the University of Washington Medical Center Brain Bank (Seattle, WA). Control and PD cases were closely matched in age (control: 72.3+/-4.7, PD: 71.0+/-7.0 years) and gender (all male except one of control case). Sections were deparaffinized in two 5-minute washes in xylene and rehydrated in decreasing concentrations of ethanol. Standard immunohistochemistry procedures followed (see above for details).

FFN206.

HEK293 cells were cultured in DMEM containing 10% fetal bovine serum, and 1% penicillin streptomycin. Cells were plated on to PDL-coated Greiner 96-well half area, black walled plates at 40,000 cells/well. Rapid transfection with pcDNA3.1, hSV2C in pcDNA3.1, hVMAT2 in CMV, or hSV2C mutant constructs in pcDNA3.1 using Lipofectamine 2000 (Invitrogen) was conducted according to manufacturer's protocols.

The FFN206 assay was conducted approximately 24 hours later as described by Hu et al. (2013): Media was replaced with 90uL/well of experimental media (DMEM without phenol red, 10% FBS, 1% 200mM L-glutamine) and control wells were treated with 10 uM/well tetrabenazine (TBZ). After a 30-minute incubation at 37C, all wells were treated with 1 uM/well FFN206. After an hour of incubation at 37C, cells were washed with pre-warmed PBS, filled with 90uL of PBS, and fluorescence measured on a Victor3 plate reader (PerkinElmer) using the Elmer 2030 Workstation software.

Generation of SV2C-KO mice.

We obtained 3 lines of SV2C-KO C57BL/6 ES cells from the European Conditional Mouse Mutagenesis Program (EUCOMM). This construct contains a FRT-flanked cassette inserted into intron 2. The cassette contains a IRES:lacZ trapping sequence and a floxed neomycin resistance gene that disrupts gene function and results in a knockout of protein expression. Two additional loxP sites flank intron 2. ES cells containing the construct were injected into blastocysts, and we chose chimeric mice to breed with WT C57BL/6 mice. We achieved germline transmission of the construct in the resulting offspring, and chose these mice to found the SV2C-"knockout first" line. Currently, we are breeding animals from the SV2C-knockout first line with Flip-recombinase (FLP) mice to excise the floxed cassette and restore the functional gene with intron 2 flanked by loxP sites. Subsequent crossing of these animals with mice containing inducible or promoter-driven Cre will result in conditional or inducible KO, giving us temporal and spatial control of SV2C expression.

Vesicular [^{3}H]- dopamine uptake.

Synaptic vesicles were isolated by differential centrifugation from whole brain homogenates in buffer (4 mM HEPES, 0.32 M sucrose, pH 7.4): 1000xg for 10 minutes and the resulting supernatant at 20,000xg for 20 minutes. Vesicular [^{3}H]-dopamine uptake was conducted as described previously (Caudle et al., 2007). Samples were harvested with a Brandel cell harvester and counted using Beckman LS6500 Liquid Scintillation Counter (Beckman Instruments).

Site-directed mutagenesis.

Using a human SV2C construct in pcDNA3.1 (pcDNA3.1-hSV2C) as a template, site-directed mutagenesis was conducted using the GeneArt Site-Directed Mutagenesis Kit (Invitrogen) at four of SV2C's five N-glycosylation sites (N480, N484, N534, and N565) by substitution of asparagine to alanine. Mutagenesis was confirmed by Beckman Coulter Quicklane Sequencing services.

Immunocytochemistry

Cells were plated onto LabTek II 8-well Chamber Slide (Electron Microscopy Sciences) and transfected the following day with pcDNA3.1, hSV2C in pcDNA3.1, mCherry-VMAT2 in pcDNA3.1, or SV2C mutant constructs in pcDNA3.1 using Lipofectamine 2000 according to manufacturer's protocol. Cells were fixed 24 hours later with 4% PFA. With washes in PBST between each step, cells were blocked in a solution of 3% NHS and BSA PBST, incubated overnight in rabbit anti-SV2C primary antibody (1:2500), and incubated for an hour the next day in the appropriate fluorescent secondary antibody (1:1000). Cells were imaged on the Olympus Fluoview 1000 TIRF microscope at the Emory University Integrated Cellular Imaging Core.

Statistical Analysis

All statistical analysis was performed on raw data by 1 way ANOVA or Student's t-test using GraphPad Prism. *Post hoc* analysis was performed using the Newman–Keuls test. Statistical significance is reported at $p<0.05$. All errors shown are standard error of the mean (SEM).

Results

Differential localization of SV2s

In order to characterize the expression pattern of SV2C and compare with that of SV2A and SV2B, immunohistochemistry was performed on sagittal mouse brain sections, labeling for each of the SV2s, and stained by 3-3' diaminobenzadine (DAB) (Fig. 1). While SV2A and SV2B are ubiquitously expressed throughout the brain, SV2C expression is strikingly restricted. SV2C expression is particularly enriched in the basal ganglia and mesolimbic reward pathway, including the striatum (CPu), nucleus accumbens (NAc), ventral pallidum (VP), globus pallidus (GP), and substantia nigra (SN).

SV2C localization in striatum and midbrain

Considering the relevance to PD pathology, we focused our further analysis on SV2C in striatal and midbrain regions. SV2C expression is enriched in the dorsal striatum (CPu), ventral striatum (nucleus accumbens), and VP (Fig. 2). In the midbrain, the fibers of the substantia nigra reticulata (SNr) and the cell bodies of the SNc and ventral tegmental area (VTA) also show strong SV2C immunoreactivity.

Colocalization of TH and SV2C in midbrain

To see if SV2C is expressed in dopaminergic neurons of the midbrain, SV2C and tyrosine hydroxylase (TH) were labeled by double immunofluorescence on mouse midbrain sections (Fig. 3). TH catalyzes the conversion of L-tyrosine to L-DOPA, the precursor to dopamine, and thus, is selectively expressed in catecholaminergic neurons. We used TH as a marker for dopamine neurons in the midbrain. Strong TH immunoreactivity (Fig. 3, green) is detected in the cell bodies of the VTA and SNc as expected, indicating these cells as dopaminergic. SV2C expression (Fig. 3, red) is also observed in the cell bodies of this region. TH and SV2C were found to colocalize in many of these cells. Our data confirms the findings of Dardou et al. (2011), which showed that SV2C is expressed in approximately 70% of dopaminergic cells of the VTA and SNc.

SV2C expression in human PD striatum

To investigate if expression of SV2C could be affected in PD, striatal tissue of human post-mortem PD patients were analyzed by immunohistochemistry (Fig. 4). Striatal brain sections of four control and two PD cases were obtained from the Center for Neurodegenerative Disease Brain Bank at Emory University for this purpose. Control and PD cases were closely matched in gender and age (average of control: 72.3±4.7; average of PD cases: 71.0±7.0).

In control striatum, SV2C is expressed diffusely throughout the caudate and putamen, which is likely staining of the terminals of the dopaminergic neurons projecting

from the substantia nigra. Strong SV2C immunoreactivity is also found in cell bodies of these regions, which are likely medium spiny neurons.

Strikingly different is SV2C expression in PD striata. In the caudate and putamen of each of the PD cases, terminal-like SV2C staining appears to be diminished, and instead of cell bodies, SV2C stains for punctate, aggregate-like structures throughout the region. Double immunofluorescence labeling with SV2C and ubiquitin reveal that these SV2C-positive puncta are also strongly immunoreactive for ubiquitin (Fig. 5), suggesting that these aggregates are pathological inclusions. Unpublished immunohistochemical analysis conducted by a graduate student in our laboratory has shown that the puncta appear to be in vesicular GABA transporter (VGAT)-positive GABAergic cell bodies. Notably, other unpublished data from our lab has shown that striatal tissue of cases of other neurodegenerative diseases including multiple system atrophy (MSA) with and without dementia with lewy body (DLB), Alzheimer's disease (AD), and progressive supranuclear palsy (PSP), do not exhibit such a disruption in SV2C expression.

SV2C overexpression increases vesicular uptake in-vitro

To study the function of SV2C in vesicular uptake, we utilized FFN206, a fluorescent false neurotransmitter that imitates the action of dopamine inside a presynaptic terminal. FFN206 is a VMAT2 substrate that can be used in a high-throughput format *in-vitro* assay to quantitatively measure vesicular uptake activity (Hu et al., 2013). When FFN206 enters a cell, it is taken up into vesicles through VMAT2, like dopamine, where it fluoresces in response to an acidic environment. This fluorescence is measured as an indicator of vesicular uptake level.

In this experiment, HEK293 cells stably expressing VMAT2 (VMAT-HEK cells) were transfected with pcDNA3.1 or pcDNA3.1-hSV2C. VMAT-HEK cells expressing SV2C had significantly increased vesicular uptake via VMAT2 as compared to cells not expressing SV2C (Fig. 6, $p<0.05$). This data supports our hypothesis that SV2C facilitates vesicular uptake.

Genetic loss of SV2C decreases vesicular dopamine uptake in-vivo

To study the role of SV2C in striatal dopaminergic synaptic vesicles *in-vivo*, we utilized SV2C-KO mice (Fig. 7a). We obtained C57BL/6 embryonic stem cells containing EUCOMM knockout-first allele constructs that contain FRT-flanked cassettes in intron 2 of the SV2C gene; this disrupts gene translation and results in functional knockout of the SV2C gene. We confirmed germ line transmission of the construct into mice using PCR and southern blotting. The mice used in this study were the homozygous knockout mice bred from these animals. Western blotting and immunohistochemistry confirmed the loss of SV2C expression (Fig. 7b, c). SV2A and SV2B expression in SV2C-KO mice did not differ from WT control (Fig. 7b), suggesting that genetic SV2C deletion does not affect expression of other SV2 isoforms.

To examine the effect of SV2C-KO in vesicular dopamine uptake, we conducted a radioactive dopamine uptake assay (Fig. 7d). Synaptic vesicles were isolated from striatal homogenates of WT and SV2C-KO mice (n=2 per group) and uptake of [^{3}H]-dopamine was allowed to occur. After washes with buffer, radioactivity was measured as an indicator of DA uptake level. SV2C-KO mice were found to have a trend of decrease

in vesicular dopamine uptake (n=2, p=0.068). Consistent with our *in-vitro* data, this may support our hypothesis that SV2C facilitates vesicular dopamine uptake.

Role of SV2C N-glycosylation in vesicular localization and dopamine uptake

Generation of SV2C glycosylation mutant constructs. To investigate the underlying molecular mechanism as to how SV2C may enhance vesicular uptake, we focused on SV2C's intraluminal glycosylations (Fig. 8). Glycosylations of SV2 have been shown to be a core component of an intravesicular matrix and acts to adsorb and immobilize neurotransmitters inside vesicles (Reigada et al., 2003). By taking dopamine out of solution, SV2C glycosylation may act to decrease the dopamine gradient and allow for greater packaging of dopamine. Another possibility is that by adsorbing dopamine, SV2C glycans may prevent transmitter efflux out of the vesicle, and therefore facilitate increased storage of dopamine.

To test if intraluminal glycosylation underlies SV2C's ability to facilitate dopamine uptake, we generated hSV2C-pcDNA3.1 constructs that contain point mutations at the N-glycosylation sites (Fig. 8). We designed the constructs so that the asparagine (AAC/AAT) residues, that are the site of glycosylation, are substituted to alanine (GCA/GCC/GCT/GCG). Alanine was chosen because its methyl functional group is small and chemically inert. At this time, mutated constructs for only four out of 5 glycosylation sites could be generated due to technical difficulties.

SV2C N-glycosylation mutants show proper vesicular localization Before testing the effects of these mutations on vesicular uptake, we tested whether localization of SV2C is affected because glycosylation is often important for proper trafficking of

proteins (Fig. 9). We conducted immunocytochemistry of HEK293 cells transfected with the SV2C mutant constructs. Cells were imaged on a TIRF microscope that allows for selective fluorescence excitation of regions close to a solid surface without exciting fluorescence from regions farther from the surface; hence cellular environments near the plasma membrane can be visualized at high resolution with low background fluorescence (Axelrod, 2001).

VMAT2 expression is observed throughout the whole surface of the cell in a punctate pattern, indicative of vesicular localization (Fig.9). Wildtype hSV2C also has a similar expression pattern, and strong coexpression with VMAT2 is observed in cells transfected with both proteins. It should be noted that our SV2C antibody shows some non-specific staining, as demonstrated by the green SV2C signal that is detected in cells not transfected with SV2C. This signal however, does not colocalize with VMAT2 expression, indicating this as non-specific to SV2C. Cells transfected with each of the four SV2C glycosylation mutants also showed strong colocalization of SV2C with VMAT2, suggesting normal vesicular localization. This indicates that SV2C glycosylation is not individually required for proper trafficking of SV2C to the vesicle.

SV2C N-glycosylation mutants show significantly decreased uptake. Using these constructs, we conducted an FFN206 assay to investigate whether these N-linked glycosylation site mutations affect vesicular uptake (Fig. 10). HEK293 cells transfected with VMAT2 and wildtype SV2C showed significantly increased vesicular uptake through VMAT2 by approximately 25%, as compared to cells transfected only with VMAT2 ($p<0.05$). This is consistent with our observation from past experiments that SV2C expression consistently confers a 20-30% increase in uptake. HEK cells

transfected with VMAT2 and any one of N480A, N484A, N534A, or N565A SV2C, all showed significantly lower vesicular uptake via VMAT2 as compared to cells expressing wild type SV2C ($p<0.01$). There were no statistically significant differences in uptake levels among the mutants of the different glycosylation sites ($p>0.05$). Uptake levels of cells with mutant SV2C was not statistically different with that of cells only expressing VMAT2 and not WT SV2C ($p>0.05$). Thus, loss of an intraluminal glycosylation appears to prevent SV2C from facilitating vesicular uptake. This supports our hypothesis that the intraluminal glycosylations are the molecular basis for SV2C's ability to facilitate vesicular uptake.

Discussion

SV2C represents a novel target for modulating dopaminergic vesicular functioning. As other studies have reported in the past, we showed here that SV2C has a strikingly restricted expression pattern that is uniquely distinct from that of SV2A and SV2B (Janz and Sudhof 1999, Dardou et al., 2011 and 2013). SV2C is especially enriched in the dopaminergic brain regions of the basal ganglia and the mesolimbic reward pathway. In particular, a majority of dopamine neurons of nigrostriatal pathway appear to express SV2C. This supports a finding recently made by Dardou *et al.* (2011), in which they measured that at least 75% of TH-positive cell bodies co-express SV2C in the SNc. SV2C's localization to these neurons is important because impaired vesicular handling of dopamine in the terminals of the striatum has been proposed as mechanism that may underlie selective neuronal degeneration in PD pathology. In fact, striatal

dopaminergic synaptic vesicles of human PD patients have been shown to be impaired in their ability to sequester dopamine (Pifl et al., 2014).

We further show that in the human striatum, SV2C is normally expressed in terminal regions and cell bodies, but that this is strikingly disrupted in PD. Terminal expression appears to be decreased, cell body staining is absent, and SV2C seems to have formed pathological ubiquitin-positive aggregates (fig. 4 and 5). Our data strongly suggest a role for SV2C in PD pathogenesis. The recent report linking SNPs of the SV2C gene to differential risks for PD in cigarette smokers is further evidence for SV2C's involvement in PD pathogenesis; in fact, cigarette smokers homozygous for the most common alleles of SV2C are at a 50% decreased risk for PD, which points to a potentially neuroprotective role for SV2C in PD (Hill-Burns et al., 2012).

While the function of SV2C is not known, studies have suggested that proteins of the SV2 family may enhance neurotransmitter loading (Reigada et al., 2003). Indeed, we see that cells expressing SV2C have a significantly higher capacity for dopamine uptake than cells without SV2C (Fig. 6). Striatal synaptic vesicles of SV2C-KO mice were also shown to have a trend of decreased vesicular dopamine uptake (Fig. 7d). While this trend is not statistically significant, Collectively, we provide evidence that SV2C may facilitate vesicular uptake of dopamine.

To identify the molecular underpinnings that allow for SV2C to enhance vesicular uptake, we focused on examining its intraluminal N-linked glycosylations. It has been proposed that the glycosylations of SV2 proteins act to adsorb neurotransmitter. Thus, SV2C may effectively decrease the intravesicular dopamine gradient by taking dopamine out of solution inside of the vesicle and promote further uptake. It is also possible that by

adsorbing dopamine, SV2C facilitates retention of dopamine and prevents its efflux out of the vesicle. In this study, we studied the effect of loss of glycosylation at Asn-480, Asn-484, Asn-535, or Asn-565 in-vitro. Mutants of each of the four glycosylation sites showed proper vesicular localization (Fig. 9), suggesting that there is no single site where glycosylation is indispensable for proper trafficking of SV2C to vesicles.

Most interestingly, N480A, N484A, N534A, and N565A all showed similar degrees of statistically significant decrease in vesicular uptake via VMAT2 (Fig. 10). Their uptake levels were similar to that of cells without SV2C expression ($p>0.05$). Although experiments assessing functionality need to be repeated to confirm our findings, our data so far would suggest that each of the four N-glycans studied here are indispensable for SV2C to facilitate vesicular uptake. A possible mechanism of interaction between SV2 and neurotransmitters has been proposed in a study conducted on cholinergic synaptic vesicles (Reigada et al., 2003): it was suggested that the negative residues of the SV2 glycans could bind to the positively charged quaternary amine of acetylcholine (Reigada et al., 2003). The study also showed that SV2 glycans could bind to ATP that has a negatively charged residue, via a strong cation intermediate such as calcium. It is plausible that dopamine's hydroxyl groups allows for binding to SV2C glycans via cations, much like as suggested for ATP; yet, further investigation is necessary to test this idea.

We would also be interested to see the effect of mutations at all or various combinations of the glycosylation sites on SV2C localization and vesicular uptake. Kwon et al. (2012) has shown that single point mutations at each of SV2A's three glycosylation sites do not affect localization, but by mutating two at a time, they show that only the

glycan at the third site is required for proper targeting of SV2A to the synaptic vesicle. This third site alone is also sufficient for botulinum neurotoxin E, a ligand of SV2A, to enter the neuron (Dong et al., 2008). This third site of SV2A is homologous to SV2C's fourth site at Asn-559. We were unable to study the effect of mutation of this site at this point, but experiments are currently underway. Furthermore, SV2 glycans have also been proposed to play a role in vesicular release or exocytosis (Reigada et al., 2003; Vautrin et al., 2009). We plan on assessing how vesicular release levels are affected by the mutation of the glycosylation sites by conducting a [^{3}H]-dopamine uptake and release assay in a cell line such as PC12 that is capable of vesicular release.

It is critically important that synaptic vesicles function properly to package dopamine, not only for effective neurotransmission, but also to prevent dopamine from autoxidizing in the cytosol and damaging the cell. Studies in human and animal models have shown that enhanced vesicular sequestration of dopamine is neuroprotective, but impaired vesicular function leads to progressive dopaminergic cell death and parkinsonism (Glatt et al., 2006; Goldstein et al., 2012; Rilstone et al., 2013). In fact, dopamine synaptic vesicles of Parkinson's disease patients have recently been shown to be impaired in its ability to take up dopamine (Pifl et al., 2014). We have also recently shown that enhanced dopamine vesicle function – that is, increased vesicular capacity for dopamine by the overexpression of the vesicular monoamine transporter 2 (VMAT2) – is protective (Lohr et al., 2014). Such evidence indicates that enhancing dopamine vesicular uptake functioning would protect dopaminergic neurons from toxicity in PD and effectively prevent cell death while also promoting dopaminergic neurotransmission.

As a novel modulator of dopamine vesicular function, SV2C requires further investigation. SV2A is the target for the widely prescribed anti-epileptic, levetiracetam, and thus, we predict SV2C to also be pharmacologically targetable. It is highly plausible that targeting SV2C to enhance its function will improve the handling of dopamine and protect neurons from damage. In PD, this may prevent or slow the progressive loss of dopaminergic neurons.

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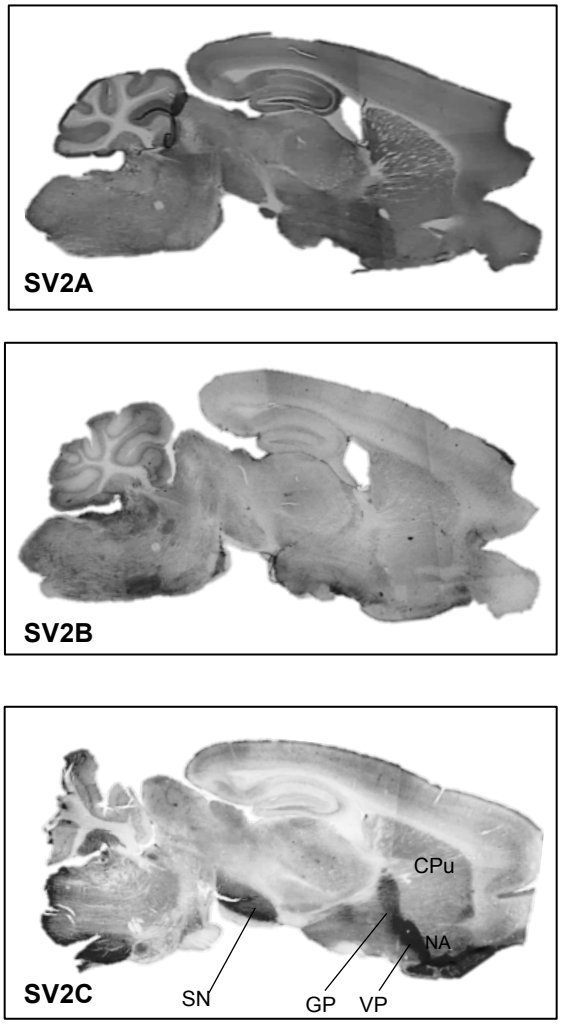

Figure 1. Differential localizations of SV2s. Sagittal mouse brain sections stained by 3-3' diaminobenzadine (DAB) show differential expression patterns of the SV2s. While SV2A and SV2B are expressed ubiquitously throughout the brain, SV2C presents a uniquely restricted localization. SV2C is particularly enriched in the basal ganglia and mesolimbic reward pathway, including the striatum (CPu), nucleus accumbens (NA), ventral pallidum (VP), globus pallidus (GP) and substantia nigra (SN).

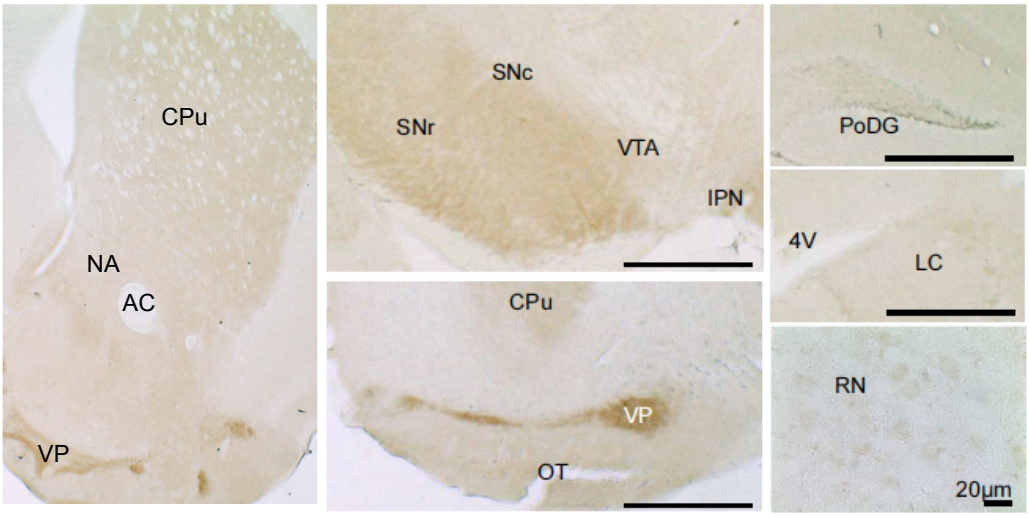

Figure 2. SV2C localization. DAB staining of SV2C on coronal mouse brain sections show brain regions where SV2C expression is observed. SV2C is expressed in caudate putamen (CPu), nucleus accumbens (NA), ventral pallidum (VP), substantia nigra pars reticulata and compacta (SNr, SNc), ventral tegmental area (VTA), dentate gyrus of the hippocampus (PoDG), locus coeruleus (LC), and red nucleus (RN). Scale bar = 500 μm except where noted.

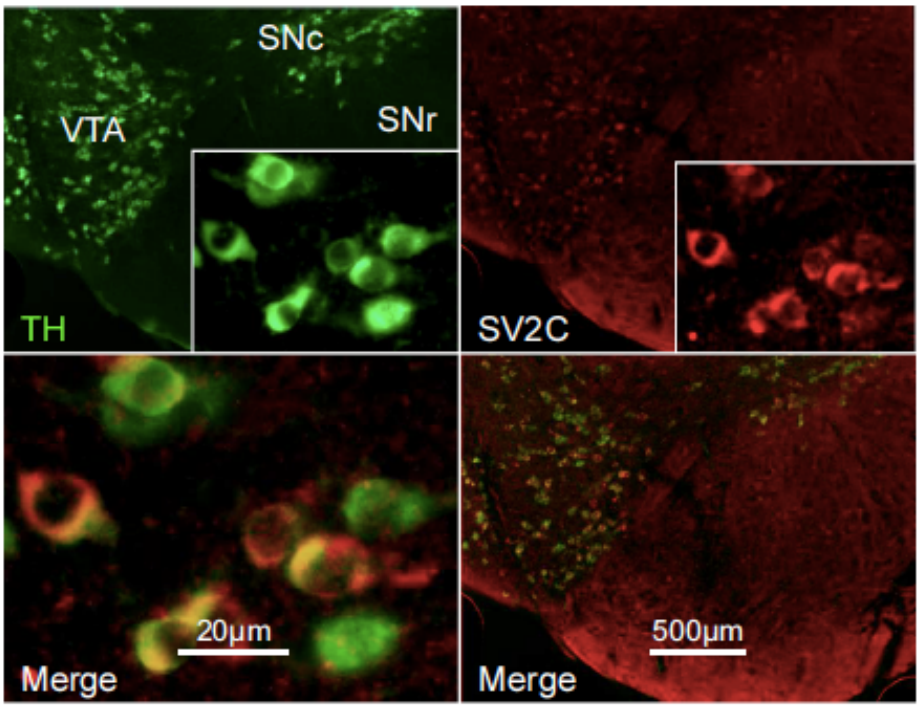

Figure 3. Colocalization of TH and SV2C in the midbrain. Immuno-fluorescence staining of SV2C and tyrosine hydroxylase (TH) on mouse midbrain sections show strong colocalization in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc). SV2C appears to be expressed in a majority of dopaminergic neurons of this region.

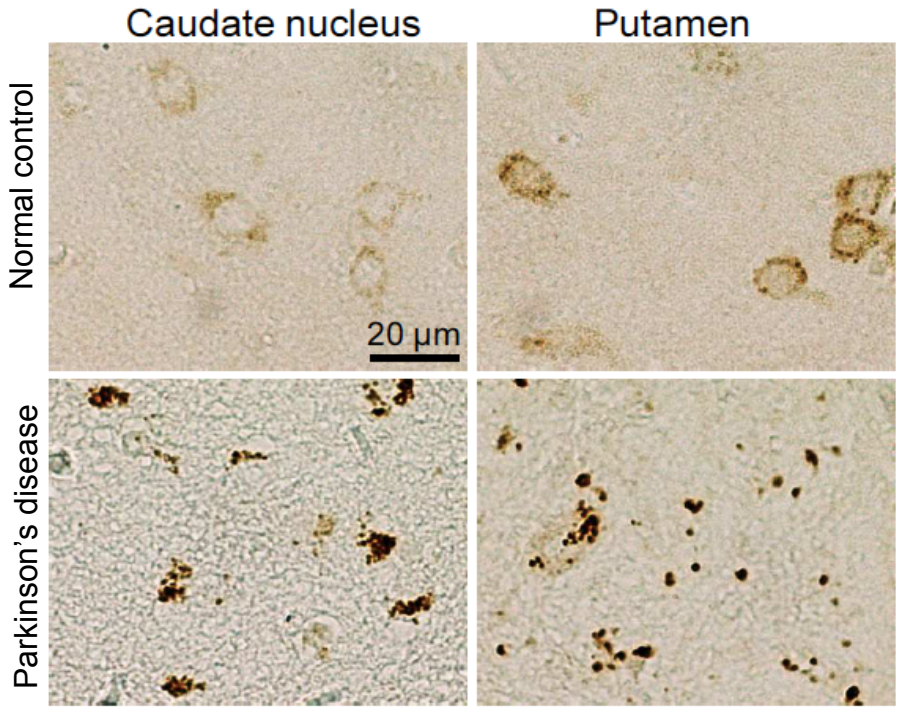

Figure 4. SV2C expression is disrupted in human Parkinson's disease caudate and putamen. Immunohistochemical analysis of SV2C expression in the caudate and putamen of normal control (age-matched) and Parkinson's disease patients was conducted; shown are representative images. In the caudate and putamen of controls, SV2C is expressed diffusely throughout the area, which is likely staining for the terminals of dopaminergic nigrostriatal projection neurons, and also strongly in cell bodies. However, in Parkinson's disease brains, terminal-like SV2C staining appears to be decreased and staining for cell bodies is absent. Instead, we observe SV2C in punctate and aggregate-like structures. Scale bar = 20um.

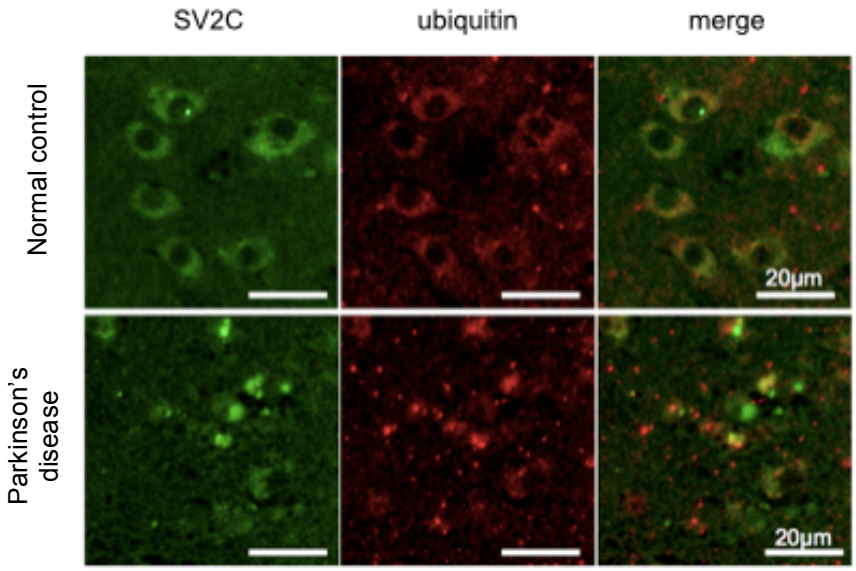

Figure 5. SV2C aggregates are ubiquitin-positive. Immunofluorescence analysis of SV2C in the putamen of normal (age-matched) and Parkinson's disease cases show that these SV2C puncta are also positive for ubiquitin. Scale bar = 20 um.

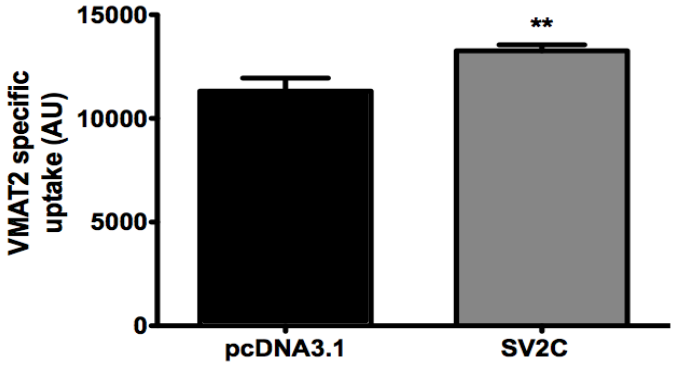

Figure 6. SV2C expression increases vesicular uptake via VMAT2. HEK293 cells stably expressing VMAT2 were transfected with pcDNA3.1-hSV2C. These cells showed significantly higher vesicular uptake of fluorescent false neurotransmitter 206 (FFN206) via VMAT2 than cells transfected with pcDNA3.1 vector. FFN206 is a pH sensitive VMAT2 substrate that fluoresces upon entering acidic intracellular organelles. Fluorescence was measured as an indicator of vesicular uptake. All values are normalized to tetrabenazine-treated conditions. (**, $p<0.05$, Student's t-test).

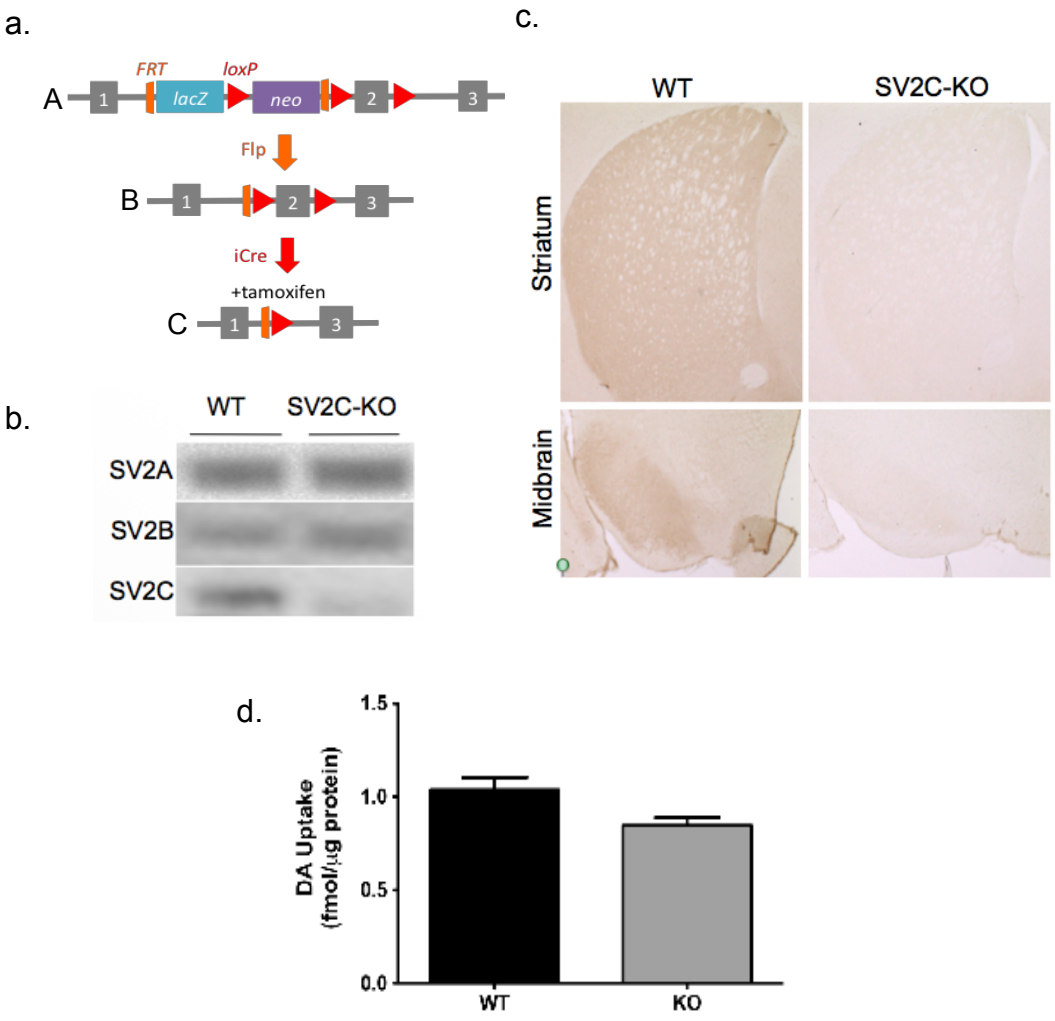

Figure 7. Genetic loss of SV2C reduces vesicular [^{3}H]-dopamine uptake. Generation of SV2C-KO mice. (a-c). **(a)** Construct A contains a FRT-flanked cassette in intron 2, which disrupts gene translation and results in functional knockout of the SV2C gene. Mice expressing this construct were used in this study. These mice will be crossed with Flip-recombinase (FLP) mice and subsequently with mice expressing inducible or promoter-driven Cre. This will result in conditional or inducible knockout of SV2C. (b) Western blot of the striatum of wildtype and SV2C-KO mice show loss of SV2C but not SV2A and SV2B expression. (c) DAB staining of SV2C also confirms loss of SV2C expression in the striatum and midbrain of SV2C-KO mice. (d) Synaptic vesicles isolated from the striatum of two SV2C-KO mice have a trend of decrease in vesicular [^{3}H]-dopamine uptake (n=2 striata per genotype; p=0.068; Student's t-test).

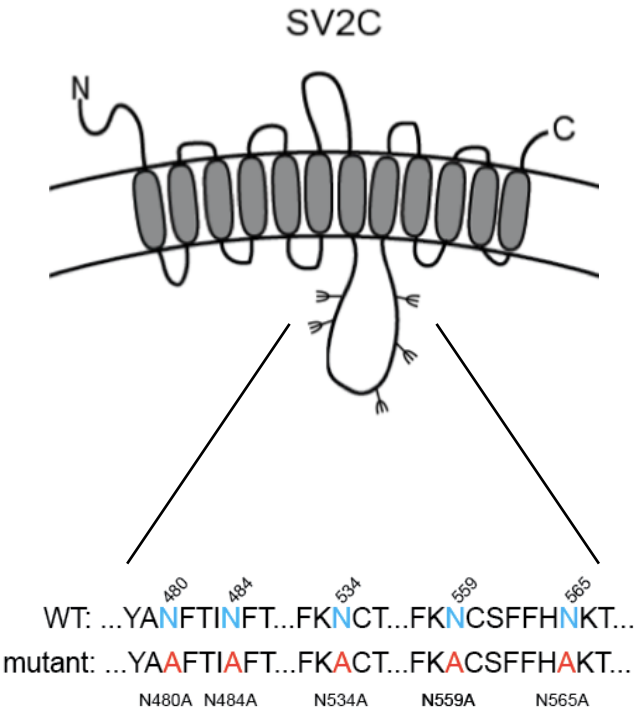

Figure 8. Schematic of SV2C's N-glycosylation sites that were disrupted by point mutations in this experiment. SV2C's fourth intraluminal loop in between the 7th and 8th transmembrane domains have five N-linked glycosylation sites. Point mutations at four of these sites (N480A, N484A, N534A, and N565A) were conducted. (Figure adapted from Kwon et al., 2012.)

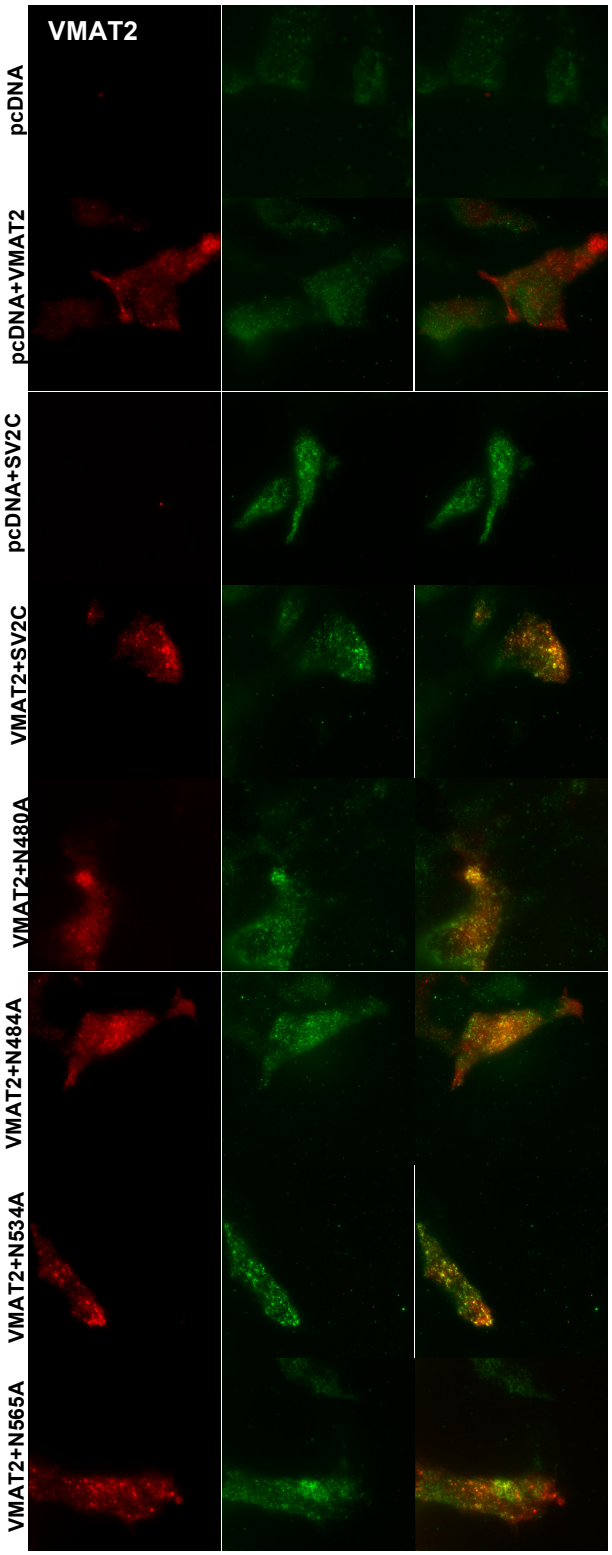

Figure 9. Each SV2C N-glycan is not individually required for proper vesicular localization. Shown are representative images from immunocytochemical analysis conducted on HEK293 cells transfected with pcDNA3.1, pcDNA3.1-hSV2C, pcDNA3.1-mCherry-hVMAT2, or N480A/N484A/N534A/N565A SV2C-pcDNA3.1. Cells were imaged on a TIRF microscope to allow for visualization only of cellular regions closest to the surface of the cell. hVMAT2 is expressed in puncta across the surface of the cell, which are likely vesicles. WT hSV2C also shows a similar expression pattern wherein high colocalization is observed between the two. All mutant forms of hSV2C also show similar degree of colocalization with VMAT2 as WT hSV2C indicating normal vesicular localization. Note that there appears to be substantial autofluorescence of GFP; this appears nonspecific to SV2C as the signal does not colocalize with VMAT2 signal.

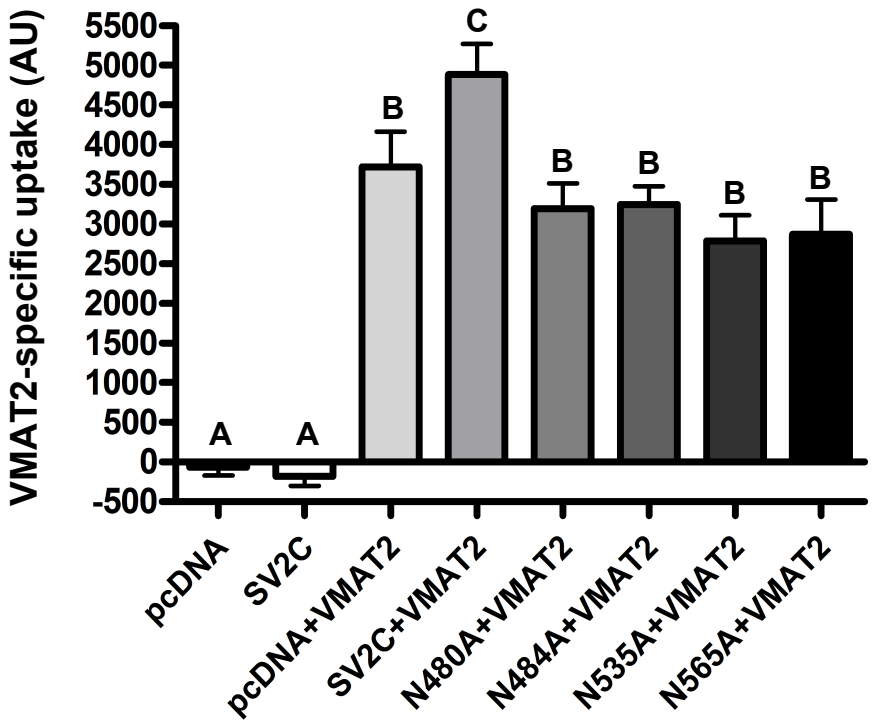

Figure 10. SV2C N-glycosylation mutants show significantly reduced vesicular uptake via VMAT2. FFN 206 assay was conducted on HEK293 cells transfected with pcDNA3.1, pcDNA3.1-hSV2C, pcDNA3.1-mCherry-hVMAT2, or N480A/N484A/N534A/N565A SV2C-pcDNA3.1. (All values are normalized to tetrabenazine-treated conditions.) Cells transfected with wildtype SV2C showed significantly increased vesicular uptake through VMAT2 by approximately 25%, as compared to cells without SV2C and only VMAT2 ($p<0.05$). Cells transfected with N480A, N484A, N534A, or N565A SV2C each showed significantly lower vesicular uptake via VMAT2 as compared to cells expressing wild type SV2C ($p<0.01$). There were no statistically significant differences in uptake levels among the mutants of the different glycosylation sites ($p>0.05$). Uptake levels of cells with mutant SV2C was not statistically different with that of cells only expressing VMAT2 and not WT SV2C ($p>0.05$). A vs. B/C, $p<0.001$; B vs. C, $p<0.01$ (except pcDNA+VMAT vs. C, $p<0.05$). Groups with same letters are not statistically significantly different from each other ($p>0.05$). (n=1; one-way ANOVA; Neman-Keuls test).