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A role for the synaptic vesicle glycoprotein 2C (SV2C) in dopamine homeostasis and Parkinson's disease

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A role for the synaptic vesicle glycoprotein 2C (SV2C) in dopamine homeostasis and Parkinson's disease

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Abstract

A role for the synaptic vesicle glycoprotein 2C (SV2C) in dopamine homeostasis and Parkinson's disease

By Amy Rose Dunn

The synaptic vesicle plays two important roles in dopamine neurons by: (1) packaging transmitter for neurotransmission, and (2) sequestering cytosolic toxicants from the rest of the cell. Impaired storage of dopamine occurs in Parkinson's disease (PD), and genetic mutations in the vesicular monoamine transporter 2 (VMAT2) lead to parkinsonism. Mutations in other vesicle-associated proteins, such as α-synuclein and LRRK2, are common causes of both familial and sporadic PD. Characterizing additional modulators of dopamine vesicle function may be important in further studying and identifying potential therapeutic targets in PD. The synaptic vesicle glycoprotein 2C (SV2C), a vesicular protein enriched in the basal ganglia, was recently identified as a genetic modifier of PD risk in smokers, suggesting an important role for SV2C in dopaminergic neurons. Polymorphisms in SV2C also alter patient sensitivity to L-DOPA, the primary pharmacotherapy for PD. SV2C may represent a novel mediator of basal ganglia neurotransmission; though its molecular function and potential role in PD is unknown. In the experiments described herein, I designed and optimized a specific SV2C antibody and detailed SV2C expression in rodents, nonhuman primates, and humans. In order to better understand the role of SV2C in dopamine homeostasis, neuronal vulnerability to degeneration, and PD pathogenesis, our laboratory developed and characterized mice lacking SV2C (SV2C-KO). Genetic ablation of SV2C resulted in a significant reduction in stimulated dopamine release as measured by fast-scan cyclic voltammetry, reduced striatal dopamine content, mild motor deficits, an altered neurochemical response to nicotine, and disrupted expression of α-synuclein. Further, SV2C-KO resulted in increased susceptibility to dopaminergic degeneration after intoxication with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a specific dopaminergic toxicant. I also present immunohistochemical data detailing SV2C's expression in human PD and in animal models of PD. SV2C expression was specifically disrupted in PD but preserved in other neurodegenerative diseases including Alzheimer's disease, progressive supranuclear palsy, and multiple system atrophy. A similar disruption in SV2C expression was observed in the striata of mice overexpressing mutated human α-synuclein. The results from our experiments identify SV2C as an important mediator of dopamine function and a potential contributor to dopamine cell death and PD pathogenesis, and suggest an important interaction between SV2C and α-synuclein.

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I. Chapter 1. Introduction: Vesicular function, Parkinson's disease, and the potential of SV2C as a novel vesicular target for Parkinson's disease pharmacotherapy

BACKGROUND

Parkinson's disease: etiology and pathogenic mechanisms

Parkinson's disease (PD) is the second most-common neurodegenerative disease, affecting approximately one million Americans. It was first described by Dr. James Parkinson in 1817 as the "Shaking Palsy" for its recognizable motor features, including tremor (Parkinson 1817). The disease is characterized neuropathologically primarily by a progressive loss of dopaminergic cells of the substantia nigra pars compacta (SNc) and aggregation of the protein alpha-synuclein into intracellular inclusions called Lewy bodies. Motor symptoms become apparent to the patient once dopaminergic cell loss reaches approximately 75%, though nonmotor symptoms—such as loss of smell, sleep and gastrointestinal disturbances, and mood disturbances almost always occur in the years and perhaps decades prior to motor symptom onset (Lee 2016; Lees 2009; Wirdefeldt et al. 2011).

Disease etiology is complex, with a host of genetic and environmental factors mediating risk for PD. Over a dozen loci at several genes have been found to cause familial or spontaneous PD, including: mutations in the gene encoding the leucine-rich repeat kinase 2 (LRRK2; *PARK8*); missense mutations, duplications or triplications in the gene encoding for alpha-synuclein (*SNCA*); and mutations in protein deglycase DJ-1 (DJ-1, *PARK7*), Parkin (*PARK2*), synaptojanin-1 (SYNJ1), and PTEN-induced putative kinase (*PINK1*). Additionally, variants in various genes may alter age of onset of PD or susceptibility to PD. Loss-of-function polymorphisms in *SNCA*, LRRK2/*PARK8*, the

vesicular monoamine transporter 2 (VMAT2; *SLC18A2*), and microtubule associate protein tau (*MAPT*) increase susceptibility to PD, with additive effects on a person's disease risk odds ratio if more than one loss-of-function polymorphism in any of these genes is present (Brighina 2013; Glatt 2006; Lesage 2009; Dettmer 2015; Funayama 2005; Gaig 2007; Hasegawa 1997; Krebs 2013; Hill-Burns EM 2016; Quadri 2013). Conversely, gain-of-function polymorphisms in *SLC18A2* (VMAT2) are protective against developing PD (Glatt 2006).

In addition to the genetic mediators of PD risk and development, there are also significant environmental contributors to PD risk. Various compounds in a person's environment can cause damage to dopamine neurons and increase risk for PD or parkinsonism, including occupational and accidental exposure to pesticides, polychlorinated biphenyls (PCBs), manganese and other metals (reviewed in (Caudle 2012; Hatcher 2008). Exposure to pesticides such as organochlorines, pyrethroids, rotenone and paraquat can directly cause damage to dopamine-producing neurons and is associated with increased incidence of PD. Excessive and chronic exposure to manganese, as a result of exposure in welders or via nutritional supplement, is toxic to cells within the basal ganglia and is associated with the development of "manganism" and parkinsonism (Bouabid 2015; Racette 2017; Schuh 2016). Traumatic brain injury and repeated concussions is associated with increased PD risk (Crane PK 2016; Rumalla 2017), as is vitamin D deficiency (reviewed in (Newmark 2007)). Interestingly, there are also multiple environmental sources of *protective* factors against PD. Caffeine consumption is protective against PD, with a dose-dependent level of protection (Ascherio A 2001; Checkoway 2002; Hernan 2002). Non-steroidal anti-inflammatory drug (NSAID) treatments have also been

found to reduce PD risk by up to 50% (Chen H 2005; Chen H 2003; Hernan 2006). The most robust protective mediator of PD risk, though, is nicotine. People who smoke cigarettes, use chewing tobacco, or consume high levels of nicotine-containing produce are strongly protected against the development of PD (Checkoway 2002; Godwin-Austen 1982; Grandinetti 1994; Hernán 2001; Kelton MC 2000; Nielsen 2013; O'Reilly et al. 2005). There is some evidence for additive effects of these protective factors, as well, where exposure to caffeine, nicotine, and NSAIDs is more protective than any one or two alone (Powers KM 2008). Genetic mediators of these effects have been identified, as well, with polymorphisms in the gene encoding a glutamate receptor, GRIN2A, mediating the protective effect of caffeine, and polymorphisms in the synaptic vesicle glycoprotein 2C (SV2C) mediating the protective effect of smoking (Hamza TH 2011; Hill-Burns 2012). These known environmental and genetic contributors to PD have given us valuable clues toward possible pathogenic mechanisms of the disease. Due to the complex etiologies of PD, a relevant theory of pathogenesis is known as the "multiple hit hypothesis" (Sulzer 2007), where multiple factors (genetic, environmental, biological) may converge in an additive or synergistic way to lead to disease development; the unique properties of dopamine neurons to exogenous "hits" may contribute to this vulnerability.

The synthesis and breakdown of dopamine is particularly important to consider in the context of PD, and contributes to a unique vulnerability to dopamine neurons to degeneration. Dopamine is synthesized in the cytosol from the amino acid tyrosine. Tyrosine is converted to L-DOPA enzymatically by tyrosine hydroxylase (TH), and L-DOPA is then converted to dopamine by the enzyme dopa-decarboxylase. Cytosolic dopamine readily undergoes auto-oxidization, forming dopamine quinones and other free

radicals (Burke 2004). Cytosolic dopamine is also metabolized by mitochondrial monoamine oxidase (MAO), forming DOPAC and hydrogen peroxide as a byproduct. These reactive byproducts of dopamine metabolism are harmful to cellular proteins, lipids, and nucleic acids, leading to cell damage and eventual cell death (Zahid 2011). Due to the production of these reactive oxygen species, cytosolic dopamine is normally maintained at very low concentrations, with the vast majority of dopamine molecules being sequestered from the cytosol within synaptic and dense core vesicles. The enhanced oxidative stress experienced by dopamine cells due to the nature of the dopamine molecule is thought to be a contributing factor to the vulnerability of the SNc to degeneration in PD (Ben-Shachar 1995; Goldstein 2013; Jenner 2003; Sulzer 2000b, 2000a).

For this reason, and perhaps more so than in any other transmitter system, dopaminergic vesicles are vitally important to the function and viability of the neuron. Their role in packaging dopamine to prepare for rapid synaptic transmission of the transmitter upon arrival of an action potential to the neuron terminal is crucial to dopamine transmission. Their role in sequestering dopamine from the cytosol promotes cell health. The function of dopaminergic vesicular function is mediated by various vesicle-associated proteins. These proteins can be grouped according to various criteria, though they all have roles at distinct stages of the vesicular cycle.

Vesicle function and the vesicular cycle

Vesicle formation

Synaptic vesicles are small and relatively simple organelles bound by a lipid bilayer with a few dozen classes of associated proteins (Sudhof 2004; Takamori S1 2006). The surface of synaptic vesicles is comprised of about one-third proteins and two-thirds phospholipids (Benfenati 1989). Most vesicular-associated proteins only transiently associate with the vesicle due necessarily to the vesicle's small size, such that the majority of proteins housed constitutively on vesicles must be critical to either vesicular trafficking, filling, or exocytosis and endocytosis. Vesicles generally originate from the Golgi apparatus and the endosome, and the endosome may also act to recycle defective vesicles. Alternatively, following full fusion of the vesicle to the membrane, vesicles may also be derived by the cell membrane during clathrin-mediated endocytosis.

Once vesicles have been formed, their lumens are acidified by proton pumps; in the case of monoaminergic vesicles, the V-type ATPase pumps protons from the cytosol into the vesicle. This rapidly lowers the pH of the lumen of the vesicle, which creates an electrochemical gradient that then drives transmitter transport into the vesicle. Transmitters are loaded into the vesicle with vesicular transmitter transporters (e.g., VMAT, VGAT, VAchT for monoamines, GABA, and acetylcholine, respectively).

Vesicle pools & trafficking

Vesicular trafficking is regulated within the neuron and terminal by kinesin-type motor proteins. Generally, they may be trafficked from their point of origin to the active zone to prepare for release. There are three major pools of vesicles within the terminal: the readily-releasable pool (RRP), the recycling pool, and the reserve pool. Traditionally, these vesicle pools have been defined by the stimulation required to elicit them (Rosenmund 1996).

The RRP, as its name suggests, is comprised of vesicles that are able to rapidly release their contents upon the arrival of an action potential to the terminal. Generally, the RRP is a minority of vesicles within a neuron terminal, but the number and proportion of vesicles within the RRP varies by neuron type. For example, the glutamatergic calyx of Held in the auditory system has approximately four vesicles in the RRP in each terminal (out of possibly thousands within the terminal), but other synapses within the central nervous system can have anywhere between four and 20+ vesicles within the RRP (Harris 1992; Xu-Friedman 2001; Taschenberger 2002; Satzler 2002). The proportion of vesicles within the RRP at the neuromuscular junction, conversely, is much larger comprising about 20% of all vesicles within the terminal. The RRP can be elicited by an action potential, though the likelihood of transmitter release for any given docked-and-primed vesicle is only about 25-30% upon arrival of the action potential (reviewed in (Sudhof 2004)). The entire RRP may be released *in vitro* or *ex vivo* with the application of hypertonic potassium or sucrose solution (Richmond 2002; Rosenmund 1996).

The majority of vesicles within the terminal comprise the reserve pool. The number of vesicles within the reserve pool depends again on neuron type, but ranges from the low hundreds (~200 vesicles within the neuromuscular junction) to thousands (in the calyx of Held) (Harris 1992; Schneggenburger 2002; Xu-Friedman 2001). The reserve pool serves to replenish vesicles to the RRP, and the total number of vesicles that participate in endo- and exo-cytosis are called the recycling pool. The majority of the recycling pool is only elicited after prolonged or high-intensity stimulation.

Defining each pool of vesicles can be challenging. As mentioned above, vesicular pools are typically defined functionally by the intensity of stimulation that elicits their

release. However, a more precise definition may depend on their respective sensitivities to calcium-induced exocytosis. In this case, the boundaries of the RRP would vary based on vesicles' physical distance from calcium channels, as well as the intracellular concentration of calcium. Alternatively, vesicular pools may be delineated visually by electron microscopy. In this case, physical proximity to the active zone may define the RRP; however, this may be an unreliable measure. Electron-dense dyes taken up by vesicles during endo- and exocytosis may be used to more precisely identify those vesicles that are participating in transmission activity.

Vesicle docking & priming

Once vesicles have arrived to the active zone, they are docked and primed for release. Docking is mediated by a variety of proteins, including Munc13 and Munc18. Munc proteins bind to syntaxin, a membrane-associated t-SNARE, to regulate SNARE complex formation; namely, Munc18 prevents syntaxin from complexing with vesicular SNAREs (i.e., synaptobrevin) until Munc13 binds to the complex, inducing a conformational change in syntaxin. At this point, syntaxin is available to bind to synaptobrevin where Munc18 functions to stabilize the complex (reviewed in (Jahn 2003)).

Vesicle fusion and exocytosis

Upon arrival of an action potential to the synaptic terminal, there is a rapid influx of calcium ions into the cytoplasm via voltage-gated calcium channels. An intracellular calcium concentration of >5uM induces fusion of vesicles with the cell membrane and subsequent release of transmitter into the synaptic cleft (Bollmann 2000). Synaptotagmin

is the presumed "calcium sensor" that alters its conformation in the presence of calcium to induce vesicular fusion. Specifically, synaptotagmin-1 has five calcium binding sites and is normally bound to SNARE complexes. When calcium ions bind to synaptotagmin, the protein undergoes a conformational change and binds to nearby phospholipids. This destabilizes the membrane and the protein fusion complex intermediary, which allows for fusion of the vesicular membrane to the cell membrane and the opening of a fusion pore through which transmitter molecules may flow (reviewed in (Meinrenken 2003)).

There are various types of fusion and exocytosis events. Under typical, low-intensity stimulation, the fusion pore opens and closes without significant disruption to the structure of the vesicle. Often, the vesicle then dissociates from the cell membrane but remains at the active zone. This is known as "kiss and stay" fusion. In "kiss and run" fusion, the fusion pore closes and the vesicle is transported away from the active zone for recycling or refilling. With high-intensity stimulation, full fusion events become more common (Gandhi 2003). In this case, the vesicle fully collapses and is integrated into the cell membrane. The vesicle may then undergo endocytosis, in which clathrin molecules and others assemble around the membrane and interact with other vesicular-associated proteins, such as synaptojanin-1 to re-form the vesicle. These vesicles may then be translocated for refilling or recycling through the endosome.

Vesicular proteins as viable targets for PD therapy

Various vesicle-associated proteins have been linked to PD, including alpha-synuclein, LRRK2, VMAT2, and synaptojanin-1. Vesicular dysfunction may be a common pathway to dopamine degeneration and disease pathogenesis.

Alpha-synuclein, the protein that accumulates in pathological Lewy bodies in PD, associates with synaptic vesicles though its exact function is not clear. Mutations in alpha-synuclein can also lead to PD pathogenesis (Polymeropoulos MH 1997; Singleton AB 2003). Disruptions in alpha-synuclein (including mutations, overexpression and underexpression) can lead to vesicular and endosomal dysfunction, as well as to deficits in vesicular mobility and vesicular docking at the active zone in mice and *in vitro* (Gaugler 2012; Lin 2012; Scott 2012; Brown 2010; Dettmer 2015; Roberts 2015a). Oligomeric alpha-synuclein is considered the most toxic form of alpha synuclein. An increase in the ratio between oligomeric and monomeric forms of alpha-synuclein is seen in PD and mouse models of PD (Choi 2013; Danzer 2007; Pieri 2012; Roberts 2015b; Waxman 2009). Alpha-synuclein fibrils may act to perforate vesicles and impair their ability to store dopamine by enhancing transmitter leak from the vesicles (Volles 2002). Further, oxidized dopamine molecules confer stabilization to the fibrillar form of alpha-synuclein (Conway 2001), indicating that PD-related pathologies may beget one another.

Additionally, mutations in the gene encoding the leucine-rich repeat kinase 2 (LRRK2) protein are the most common causes familial PD. *Ex vivo*, inhibiting LRRK2 leads to deficits in vesicular trafficking, impaired synaptic excitability and impairs transmitter release (Belluzzi 2012; Paisan-Ruiz 2008). Together with vesicular disruptions seen in models of alpha-synuclein dysfunction, these data suggest that some of the most

common genetic causes of PD may be acting on vesicular function to increase vulnerability to degeneration.

Genetic variation in the dopaminergic vesicle protein VMAT2 have also been identified as mediating PD risk. Mutations in VMAT2 can lead to juvenile parkinsonism, while polymorphisms within the gene can increase or decrease a person's risk for PD (Brighina 2013; Glatt 2006; Rilstone 2013). In humans with PD, synaptic vesicles isolated from postmortem striata are deficient in their capacity to store dopamine, which is likely a significant contributing factor to dopamine cell death (Pifl 2014; de la Fuente-Fernandez et al. 2009).

Similarly, our laboratory has demonstrated that varying levels of VMAT2 in mice determine vulnerability of dopamine neurons to degeneration. Specifically, mice with a 95% reduction in VMAT2 protein (VMAT2-LO) have increased cytosolic dopamine, enhanced oxidative stress, and progressive loss of SNc dopamine neurons with concurrent motor function decline. These animals are also more vulnerable to degeneration induced by dopamine-specific toxicants, such as MPTP and methamphetamine (Caudle 2007; Guillot 2008b; Lohr 2016; Mooslehner 2001; Taylor 2009, 2014; Taylor, Caudle, and Miller 2011). Conversely, animals expressing enhanced levels and function of VMAT2 genetically (VMAT2-HI) or pharmacologically (following PACAP38 administration) have enhanced vesicular storage of dopamine, elevated dopamine levels, larger dopamine vesicles, and are significantly protected against degeneration induced by MPTP or methamphetamine (Guillot 2008a; Lohr 2014, 2015).

Thus, in mice and humans, impaired dopamine vesicular function can result in enhanced vulnerability to cell death, while enhanced vesicular sequestration of dopamine can confer resistance to age-related or environmentally-mediated degeneration.

SV2C as a novel target for PD research

This dissertation describes a previously under-characterized protein, the synaptic vesicle glycoprotein 2C (SV2C) and its role in dopamine homeostasis, neuronal degeneration, and Parkinson's disease. SV2C is one of three members of the SV2 family of proteins. SV2A, and to a lesser extent SV2B, has been extensively studied for more than two decades. SV2 was first identified in 1985, and cloned in 1992 (Buckley 1985; Feany 1992; Gingrich 1992). At the time, the homology between SV2 and glucose transporters led to the conclusion that SV2 was a novel type of vesicular transporter. Despite the decades' worth of efforts by various labs, however, no transporter activity or substrate of SV2s has been identified in mammalian cells.

SV2 background

SV2s are 12-transmembrane proteins present on every secretory vesicle, including synaptic vesicles, in vertebrates. The three family members have over 80% structural similarity, with SV2C having 63.4% and 60.8% amino acid sequence homology with SV2A and SV2B, respectively (Table 1-1). Each family member has a large, evolutionarily conserved glycosylated intraluminal loop (Fig. 1-1). The cytosolic N-terminus shows the least conservation across the SV2 family (Bajjalieh 1994). SV2 proteins are closely related to

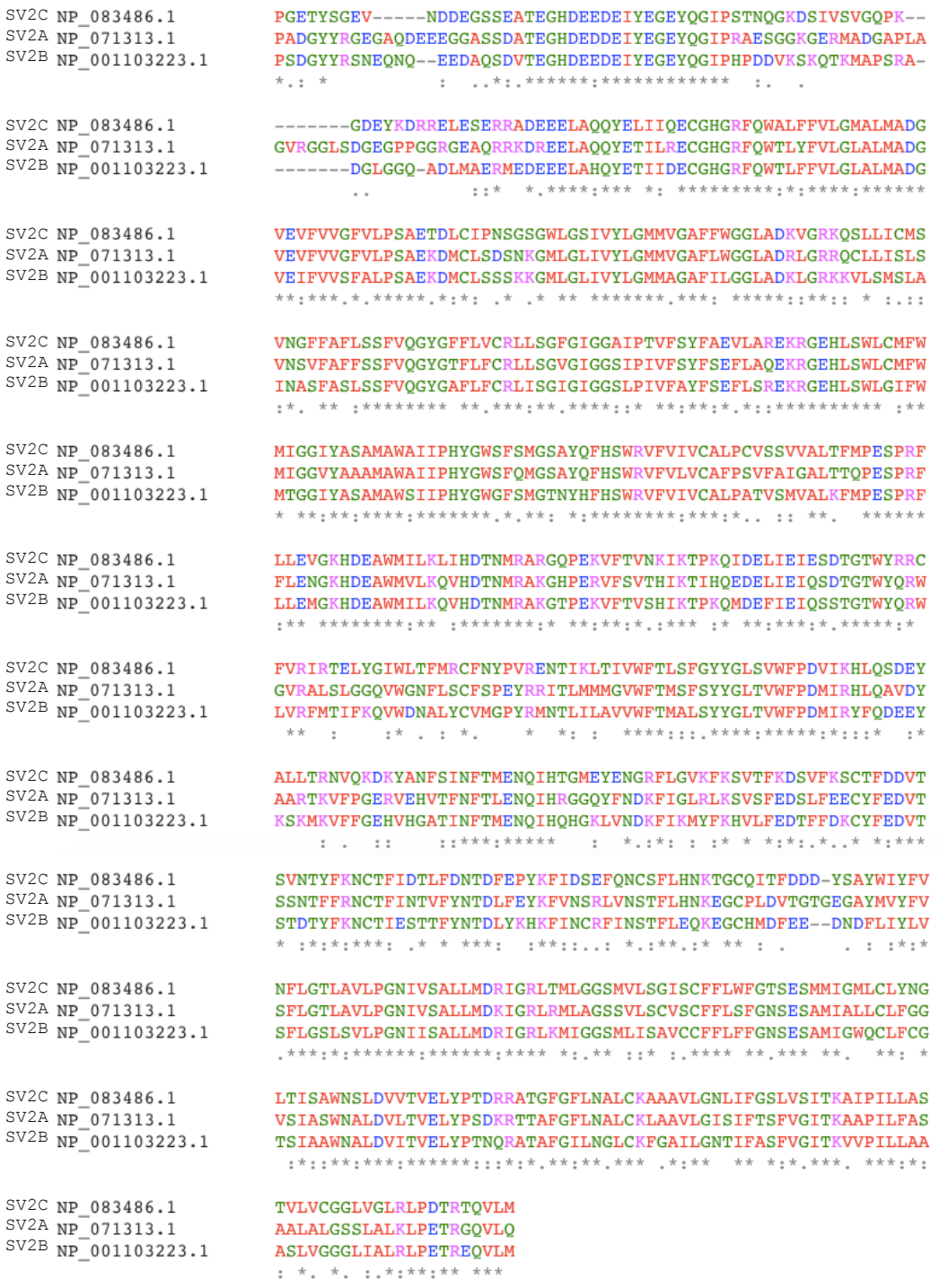

Table 1-1. SV2 sequence homology. Alignments of amino acid sequences of SV2C, SV2A, and SV2B (generated using Clustal Omega).

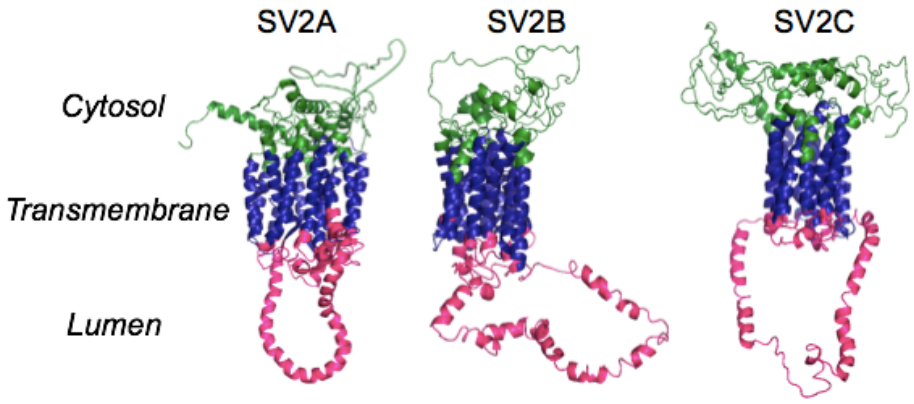

Figure 1-1. SV2 structure similarity. Predicted structure of SV2A, SV2B, and SV2C (generated using MacPyMol and adapted from Stout, et al. (unpublished; in prep)).

another vesicular protein, SVOP, which lacks a glycosylated intraluminal loop. It is thought that SVOP is the evolutionary precursor to SV2, as invertebrates express an SVOP homologue, but not an SV2 homologue.

The precise function of SV2 proteins remains elusive, though significant effort has gone toward elucidating its molecular mechanism on the vesicle. Research into the function of SV2s have focused primarily on SV2A, the dominant isoform, and SV2s are thought to be involved in transmitter release in multiple ways. First, SV2 modulates the size of the readily releasable pool of vesicles. Deletion of SV2A reduces the size of the RRP by about 50% and impairs SNARE complex formation (Xu 2001). Additionally, SV2 enhances recovery of the RRP following stimulus-induced depletion (Vogl 2012; Wan 2010). Second, each member of the SV2 family directly binds to synaptotagmin. The binding of SV2A and SV2C uniquely modulate the calcium sensitivity of synaptotagmin. Further, SV2s are co-trafficked with synaptotagmin, and knocking out SV2A reduces localization of synaptotagmin to the vesicle (Lazzell 2004; Schivell 2005). This interaction between synaptotagmin and SV2 likely underlies the contribution of SV2 to calcium-induced exocytosis. Genetic ablation of SV2A leads to impaired neuronal excitability and reduced synaptic release, and impaired responsiveness of primed vesicles to calcium (Crowder 1999; Custer 2006; Iezzi 2005; Janz 1999a; KM et al. 1999; Nowack 2012; Yao 2010). Finally, it has been hypothesized that SV2 proteins maintain chemo-osmotic stability of the vesicle lumen with their heavily glycosylated intraluminal loops (de Toledo 1993; Reigada 2003; Vautrin 2009). The intraluminal loops of SV2s are thought to comprise the intra-vesicular proteoglycan “gel” matrix, which can be visualized by electron microscopy (Harlow 2013). This matrix is possibly capable of facilitating transmitter loading or

preventing transmitter leak from the vesicle, though this has never been experimentally demonstrated (Crowder 1999; Custer 2006; Janz 1999a).

It is unclear to what extent the function of SV2 proteins are redundant. Each SV2 family member has a distinct expression pattern, with SV2A being expressed widely throughout the nervous system and SV2B showing a more restricted distribution within the brain and retina. SV2C has the most discrete expression pattern and is enriched in the basal ganglia (Bajjalieh 1994; Dardou D 2010; Janz 1999b). Each vesicle contains approximately two to five copies of SV2s, and in some systems, deletion of one or two SV2 isoforms results in an upregulation of expression of the remaining isoform(s) (Crowder 1999; Mutch 2011; Takamori S1 2006; Xu 2001). However, any compensation in expression level with other isoforms following the loss of one or two SV2s is unable to overcome the resulting functional deficits. Thus, it seems as though the function of the different isoforms of SV2s is not interchangeable, and there may be isoform-specific functional specialization.

SV2s in neurological disease

The following section is a portion of a manuscript in final preparation for publication:
Kristen A. Stout, Amy R. Dunn, Carlie A. Hoffman, Gary W. Miller. The synaptic vesicle glycoprotein 2: Structure, function, and disease relevance.

SV2s have been implicated in several neurological diseases. SV2A has been the subject of extensive investigation for its role in epilepsy, and is the molecular target for the antiepileptic drug (AED) levetiracetam (LEV) and its derivatives (e.g. seletracetam and

brivaracetam, BRV). More recently, levetiracetam has shown efficacy in treating symptoms and neurological features of mild cognitive impairment (MCI) and Alzheimer's disease (AD). This, combined with some evidence of alteration of SV2A and SV2B expression in AD models, suggests a function for SV2A, either directly or indirectly, in cognitive function and cell health. SV2C, as might be expected by its enriched expression in the basal ganglia, has been increasingly implicated in Parkinson's disease (PD). The data tying SV2s to these various neurological diseases point to the importance of further development of SV2-targeting compounds as a potentially expanded route for the treatment of disease. Particularly considering their varied anatomical distributions, targeting SV2s may be a strategy to focus treatment to relevant nuclei while minimizing off-target or undesirable effects of new compounds.

SV2A and epilepsy

The efficacy of LEV as an AED was well-known before its molecular target had been identified, but the severe seizure phenotype of SV2A-KO mice has been known since 1999 (Crowder 1999). The identification of the new target SV2A by Lynch, et al. in 2004 (Lynch 2004) spurred research into the role of SV2A in epilepsy, and has led to the development of various SV2A-targeting LEV-derivatives which also show efficacy as AEDs (Bennett 2007; von Rosenstiel 2007). LEV, along with its derivatives, is unique in that it is the only AED that targets the synaptic vesicle in order to reduce hyperexcitability and epileptogenesis. The exact role SV2A plays in epilepsy has not yet been described, though various lines of evidence further tie the protein to the disease.

An observed decrease in the expression of the SV2A gene and protein has been commonly reported in epilepsy and in animal models of epilepsy (Gorter et al. 2006; van Vliet et al. 2009; Feng 2009; Shi 2015), suggesting that reduced SV2A increases vulnerability for epileptogenesis; though, SV2A may increase in expression during seizure kindling (Ohno et al. 2009). Consistent with this, low frequency stimulation in the hippocampus in pharmaco-resistant spontaneously epileptic rats led to an increase in SV2A and a subsequent decrease in seizure frequency (Wang et al. 2014). The Fepi chicken strain expresses an SV2A mRNA splice variant that results in reduced SV2A protein expression leads to a photosensitive epileptic phenotype that can be rescued with LEV (Douaud et al. 2011). In humans with glioma-associated epilepsy, response to LEV to treat seizure activity may be predicted by expression levels of SV2A in the tumor and peritumor tissue, in that patients with lower SV2A levels are more likely to be LEV non-responders (de Groot 2011). Beyond protein expression, though, there is no apparent association between genetic variation or single nucleotide polymorphisms in SV2A and risk of epilepsy or variations in LEV efficacy (Lynch et al. 2009; Dibbens et al. 2012). However, homozygosity for a recessive mutation SV2A results in epilepsy among a host of other neurological abnormalities (Serajee and Huq 2015). Interestingly, LEV was inefficacious at reducing seizures in this case. These data further indicate that normal SV2A expression and function is required to maintain proper neurotransmission, and a lack of SV2A function leads to seizure vulnerability. However, work by Nowack, et al. (2011) suggests that *over*expression of SV2A also leads to altered excitability and release probability, and that these abnormalities are also reversed by LEV (Nowack 2011).

The precise mechanism of how LEV may be acting at SV2A to rescue seizure phenotypes and abnormal excitability is still unknown. LEV enhances synaptic depression via impairing replenishment of the readily releasable pool (RRP) of vesicles, particularly during periods of heightened stimulus intensity or activity (e.g., epileptogenesis) (Garcia-Perez et al. 2015). Similarly, BRV slows vesicle recycling to the RRP, but with significantly higher affinity to SV2A than its parent drug (Yang et al. 2015). This suggests that LEV and BRV inhibit SV2A's role in regulating the RRP. Other pieces of evidence point to a likelihood of LEV inhibiting additional functions of SV2A, such as its potential galactose transport activity (Madeo 2014). However, since a reduction in SV2A results in heightened vulnerability to seizures in humans and animals, it is possible that pure inhibition of the protein would be deleterious; rather, LEV may stabilize the protein in an ideal functional conformation. Thus, the function of SV2A may have a U-shaped relationship with seizure vulnerability, in that both high and low function or expression of SV2A may alter neurotransmission in such a way to lead to a seizure phenotype. In this way, LEV may rescue deficits resultant from both reduced and overexpressed SV2A. Additionally, prophylactic LEV (i.e., LEV administered to asymptomatic, high-risk spontaneously epileptic animals) may be efficacious in preventing vulnerability to seizures, and protective against hippocampal degeneration associated with epilepsy (Sugata et al. 2011). It is important to note that administration of LEV to non-epileptic animals and humans does not produce similar hyperexcitability as seen with reduced or overexpressed SV2A.

SV2s and Alzheimer's disease.

The coincidence of seizures in Alzheimer's disease, regardless of gender, race or ethnicity, has been known for decades (Sherzai et al. 2014) (reviewed in (Chin and Scharfman 2013; Horvath 2016)). Abnormal hyperactivity within the hippocampus occurs in the prodrome period of AD, and an increase in seizures in later stage AD is associated with a significant decline in cognitive function (Vossel et al. 2013). Furthermore, various animal models of AD display altered cortical excitability and spontaneous seizure phenotypes (Siwek et al. 2015; Ping et al. 2015; Ziyatdinova 2016; Sola et al. 2015; Chan 2015). Various AEDs show efficacy in reducing seizures that occur in AD, though LEV reduces seizures more effectively than other classes of AEDs (Vossel et al. 2013). Furthermore, several studies have indicated that LEV, unlike other AEDs, is able to rescue cognitive deficits to some degree in AD, amnestic MCI (aMCI) (Cumbo and Ligori 2010) and animal models of AD that display seizures (Sola et al. 2015). In particular, LEV reduces the characteristic hyperactivity in CA3 and improves memory performance (Bakker et al. 2012). LEV has also been shown to improve cognition in non-demented epilepsy patients (Wu et al. 2009; Helmstaedter and Witt 2010), as well as in non-epileptic aged mice and some mouse models of AD (Devi and Ohno 2013), (Celikyurt et al. 2012). These data indicate that modulating SV2A activity, independent of reducing seizure activity, is effective in reversing some degree of cognitive decline in AD and aging.

The mechanism behind cognitive improvement with LEV is unknown, although the evidence points to a number of possibilities. One line of evidence supports the idea that pathological hippocampal hyperactivity in early stages of the AD prodrome period creates heightened vulnerability in CA3 cells to degeneration. In fact, asymptomatic people who are at a high genetic risk for AD (ApoE4, familial AD mutation carriers) have hyperactive

CA3 nuclei. This hyperactivity may interfere with memory formation and retrieval and increase vulnerability of the cells to degeneration; dampening this activity with LEV treatment is effective at improving memory function (Bakker et al. 2012). However, this is not likely to be the only explanation for this phenomenon, as it would be expected that any AED—not just LEV—would show similar results on cognition. LEV stimulated neuritogenesis and increased synaptic markers in an *in vivo* model of late-onset AD, possibly through direct interaction with mitochondrial SV2A (Stockburger et al. 2015). This may counter the regional loss of synapses observed in aMCI/AD progression (Sze 2000; Robinson et al. 2014), and may be an additional mechanism behind the cognitive improvement seen with LEV treatment.

SV2B may also play some role in AD pathogenesis. Heese et al. (2001) revealed an upregulation of SV2B *in vivo* after treatment of cells with the AD-associated cytotoxic Aβ42. This treatment stimulated the production of a variant SV2B mRNA transcript not seen in untreated cells. The protein product of this transcript variant is identical to SV2B, but the modification is thought to result in a more stable transcript and a protein with distinct posttranslational modifications (Heese, Nagai, and Sawada 2001). Furthermore, SV2B-KO appears to protect against toxicity and cognitive deficits in mice induced by injected amyloid beta (Aβ) oligomers (Detrait et al. 2014).

SV2C and Parkinson's disease

SV2C is the least abundant and most regionally-restricted of its family members. When it was discovered by Janz and Sudhof in 1999, the investigators immediately postulated that it may be particularly important in basal-ganglia functions and PD (Janz

1999b). Indeed, subsequent studies have supported this idea. SV2C was recently identified as a genetic modifier of PD risk in smokers, where the minor alleles at multiple loci in the promoter region of the SV2C gene confer a significantly increased risk for PD in smoker (rather than the more typical significant *protection* seen in nicotine consumption) (Hill-Burns 2012). The expression patterns of SV2C were further described by Dardou, et al. (2011), indicating that SV2C is in a majority of dopamine cells in the substantia nigra, as well as in other neuron types in the basal ganglia (Dardou 2011). This group also provided the first experimental evidence that SV2C may be linked with PD by showing that SV2C mRNA expression increases after intoxication by the dopaminergic toxicant, MPTP (Dardou 2013c).

More recently, our group has shown a more direct relationship between SV2C, basal ganglia, dopamine function, and PD. The expression of SV2C is significantly and specifically disrupted in PD. This disruption in PD may contribute to disease pathogenesis, as suggested by our studies into the effect of SV2C-KO *in vivo.* Ablation of SV2C leads to reduced dopamine release and impaired motor function in mice (Dunn 2017). SV2C, then, is positively associated with dopamine neuron function. These findings that SV2C promotes dopaminergic function are consistent with data indicating that vesicular function, particularly in dopamine neurons, is crucial for cell integrity (reviewed in (Alter 2013)). Finally, SV2C's promotion of proper vesicular and neuronal function may explain in part the observation that statins protect against PD (Bai 2016), as SV2C expression increases after treatment with statins (Schmitt 2016).

SV2C in mood disorders/psychiatric conditions

SV2C, with its localization to dopaminergic nuclei, may also play a role in other diseases where altered dopamine neurochemistry is a feature. For example, dopamine plays a significant role in mood disorders, psychiatric conditions, and substance abuse disorders. Altered vesicular storage of dopamine has been implicated in all of these. SV2C genotype has also been found to mediate response to atypical antipsychotic drugs(Ramsey 2013), which act primarily on the dopamine system.

As SV2C is preferentially expressed in the basal ganglia, its potential to impact reward behavior is readily apparent. However, little is known about the impact of SV2C in appetitive behavior. A gene array study comparing differences in rats bred for high and low drug preference found high consuming rats had significantly reduced SV2C expression in the frontal cortex (Higuera-Matas et al. 2011). Lentiviral knockdown of SV2C expression in the midbrain of adult mice reduced cocaine place preference compared to controls, whereas global SV2C knockout mice show no alteration in preference (Dardou 2013b). Our group recently demonstrated that global SV2C-KO animals have reduced preference for methamphetamine (METH), in conjunction with reduced locomotor stimulant effect (unpublished observations).

Targeting the synaptic vesicle to reduce the rewarding effect of stimulants has been explored repeatedly, and several therapeutics (most notably lobeline and its derivitives) show promising efficacy (Beckmann et al. 2012; Beckmann et al. 2010; Damaj et al. 1997; Dimatelis et al. 2012; Dwoskin and Crooks 2002; Harrod et al. 2001). However, all of these therapeutics target VMAT2. Historically, the VMAT2-targeted drug, tetrabenazine has shown promise in treating a number of diseases, but undesirable effects, presumably from concomitant depletion of other monoamines (serotonin, norepinephrine, histamine), has

limited the applicability of targeting this protein (Jankovic and Clarence-Smith 2011; Jankovic, Glaze, and Frost 1984; Kenney, Hunter, and Jankovic 2007; Kenney et al. 2006; Leung and Breden 2011; Paleacu et al. 2004). SV2C represents a novel, unexplored, and discrete therapeutic target for the treatment of addiction.

Pharmacological manipulation of SV2C may be a promising route for the treatment of mood and psychiatric conditions, either as a monotherapy or as a way to enhance efficacy of existing treatments.

RATIONALE

Synaptic vesicles are critical to the health and function of neurons, particularly dopaminergic cells. The breakdown of proper vesicle function can lead to dopamine cell death and is associated with Parkinson's disease. Enhancing our understanding of modifiers of dopamine vesicle function will help to identify novel targets for the study and treatment of Parkinson's disease. This dissertation focuses on one novel target, SV2C and its role in dopamine cell function and health.

HYPOTHESIS

Given SV2C's role in vesicular function, I hypothesized that SV2C facilitates proper vesicular dopamine handling, and that this function mediates dopamine release and vulnerability to neurodegeneration.

II. ***Chapter 2. Immunochemical analysis of the distribution of SV2C in mouse, macaque and human basal ganglia***

In preparation for publication as:

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ABSTRACT

The synaptic vesicle glycoprotein 2C (SV2C) is an undercharacterized protein with enriched expression in phylogenetically old brain regions. Its precise role within the brain is unclear, though various lines of evidence suggest that SV2C is important in the function of synaptic vesicles through regulating vesicular trafficking, calcium-induced exocytosis, or synaptotagmin function. SV2C has been linked to multiple neurological disorders, including Parkinson's disease and psychiatric conditions. SV2C is localized to various cell types--primarily dopaminergic, GABAergic, and cholinergic cells. In mice, it is most highly expressed in nuclei within the basal ganglia, though it is unknown if this pattern of expression is consistent across species. Here, we use a novel SV2C-specific antiserum to describe localization within the brain of mouse, nonhuman primate, and human, including cell-type localization and subcellular localization patterns. We confirm the localization of SV2C to the basal ganglia of rodents, macaque, and human, with strong expression of SV2C in the substantia nigra, ventral tegmental area, dorsal striatum, globus pallidus, nucleus accumbens, ventral pallidum and olfactory tubercle in mouse. In primates, the substantia nigra, ventral tegmental area, caudate nucleus, putamen, and globus pallidus are most heavily labeled. Further, we demonstrate co-localization between SV2C and markers of dopamine, GABA, and acetylcholine within these brain regions. Characterizing novel vesicular proteins such as SV2C will be an important resource moving forward in our understanding of the role of vesicle dynamics in neurological disease.

INTRODUCTION

The synaptic vesicle glycoprotein 2C (SV2C) is one of three proteins within the SV2 family, which also includes SV2A and SV2B. SV2B is expressed at moderate levels throughout the nervous system, and particularly within the retina. SV2A is the most widely expressed and most extensively characterized of this family of proteins. SV2A is present throughout the nervous and endocrine systems and, in fact, antibodies for SV2A are often used as molecular markers for axon terminals (Bajjalieh 1994). Additionally, as it is the molecular target for the commonly used antiepileptic drug levetiracetam, SV2A is particularly relevant to the epilepsy research community (Lynch 2004). Dysregulation of SV2A expression leads to seizures in mice and humans (Douaud et al. 2011; Feng 2009; Gorter et al. 2006; Nowack 2011; Ohno et al. 2009; Serajee and Huq 2015; Shi 2015; van Vliet et al. 2009; Wang et al. 2014). SV2A is known to regulate neuronal excitability, synaptotagmin trafficking, calcium sensitivity, and vesicular mobilization (Chang 2009; Crowder 1999; Custer 2006; de Toledo 1993; Iezzi 2005; Janz 1999a; Lazzell 2004; Schivell 2005; Wan 2010; Xu and Bajjalieh 2001; Yao 2010). The functions of the individual members of the SV2 family of proteins are thought to be similar, though not interchangeable, as any two isoforms cannot compensate for the loss of any one isoform.

SV2C is distinguished from SV2A and SV2B by its enriched expression within the basal ganglia. Previous rodent studies have demonstrated that SV2C is most highly expressed in the substantia nigra (pars compacta and pars reticulata), ventral tegmental area, caudoputamen, nucleus accumbens, and ventral pallidum, with minimal expression in cortical regions (Dardou D 2010; Janz 1999b). Previous reports have estimated that

SV2C localizes to approximately 70% of midbrain dopamine neurons and their striatal terminal regions. SV2C is also strongly expressed in GABAergic regions of the basal ganglia, and is present in about 30% of cholinergic neurons within the dorsal striatum (Dardou D 2010).

Antibodies previously used to characterize SV2C localization are not commercially available, and other commercially-available SV2C antibodies have not been well-characterized. Thus, we have designed and optimized two specific rabbit-anti-SV2C sera recognizing both mouse and human SV2C, respectively. We have extensively used the mouse antiserum in both fluorescent and 3-3'-diaminiobenzidine (DAB) immunohistochemistry, immunoblotting on brain homogenate and tissue culture lysate, and immunoprecipitating protein complexes from brain homogenate and tissue culture lysate. Likewise, we have optimized the use of the human antiserum in immunohistochemistry and immunoblotting. Additionally, these SV2C antisera do not recognize either SV2A or SV2B.

Here, we use the human and mouse SV2C antibodies to describe SV2C expression in mouse, macaque, and human brain using immunohistochemistry. We provide further validation of the antibody in immunoblotting, immunohistochemistry, and immunocytochemistry in cultured ventral mesencephalon neurons. As would be expected with a vesicular protein, we found that SV2C is localized to axon terminal regions in cultured neurons. However, in intact postmortem brain tissue, SV2C localizes to terminals and somata in mice and humans. In accordance with previous reports, we found that in mice, SV2C is expressed primarily in the basal ganglia and colocalizes with markers of dopamine, GABA, and acetylcholine. Similarly, SV2C expression in the macaque brain is

highly consistent with expression patterns in mice, with the greatest expression in the midbrain, caudate, putamen and ventral pallidum. In humans, SV2C is present in throughout the dorsal striatum including GABAergic medium spiny neurons (MSNs) and dopaminergic terminal regions, as well as in the substantia nigra and ventral tegmental area.

While SV2C expression is largely consistent across species, there is some variability in the degree of expression in certain cell types. This manuscript highlights the similarities and differences in expression of SV2C in rodents and primates. Given the growing importance of SV2C to Parkinson's disease in particular, a greater understanding of SV2C expression within the basal ganglia across species will be critical in future studies hoping to translate rodent studies to human disease relevance.

MATERIALS AND METHODS

Antibodies:

Polyclonal SV2C antibodies were designed by our lab and custom-produced by Covance as described previously (Dunn 2017). Briefly, immunizing peptides corresponding to amino acids 97-114 of SV2C (mouse SV2C: STNQGKDSIVSVGQPKG; human SV2C: SMNQAKDSIVSVGQPKG) were mcKMH-conjugated and injected into rabbits. Each antibody recognizes SV2C in mouse, nonhuman primate, and human tissue. Mouse SV2C antiserum (mSV2CpAb) was used for immunohistochemistry in mouse tissue, and human SV2C antiserum (hSV2CpAb) was used for immunohistochemistry in human and macaque tissue.

Affinity purification: Purification of the SV2C antibodies was performed using a commercially-available kit (Thermo) according to manufacturer's protocols. Immunizing SV2C peptides were conjugated to agarose beads and incubated in serum. Unbound serum was removed and bound antibody was eluted from the beads using a low-pH elution buffer.

Antibodies against TH (cat. AB152), DAT (cat. MAB329), and tau were purchased through Millipore. Antibody against VGAT (cat. 131-011) was purchased through Synaptic Systems. Antibody against nAchR subunit β2 was purchased through Abcam (cat. 24698). Antibody against ChAT was purchased through Chemicon (cat. AB144P), and antibody against vAchT was purchased through Novus Biologicals (cat. NB100-91347). Monoclonal hCHT anitbody (clone 62-2E8) was generously provided by Emory Center for Neurodegenerative Diseases.

Secondary antibodies were purchased through Jackson ImmunoResearch (biotinylated, HRP-conjugated) or ThermoFisher (AlexaFluor; fluorescent). Category numbers are as follows. Biotinylated: goat-anti mouse (cat. 115-065-003), goat-anti rabbit (cat. 111-065-144), goat-anti rat (cat. 112-065-003); HRP-conjugated: goat-anti rabbit (111-035-003); fluorescent: goat-anti mouse 594 (cat. A11005), goat-anti rabbit 488 (cat. A11034), goat-anti rabbit 594 (A11012), goat anti-rat 488 (A11006).

***Animals*:**

Male wildtype C57BL/6 mice were used for all mouse immunohistochemistry experiments (age 4-10mos). SV2C-KO animals were generated as described previously (Dunn 2017; Skarnes 2011). Briefly, animals were generated using the EUCOMM "knockout first allele" construct. These animals contained a *lacZ*/neomycin resistance cassette flanked by

FRT sites inserted into the *sv2c* gene. Animals were crossed with a line globally expressing Flp-recombinase to excise the cassette, resulting in a line of mice containing a floxed exon 2 of the *sv2c* gene. These mice were then crossed with a line containing a *nestin*-driven Cre-recombinase in order to achieve a neuronal knockout of SV2C. Saline-treated control male macaques from MPTP treatment experiments were used for all macaque immunohistochemistry.

Tissue culture:

Cell lines: HEK293 cells were cultured according to standard protocols and maintained in DMEM with 10% fetal bovine serum (FBS) + 1% penicillin/streptomycin. Transfections of SV2C in a pcDNA3.1 vector was performed with Lipofectamine 2000 (Invitrogen) according to manufacturer's protocols. Cells were harvested 24 hours post-transfection, lysed in RIPA buffer and total protein extraction was achieved through differential centrifugation.

Primary neurons: Ventral mesencephalon primary neurons were cultured as described previously (Bradner JM 2013). Briefly, the ventral mesencephalon was dissected from mice between postnatal days 1-3. Tissue was dissociated and plated at a concentration of 1,000 cells / microliter to a 96-well plate. Cells were maintained at 37ºC and 5% carbon dioxide in culture media (Neuralbasal-A + 1% l-glutamine + 2% B27 + 0.1% aphidicolin + 1% penicillin/streptomycin).

Western blotting:

Western blots were performed as previously described (Dunn 2017). Briefly, samples were homogenized and underwent differential centrifugation to achieve a crude protein extraction. Samples were run through SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked with 7.5% nonfat dry milk and incubated in primary antibody overnight at 4°C with gentle agitation. Secondary antibodies (HRP-conjugated) were incubated at room temperature for 1 hour. Signal was amplified using chemiluminescence (Thermo) and visualized using a BioRad UV imager.

Immunocytochemistry:

Cells were fixed with 4% paraformaldehyde and rinsed and perforated with PBS with 0.1% Triton X-100 (TPBS). Nonspecific antibody binding was blocked using 10% NHS in TPBS. Antibodies were diluted in blocking solution (tau, 1:1,000; SV2C 1:10,000) and incubated on cells overnight at 4°C with gentle agitation. Secondary antibodies were also diluted in blocking solution and incubated on cells for 1 hour at room temperature.

Immunohistochemistry (mouse tissue):

Mice were transcardially perfused with 4% paraformaldehyde. Brains were removed and placed in 4% paraformaldehyde for 24 hours at 4°C for post-fixation. Brains were then transferred to sucrose and allowed to equilibrate for an additional 48 hours, at which point they were frozen and sectioned to 40μm. Sections were stored in cryoprotectant at -20°C until staining. For immunohistochemistry, sections were rinsed with 1X PBS containing 0.02% Triton X-100 (PBST). Sections underwent antigen retrieval using Citra Antigen Retrieval Buffer (Biogenex) for one hour at 70°C. Endogenous peroxidase activity was

quenched with a 12-minute incubation in 3% hydrogen peroxide at room temperature. Nonspecific antibody binding was blocked using a 3% Normal Horse Serum (NHS) in PBST solution for one hour at room temperature. Sections incubated in primary antibody solution (1:1,000 in PBST for all antibodies, except the purified SV2C antiserum, which was used at a dilution of 1:50) overnight at 4°C with gentle agitation, followed by incubation in secondary antibody (1:1,000 for biotinylated; 1:800 for fluorescent) for one hour at room temperature.

For biotinylated secondaries, the signal was amplified using an avidin-biotin complex (VectaStain Elite, Vector Laboratories) for one hour at room temperature. Biotinylated secondary antibodies were visualized using a 3-3'-diaminobenzidine reaction for approximately 1 minute; reaction was quenched with PBST. Sections were mounted to charged slides, dehydrated in ethanol, cleared with xylenes and coverslipped.

For immunofluorescence, autofluorescence was quenched with a 7-minute incubation in a 0.1% Sudan Black B solution in 70% ethanol at room temperature. Slides were treated with hard-set mounting medium (Vector Laboratories) and coverslipped.

Immunohistochemistry (macaque tissue):

Post-fixed macaque sections underwent antigen retrieval using a low-pH glycine buffer (0.5M glycine, pH 2.0) for one hour at room temperature. Endogenous peroxide activity was quenched with a 10% hydrogen peroxide solution for 20 minutes at room temperature. Nonspecific antibody binding was blocked using a solution of 1% NHS and 1% bovine serum albumin (BSA) in PBST. Sections were incubated in primary antibody solution (1:1,000) overnight at 4°C with gentle agitation. Sections were then incubated in

secondary antibody, either biotinylated (1:200) or fluorescent (1:800) for 1 hour at room temperature. Secondary antibodies were visualized as described above for mouse tissue.

Immunohistochemistry (human tissue):

Paraffin-embedded sections were obtained through the Alzheimer's Disease Research Center Brain Bank at Emory University. Slides were deparaffinized using xylenes and rehydrated in decreasing concentrations of ethanol. Sections underwent antigen retrival using a hot citric acid buffer at 95°C for 1 hour. Endogenous peroxidase activity was quenched with a 12-minute incubation in 3% hydrogen peroxide at room temperature. Nonspecific antibody binding was blocked using 3% NHS in PBST for one hour at room temperature. Sections were incubated in primary antibody (1:1,000 in PBST) overnight at 4°C with gentle agitation. Sections were then incubated in secondary antibody solutions (1:1,000 biotinylated or 1:800 fluorescent). Secondary antibodies were visualized as described above for mouse tissue.

Micrograph visualization:

Immunohistochemistry was visualized using a Zeiss AX10 microscope equipped with a MicroBrightField camera. Images were captured using StereoInvestigator software. Immunocytochemistry was visualized using an ArrayScan (Cellomics). Colocalization analysis was performed using the Coloc 2 plugin of FIJI using background-subtracted images without thresholds or ROI delineation, except where noted.

RESULTS

Validation of SV2C antibodies

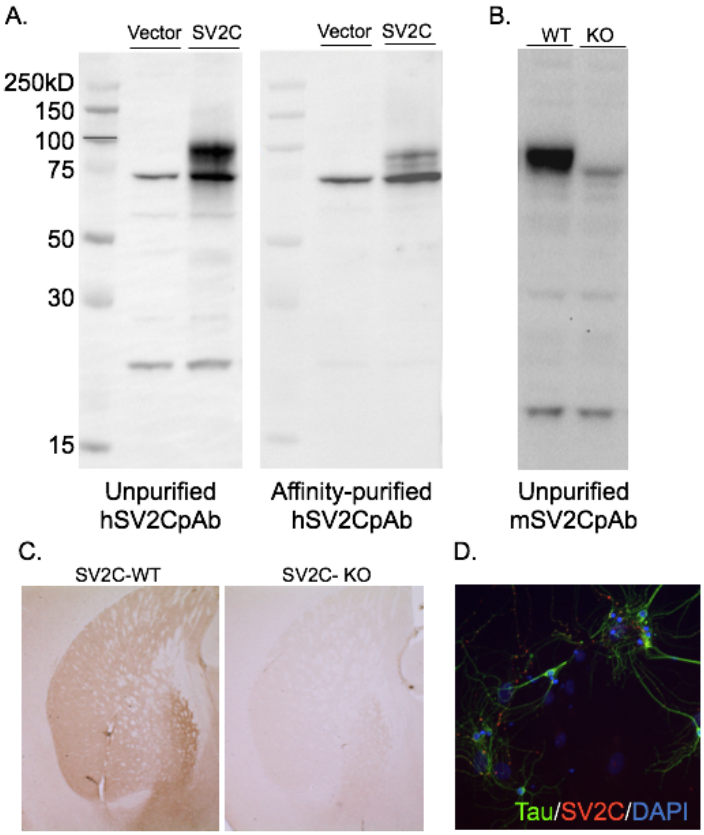

Figure 2-1. Validation of the polyclonal SV2C antibodies. (A) The polyclonal human SV2C antibody (hSV2CpAb) recognizes human SV2C in transfected HEK293 cells, a cell line that does not endogenously express SV2C. The specific band is at the expected molecular weight of 90kD. Affinity purifying the antibody results in a reduction in both the nonspecific and specific immunoreactivity of the antibody. (B) Immunoreactivity at the specific band using the polyclonal mouse antibody (mSV2CpAb) is eliminated in striatal homogenates from mice lacking SV2C (SV2C-KO), as well as by (C) immunohistochemistry. (D) Using the mSV2CpAb in immunocytochemistry of primary ventral mesencephalon neurons, we observed a punctate, terminal-like staining pattern of SV2C (red) in contrast to tau (green), which labels the entire cytoskeleton and DAPI (blue) which labels nuclei.

To validate the SV2C antibodies, we performed immunoblotting and immunohistochemistry on positive and negative controls. For hSV2CpAb validation, we transfected HEK293 cells with a pcDNA3.1 DNA vector containing either human SV2C or no additional DNA construct ("Vector") and tested immunoreactivity. The hSV2CpAb shows strong immunoreactivity at the expected molecular weight of SV2C (~90kD) in the transfected but not the untransfected cell lysates. There are several nonspecific bands in both the vector- and SV2C-transfected lysates. Most of these bands were eliminated with affinity-purification of the antibody; however, we also observed a reduction in immunoreactivity in the specific band. (Fig. 2-1A). We validated the mSV2CpAb using striatal homogenates from WT and mice lacking SV2C (SV2C-KO). As expected, we see a loss of SV2C-specific immunoreactivity in the SV2C-KO striatal homogenate (Fig. 2-2B). We were also able to validate the specificity of the mSV2CpAb via immunohistochemistry on WT versus SV2C-KO tissue, where we see a lack of immunoreactivity using the mSV2CpAb (Fig. 2-2C). Our mSV2CpAb also indicates subcellular specificity of the antibody using immunocytochemistry of primary ventral mesencephalon cells (Fig. 2-2D). SV2C appears to localize specifically to punctate terminal regions in cultured cells.

SV2C localization in mouse

We found expression in a variety of brain regions within the basal ganglia. In particular, SV2C immunoreactivity was localized to the neuropil of the caudoputamen (CPu), nucleus accumbens core and shell (NAc, NAsh), the ventral pallidum (VP), the globus pallidus (GP), substantia nigra pars reticulata (SNr), the interpeduncular nucleus (IPN), and the cell

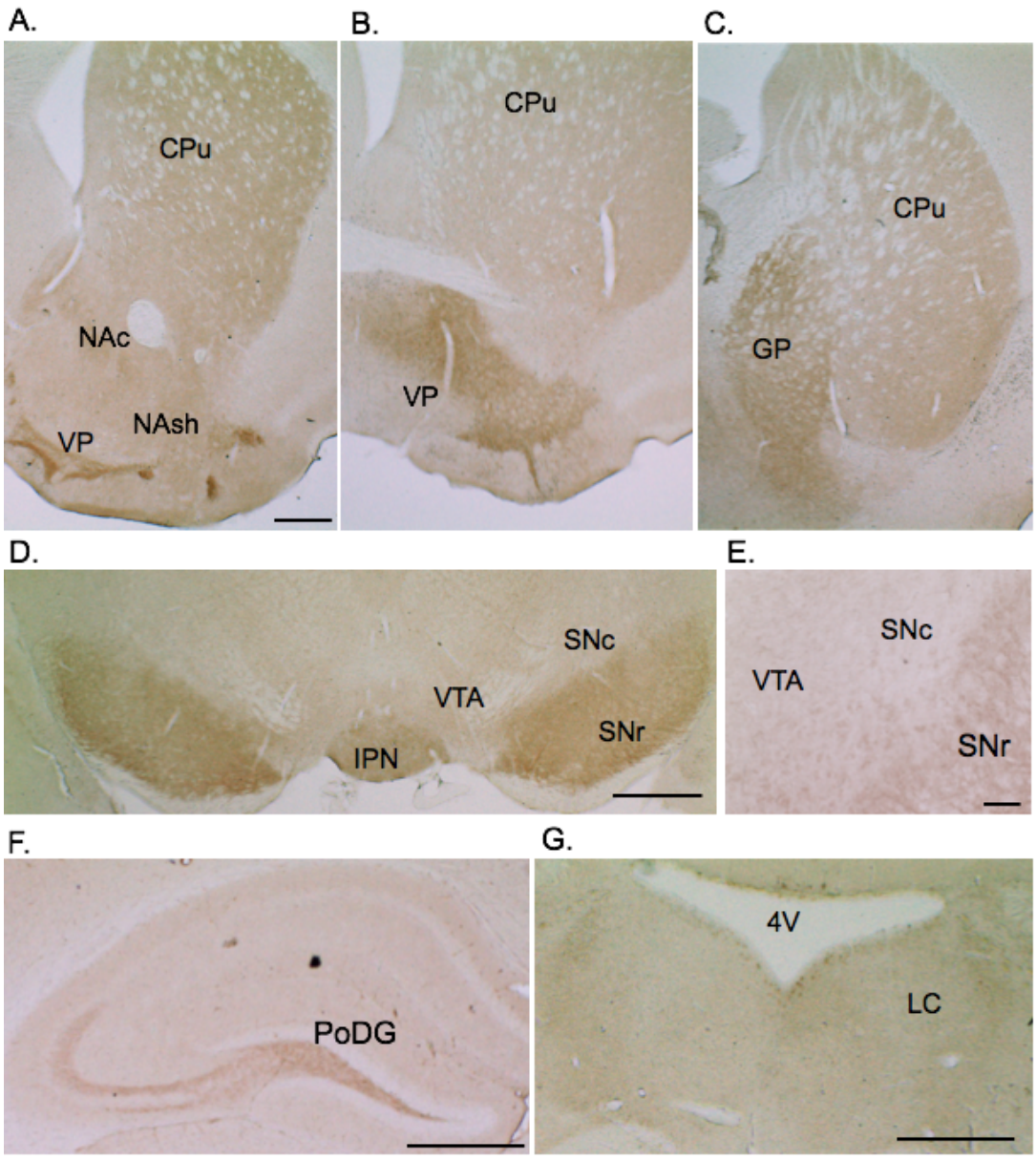

Figure 2-2. SV2C localization within the mouse brain. (A-C) SV2C is expressed at high levels in the dorsal striatum (CPu), the nucleus accumbens core and shell (NAc, NAsh), the ventral pallidum (VP), and the the globus pallidus (GP). (D-E) SV2C is expressed in the substantia nigra pars compacta and pars reticulata (SNc, SNr), the ventral tegmental area (VTA), and the interpeduncular nucleus (IPN). Outside of the basal ganglia, SV2C is expressed in the dentate gyrus of the hippocampus (PoDG), and locus coeruleus (LC). *Scale bars = 500μm for 2.5X (A-D), 100μm for 10X (E-G).*

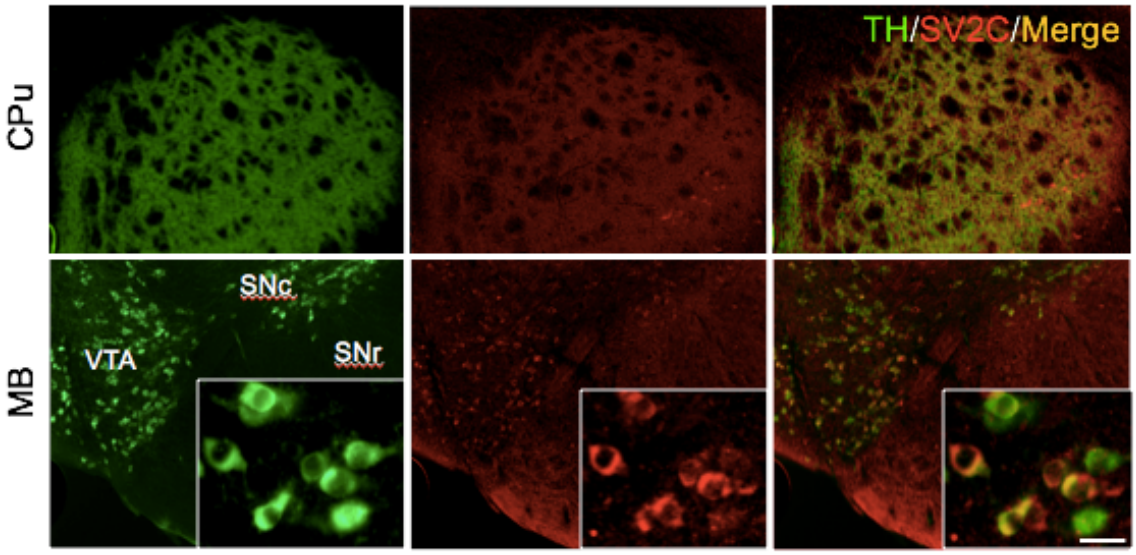

Figure 2-3. SV2C colocalizes with the dopamine marker TH in the mouse basal ganglia. SV2C is expressed in dopaminergic neurons in the ventral tegmental area (VTA) and SNc, as well as in dopamine terminal fields in the CPu. A semi-quantitative colocalization analysis using FIJI indicated a high degree of colocalization between TH and SV2C in both the CPu (Mander's M2 coefficient = 0.976) and the midbrain (Mander's M2 = 0.953). *Scale bar = 20 μm*

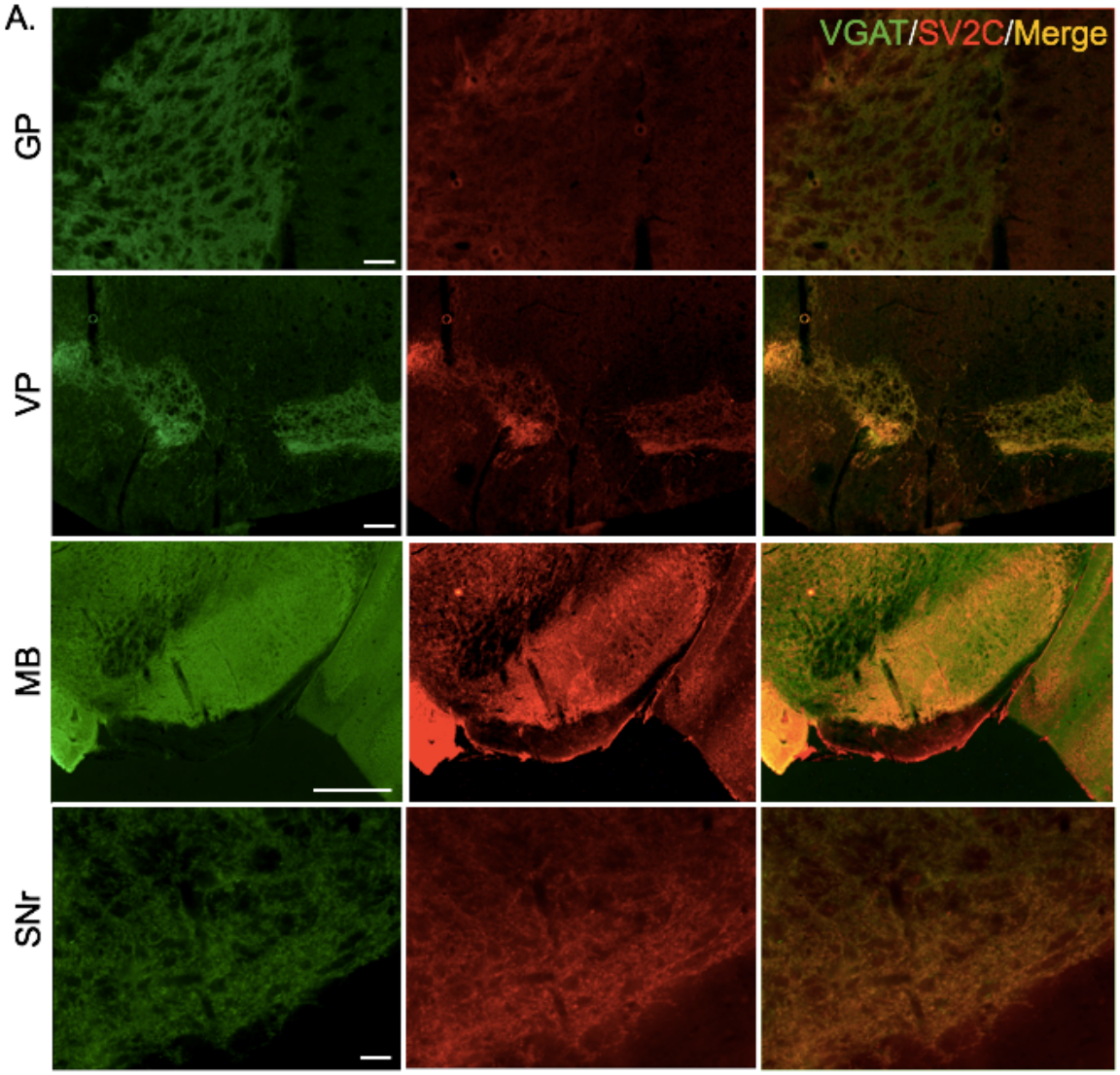

Figure 2-4. SV2C expression in GABAergic regions of the basal ganglia. SV2C and vesicular GABA transporter (VGAT) immunoreactivity is highly colocalized in the neuropil of GABAergic nuclei within the basal ganglia including the VP, GP, midbrain (MB) and SNr. *Scale bars = 500 µm (MB), 100 µm for all others*

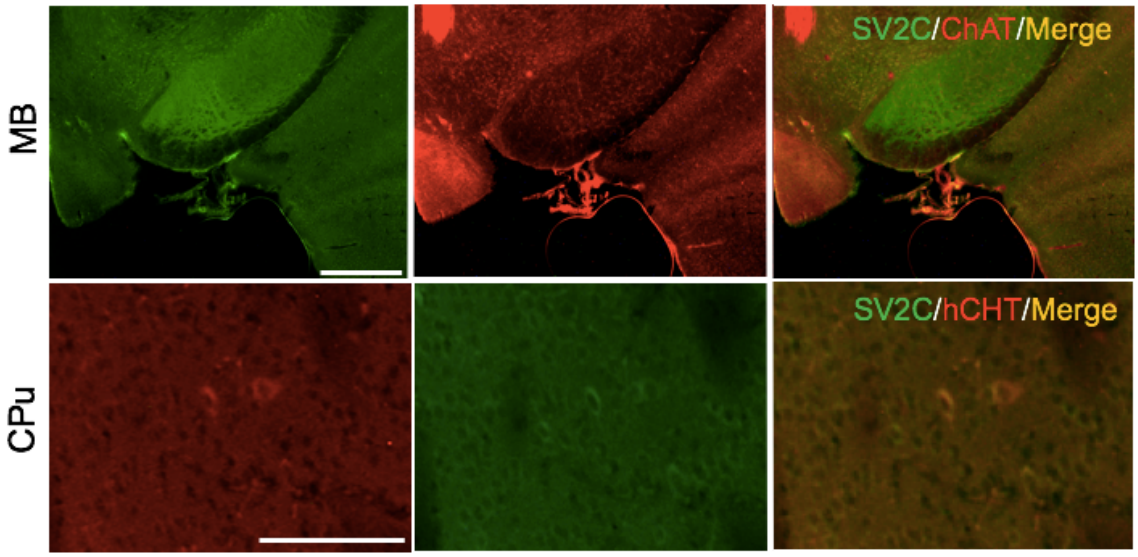

Figure 2-5. SV2C localization in cholinergic cells within the basal ganglia. SV2C colocalizes with markers for acetylcholine (choline acetyltransferase, ChAT, and the choline transporter, hCHT) in the midbrain (MB) and dorsal striatum (CPu). *Scale bars = 500 μm (MB), 100 μm (CPu)*

bodies of the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc). We previously published delineation of the VP, demonstrating high expression of SV2C in the VP (Stout 2016). A semiquantitative analysis using revealed a Mander's M2 colocalization coefficient of 0.976, indicating a substantial degree of colocalization between TH-positive and SV2C-positive neuropil of the dorsal striatum. A similar analysis of colocalization between TH and SV2C in the midbrain revealed a Mander's M2 coefficient of 0.953, with a similar degree of colocalization with the VTA (M2 = 0.97) and the SNc (M2 = 0.968). Outside of the basal ganglia, we observed light SV2C immunoreactivity in the polymorphic layer of the dentate gyrus (PoDG) in the hippocampus and the locus coeruleus (LC) (Fig. 2-2).

SV2C shows a similar pattern of expression as dopaminergic markers such as tyrosine hydroxylase (TH). SV2C and TH are colocalized in the dopamine terminal regions of the CPu, as well as in cell bodies in the VTA and SNc (Fig. 2-3). Previous reports have indicated that SV2C is present in striatal GABAergic neurons, with SV2C preferentially localizing to GABA cells over glutamatergic cells (Dardou D 2010; Gronborg et al. 2010). We found that SV2C is expressed in GABAergic regions of the basal ganglia, including terminal regions of the GP, VP, midbrain (MB), and SNr (Fig. 2-4). Given previous indications that SV2C may be a mediator of the neuroprotective and neurochemical effects of nicotine, as well as a previous record showing colocalization between SV2C and markers for acetylcholine, we also examined SV2C expression in cholinergic cells. We found coexpression of SV2C and markers of cholinergic cells, choline acetyltransferase (ChAT) and the choline transporter (hCHT) (Fig. 2-5), indicating that SV2C is present in some cholinergic cells, consistent with previous reports (Dardou 2010).

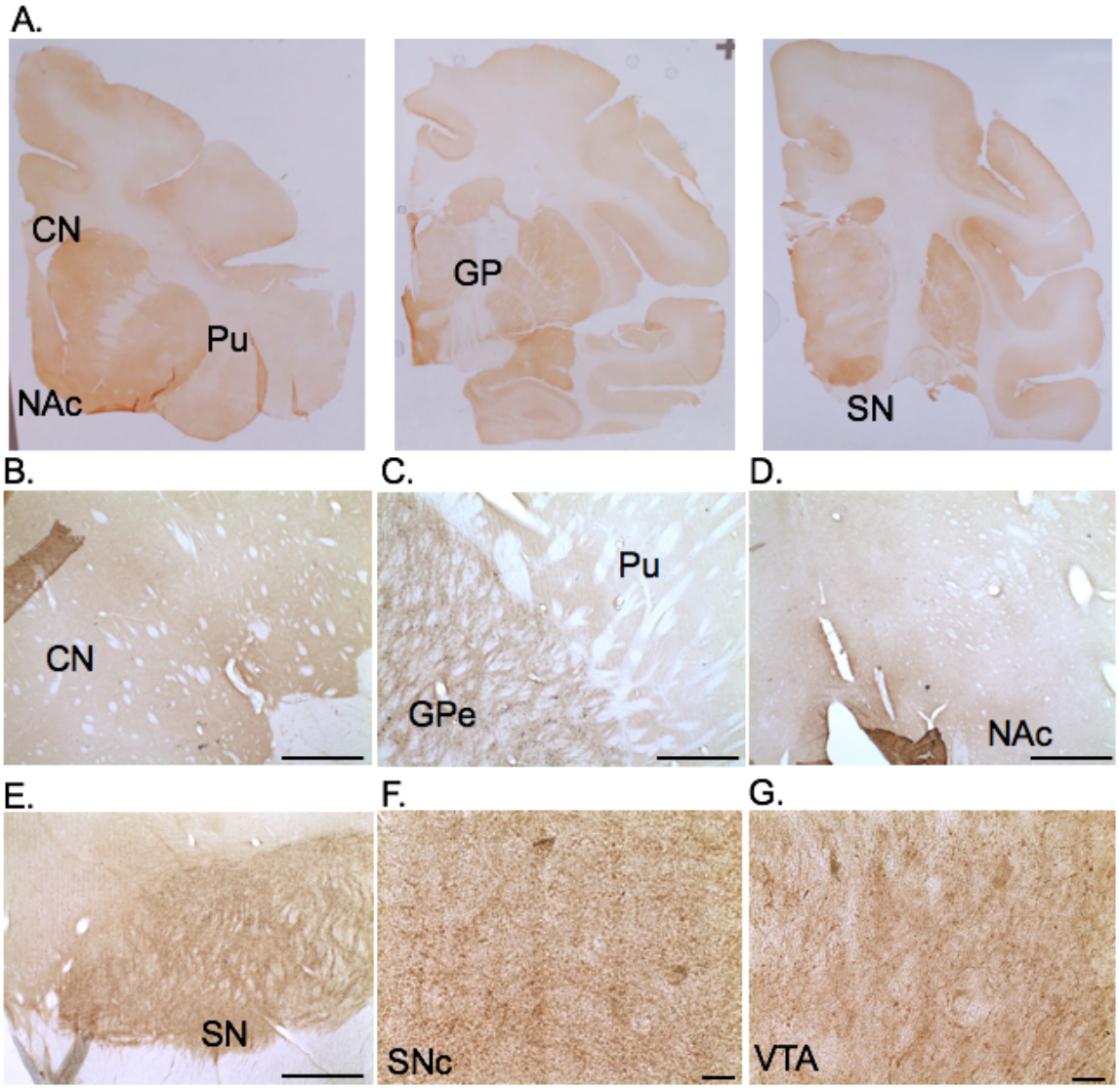

Figure 2-6. SV2C is expressed in the basal ganglia of nonhuman primates. SV2C is highly expressed in the caudate nucleus (CN), the putamen (Pu), GP, and NAc (A-D) of macaques. SV2C expression is also observed in the neuropil of the midbrain (E), and light expression in the cell bodies of the VTA (F) and SNc (G). NAc of macaques. *Scale bars = 1 mm (B-E), 20 μm (F-G)*

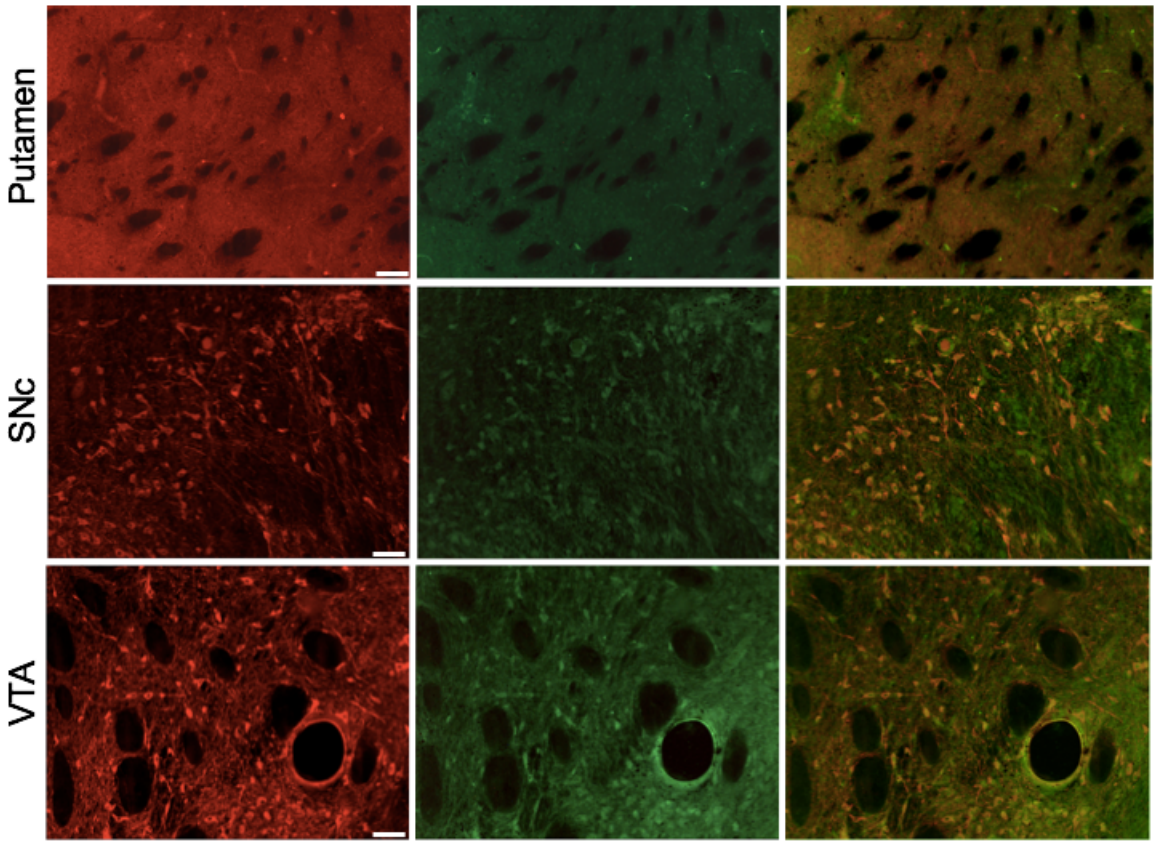

Figure 2-7. SV2C is localized to dopaminergic regions of the NHP basal ganglia. SV2C colocalizes with TH in cell bodies within the SNc and VTA, and in terminal regions of the dorsal striatum (Pu). *Scale bars = 100 μm (putamen), 20 μm (midbrain)*

SV2C localization in macaque

SV2C expression followed a similar pattern of expression in macaques as has been reported in rodents. Specifically, SV2C is expressed throughout the basal ganglia, particularly the caudate nucleus (CN), putamen, GP, SNc, and SNr (Fig. 2-6). The expression pattern of SV2C in macaques, as in mice, appears to be in neuropil throughout the basal ganglia. Further, SV2C localizes to dopaminergic cell bodies within the midbrain and dopaminergic terminals in the putamen (Fig. 2-7). A semiquantitative analysis of colocalization of TH and SV2C within the SNc and VTA revealed a Mander's M2 coefficient of colocalization of 0.92 and 0.988 respectively, indicating a high degree of localization of SV2C to midbrain dopaminergic neurons.

SV2C localization in human

SV2C expression in humans follows a similar, though not identical, pattern of expression as compared to rodents and NHPs. Similar to rodents, SV2C immunoreactivity in human tissue was present throughout the basal ganglia (Fig. 2-8). SV2C is expressed in the SNr and SNc, as well as in terminal and cell body regions of the dorsal striatum (Fig. 2-8). We observed light SV2C immunoreactivity in neuromelanin-containing cells of the SNc, as contrasted to an unstained (no primary antibody) control SNc section. SV2C immunoreactivity is greater in the putamen than in the caudate nucleus, particularly in the diffuse terminal regions. In these regions, diffuse staining of SV2C has a similar pattern of expression as DAT (Fig. 2-9A). SV2C is expressed in cell bodies throughout the striatum, including in the caudate nucleus, putamen and GP (Fig. 2-8B). SV2C is colocalized with

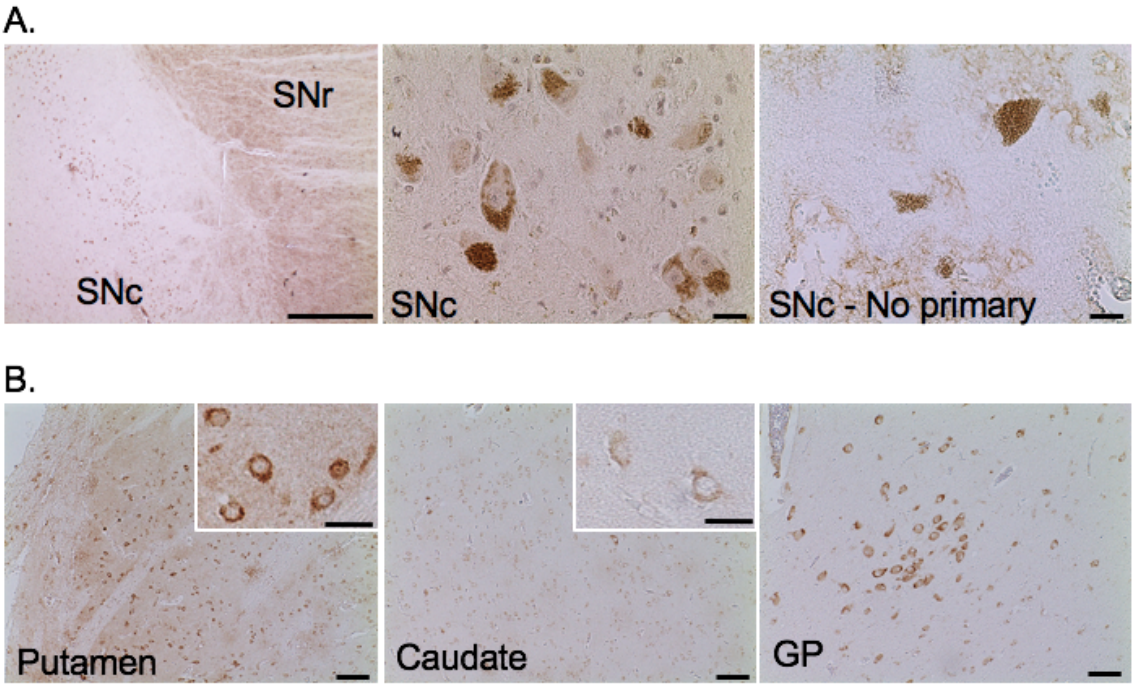

Figure 2-8. SV2C is localized to the basal ganglia in the human brain. (A) SV2C is expressed in the SNc and SNr. We observed light SV2C immunoreactivity in the somata of neuromelanin-containing SNc cells, which is contrasted to the lack of immunoreactivity in an unstained SNc section. Neuromelanin is visible in the unstained tissue. (B) SV2C is expressed in the somata and neuropil of the putamen and, to a lesser extent, the caudate nucleus. We also observed immunoreactivity in the somata of the GP. *Scale bars = 500 μm (A, low magnification), 100 μm (B low magnification), 20 μm (A, high magnification and B insets)*

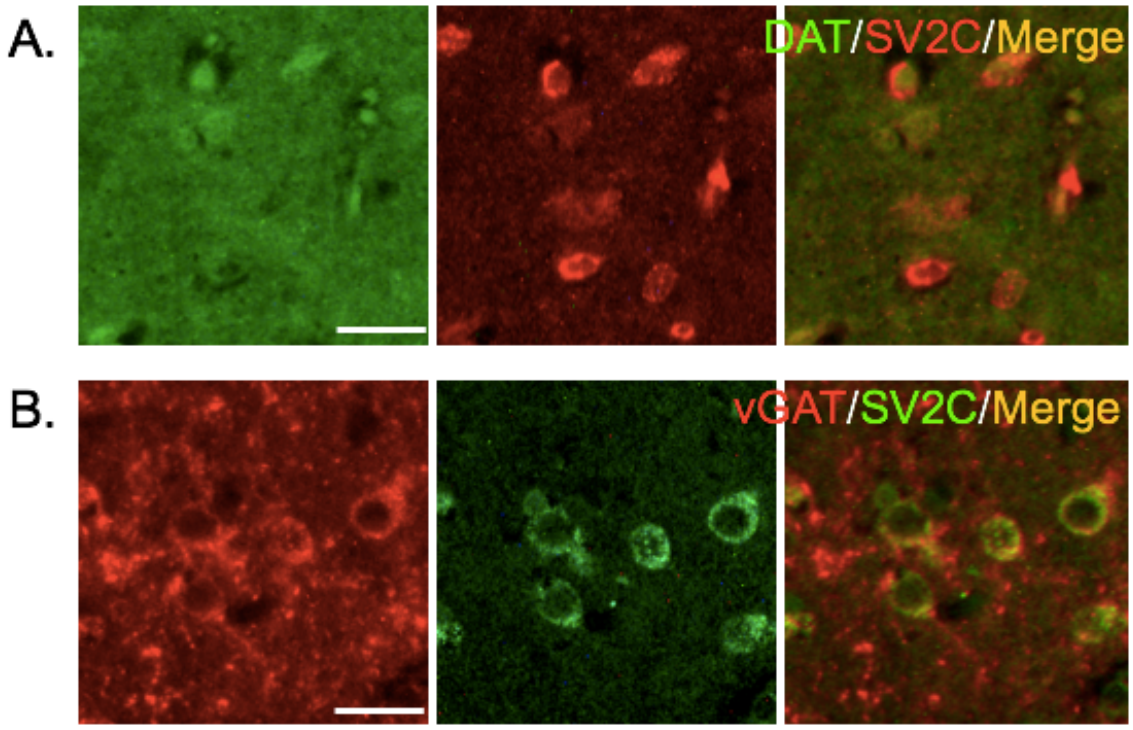

Figure 2-9. SV2C is present in dopamine terminals and GABAergic cells in the human putamen. (A) DAT and SV2C immunoreactivity show similar expression in the putamen, though nonspecific cell body staining by the DAT antibody in the putamen reflects the unreliability of staining human tissue. (B) SV2C is also highly colocalized with the vesicular GABA transporter (vGAT) in medium spiny neurons of the putamen. *Scale bars = 20 µm*

the vesicular GABA transporter (VGAT), indicating SV2C expression within GABAergic medium spiny neurons (MSNs, Fig. 2-9B).

DISCUSSION

SV2C is a vesicular protein with a distinct basal-ganglia expression pattern. Previous immunological investigations into its localization focused exclusively on the mouse brain using antibodies that are not commercially available. Here, we use two novel polyclonal SV2C antisera recognizing mouse and primate SV2C. Further clarification of the expression pattern of SV2C, particularly with respect to similarities across species, will be invaluable in interpreting future experimental results and their translatability to human relevance, and this pair of novel antibodies for SV2C will be important tools in these studies.

Specificity of the SV2C antibody.

We previously demonstrated that the hSV2CpAb is specific to SV2C and does not recognize other proteins within the SV2 family (SV2A, SV2B) (Dunn 2017). Here, we further demonstrate that these antibodies recognize SV2C by immunoblot of cell lysates and striatal homogenates, as well as by immunocytochemistry and immunohistochemistry of mouse tissue. We observe a loss of SV2C immunoreactivity throughout the brain of SV2C-KO animals that corresponds to an expected lack of SV2C protein, and SV2C protein has expression patterns consistent with previous reports. This antiserum results in a relatively strong nonspecific band at approximately 70kD. We attempted to affinity-

purify the antisera. Unfortunately, affinity-purification resulted in a reduction in the strength of the SV2C-specific band in addition to a reduction in the nonspecific bands. This is presumably due to the inability of the elution buffer to remove the most strongly-bound antibody clones, thus resulting in a loss of the specific immunoreactivity in the purified antiserum. Ultimately, the unpurified antiserum provided the optimal specific immunoreactivity for our experiments, and we completed all further experimentation with the unpurified antiserum.

Anatomical and subcellular distribution of SV2C.

SV2 is frequently used as a marker for synaptic terminals, and SV2A and SV2B are generally not expressed in the cell body (Bajjalieh 1994; Stockburger et al. 2015). There is some evidence for mitochondrial SV2 expression (Stockburger 2015). SV2C expression specifically varies from the traditional terminal-only expression pattern of the other SV2s, and based on previous reports, we expected to see SV2C expression in synaptic terminals and cell bodies. Indeed, we see SV2C expressed in a punctate, characteristically synaptic distribution in primary mouse neurons. In tissue sections, SV2C is expressed in a diffuse pattern throughout various nuclei in the basal ganglia, suggesting localization to synaptic terminals of dopaminergic and GABAergic neurons. We also see localization of SV2C to the somata of various cell types, including midbrain dopamine neurons, striatal GABAergic medium spiny neurons, and striatal cholinergic cells. The contrast of subcellular and cell-type specific distribution of SV2C versus what has been reported for SV2A and SV2B suggests a distinct role of SV2C within these cells.

***Similarity of SV2C distribution across species*.**

In each species examined, SV2C is expressed in a clear basal-ganglia enriched pattern. The areas of highest expression are the substantia nigra, the ventral tegmental area, the caudate nucleus, the putamen, the globus pallidus and the ventral pallidum. There is minimal SV2C expression in the cortex of all species. The similar expression patterns of SV2C across species suggests a preserved function and role for SV2C in these brain regions. SV2C was previously found to regulate dopamine expression in mice, and because SV2C expression is in similar brain regions, it is likely that SV2C also regulates dopamine release in primates. This provides support to the translatability and human relevance of experimental results of SV2C function in mice.

Key differences of SV2C expression in humans versus other species.

SV2C is strongly expressed in medium spiny neuron somata throughout the striatum of humans. This contrasts with what we and others have observed in rodent tissue, and suggests an elevated importance of SV2C in GABA transmission in humans. However, the SV2C antibody strongly labels the SNr in all species examined. Given that the main GABAergic input to the SNr are the MSNs from the dorsal striatum, it is possible that only the relative expression in the somata versus the synaptic terminals is the primary difference. Additionally, immunochemical staining of human tissue is characteristically problematic, given the inconsistencies and difficulties of preserving and processing the tissue postmortem. Nonetheless, these experiments are valuable in demonstrating consistencies in SV2C expression across various species. Additionally, it will be important to consider

the possible differences in the contribution of SV2C in GABA transmission in humans when interpreting mouse data.

Overall, we demonstrate that SV2C expression is relatively consistent throughout various species, with a basal-ganglia enriched distribution. We introduce a novel pair of SV2C antisera that can be used in a variety of immunological methods in various species. These data will be useful tools in future investigations into the function of SV2C and its relevance in associated neurological diseases.

III. Chapter 3. SV2C modulates dopamine release and is disrupted in Parkinson's disease

A version of this chapter was published as part of a manuscript:

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ABSTRACT

Members of the synaptic vesicle glycoprotein 2 (SV2) family of proteins are involved in synaptic function throughout the brain. The ubiquitously expressed SV2A has been widely implicated in epilepsy, though SV2C, with its restricted basal ganglia distribution, is poorly characterized. SV2C is emerging as a potentially relevant protein in Parkinson disease (PD), as it is a genetic modifier of sensitivity to L-DOPA and of nicotine neuroprotection in PD. Here we identify SV2C as a mediator of dopamine homeostasis and report that disrupted expression of SV2C within the basal ganglia is a pathological feature of PD. Genetic deletion of SV2C leads to reduced dopamine release in the dorsal striatum as measured by fast-scan cyclic voltammetry, reduced striatal dopamine content, disrupted alpha-synuclein expression, deficits in motor function, and alterations in neurochemical effects of nicotine. Further, SV2C expression is dramatically altered in postmortem brain tissue from PD cases, but not in Alzheimer disease, progressive supranuclear palsy, or multiple system atrophy. This disruption was paralleled in mice overexpressing mutated alpha-synuclein. These data establish SV2C as a novel mediator of dopamine neuron function and suggest that SV2C disruption is a unique feature of PD that likely contributes to dopaminergic dysfunction.

Significance statement:

Here we describe a role for the synaptic vesicle glycoprotein 2C (SV2C) in dopamine neurotransmission and Parkinson disease (PD). SV2C is expressed on the vesicles of dopamine-producing neurons and genetic deletion of SV2C causes a reduction in synaptic release of dopamine. The reduced dopamine release is associated with a decrease in motor activity. SV2C is suspected of mediating the neuroprotective effects of nicotine and we show an ablated neurochemical response to nicotine in SV2C-knockout mice. Lastly, we demonstrate that SV2C expression is specifically disrupted in mice that express mutated alpha-synuclein and in humans with PD. Together, these data establish SV2C as an important mediator of dopamine homeostasis and a potential contributor to PD pathogenesis.

INTRODUCTION

Synaptic vesicles, particularly those within the dopaminergic nigrostriatal pathway, have two important roles: packaging transmitter for neurotransmission and sequestering compounds that may have adverse effects on the cell, such as cytosolic dopamine (Goldstein 2013; Mosharov 2009; Sulzer 2000b). Ineffective sequestration of dopamine leads to progressive cell loss and heightened vulnerability to dopaminergic toxicants in mice (Caudle 2007; Fon 1997; Mooslehner 2001; Takahashi 1997; Taylor 2009; Guillot 2008b; Taylor 2014). Mutations in the gene encoding the vesicular monoamine transporter 2 (VMAT2) lead to infantile parkinsonism (Rilstone 2013), while increased expression of

VMAT2 is associated with decreased risk for Parkinson disease (PD) (Glatt 2006; Brighina 2013). Dopamine vesicle function is impaired in patients with PD, suggesting that dysfunctional dopamine handling is fundamental to the disease (Pifl 2014). Genetic mutations in several other vesicle-associated proteins, such as α-synuclein, LRRK2 and synaptojanin-1, can lead to disrupted presynaptic dopamine handling and are commonly linked to PD (Belluzzi 2012; Krebs 2013; Lotharius and Brundin 2002; Volles 2002; Cirnaru 2014; Nemani 2010; Piccoli 2011). Disrupted vesicle function may represent a common pathway to degeneration and identifying novel mediators of vesicular function could provide insight to our understanding of disease pathogenesis.

Proteins within the synaptic vesicle glycoprotein 2 (SV2) family are thought to positively modulate vesicular function in a variety of ways, possibly by aiding in vesicular trafficking and exocytosis, interacting with synaptotagmin-1, or stabilizing stored transmitter (Chang 2009; Crowder 1999; Custer 2006; Harlow 2013; Iezzi 2005; Janz 1999a; Lazzell 2004; Nowack 2012; Reigada 2003; Schivell 2005; Wan 2010; Xu 2001; Yao 2010; Vautrin 2009). SV2A has been strongly implicated in epilepsy and is the specific pharmacological target for the antiepileptic drug levetiracetam (Lynch 2004). However, despite extensive research the molecular function of each of the SV2 proteins has not been fully described. SV2C is distinguished from SV2A and SV2B by its enriched expression in the basal ganglia and preferential localization to dopaminergic cells in mice (Dardou 2011; Janz 1999b). Intriguingly, SV2C was recently identified as a genetic mediator of one of the most robust environmental modulators of PD risk: nicotine use, which is strongly protective against PD (Hernan 2002; Hill-Burns 2012). Variation within the SV2C gene was also found to predict PD patient sensitivity to L-DOPA (Altmann 2016). These data

suggest that SV2C may play a particularly important role in the basal ganglia, though there has been no experimental evidence linking SV2C to dopaminergic function, neurochemical effects of nicotine, or PD.

We first characterized SV2C expression in multiple mouse models of PD in order to establish potential alterations in SV2C following dopaminergic cell loss. To directly test the hypothesis that SV2C is involved in dopamine function within the basal ganglia, we then developed a line of mice lacking SV2C (SV2C-KO). We quantified striatal dopamine content and dopamine metabolites in SV2C-KO animals with high performance liquid chromatography (HPLC). We then investigated alterations in dopamine- and PD-related motor behavior and protein expression following genetic deletion of SV2C and evaluated a possible interaction between SV2C and α-synuclein. Next, we performed fast-scan cyclic voltammetry to measure stimulated dopamine release in the dorsal striatum of SV2C-KO and wildtype (WT) mice at baseline and in the presence of nicotine. Finally, we analyzed SV2C expression in the basal ganglia of postmortem PD cases and other neurodegenerative diseases. The data described below establish SV2C as a mediator of dopamine dynamics and provide a functional basis for a role for SV2C in PD.

MATERIALS & METHODS

Antibodies:

Two polyclonal anti-SV2C sera were raised in rabbits against peptides in the N-terminus 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 laboratory by Covance Custom Immunology Services (See Chapter 2 for antibody characterization). The following primary antibodies were purchased commercially: DAT, TH and synaptophysin (Millipore), β-actin (Sigma), SV2A (E-15) (Santa Cruz Biotechnology), SV2B and synaptotagmin-1 (Synaptic Systems), α-synuclein (BD Transduction Laboratories), and ubiquitin (Fitzgerald Industries). Secondary antibodies were purchased from Jackson ImmunoResearch (biotinylated, HRP-conjugated) or Abcam (fluorescent).

Tissue culture:

HEK293 and Neuro-2a (N2a) (ATCC) cells were cultured according to standard protocols. Growth media was either DMEM with 10% fetal bovine serum (FBS) + 1% penicillin/streptomycin (HEK293) or EMEM + 10% FBS + 1% penicillin/streptomycin (N2a). Transfections of SV2C and SV2A in pcDNA3.1 vectors were performed with Lipofectamine 2000 (Invitrogen) according to manufacturer's protocols. SV2C shRNA constructs were transfected via electroporation (Amaxa Nucleofector) according to manufacturer's protocols. Cells were harvested 24 hours post-transfection, lysed in RIPA buffer and total protein extraction was achieved through differential centrifugation.

SV2C shRNA:

Short hairpin RNAs (shRNA) against SV2C were custom-designed by Origene for our laboratory. Two SV2C-specific sequences (GACAGCATCGTGTCTGTAG and ATCGTGTCTGTAGGACAGC) significantly reduced expression of endogenous SV2C expression in N2a cells. One scrambled sequence not corresponding to any sequence in SV2C had no effect on SV2C expression.

Western blotting:

Western blots were performed as previously described (Lohr 2014). Primary antibodies were used at the following dilutions: SV2C (1:2,500), DAT (1:5,000), TH (1:1,000), synaptotagmin 1 (1:1,000), synaptophysin (1:1,000) α-synuclein (1:1,000), and β-actin (1:5,000). HRP-conjugated secondary antibodies were diluted to 1:5,000. For antigen blocking, mSV2CpAb was diluted 1:2,500 in a solution containing 1mg/mL immunizing peptide and 0.75% nonfat powdered milk for one hour at room temperature and then applied to the immunoblot.

Animals:

12-month old male and female A53T-OE mice and WT littermates, and 22-month old male and female VMAT2-LO mice and WT littermates were used for studies using genetic models of PD. Six- to 12-month old male WT C57BL/6 mice (Charles River Laboratories) were used for MPTP studies. Three to six animals were included in each group for all experiments. 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.

MPTP treatments:

Male mice were injected (s.c.) with MPTP hydrochloride (Sigma) or saline. The "terminal" lesion consisted of two injections of 15mg/kg MPTP with an inter-injection interval of 12hr. The 4x15mg/kg lesion consisted of 4 injections in 1 day of 15mg/kg MPTP with an inter-injection interval of 2hr. The "cell body" lesion consisted of five injections of 20mg/kg MPTP with an inter-injection interval of 24hr.

Transgenic mouse generation:

SV2C-KO mice: We obtained 3 lines of C57BL/6 ES cells from the European Conditional Mouse Mutagenesis Program containing the SV2C "knockout-first construct" (Skarnes 2011). ES cells containing the construct were injected into blastocysts. Chimeric mice were bred with C57BL/6 mice. We achieved germline transmission of the construct in resulting offspring, and chose these mice to found the SV2C-"knockout first" line. This line was subsequently crossed with Flp-recombinase containing mice followed by mice containing Cre under a *NES* (nestin) promoter to delete the intron and found the SV2C-KO line. PCR genotyping primers are as follows:

Sv2c (Exon 2): (Fw) TCA TCT AGA AGG GTT AAG GTC TGG, (Rev) ACC ATC ATC CCG AGG TAC AC; *LoxP*: (Fw) GCC TCA ACC AGA CCT AAG AA, (Rev) TAG GAA CTT CGG AAT AGG; *LacZ*: (Fw) GTC GTT TGC CGT CTG AAT TT, (Rev) CAT TAA

AGC GAG TGG CAA CA; *Cre*: (Fw) CCT GGA AAA TGC TTC TGT CCG TTT GCC, (Rev) GAG TTG ATA GCT GGC TGG TGG CAG ATG; *Flp*: (Fw) CAC TGA TAT TGT AAG TAG TTT GA, (Rev) CTA GTG CGA AGT AGT GAT CAG G

Back-crossed VMAT2-LO mice: Mice expressing 5% of normal VMAT2 levels were generated as described previously (Caudle 2007; Taylor et al. 2009; Taylor, Caudle, and Miller 2011) and back-crossed to a C57BL/6 genetic background (Lohr 2016).

A53T-OE mice: Mice overexpressing human PD-associated α-synuclein A53T missense mutation in nigrostriatal dopamine cells under the control of a *Pitx3* promoter were generated as described previously (Lin 2012).

Immunohistochemistry:

Paraffin-embedded human tissue. Sections were deparaffinized in xylenes and rehydrated in decreasing concentrations of ethanol. Endogenous peroxidase was quenched with 3% H_2O_2, followed by antigen retrieval in citrate buffer (pH 6.0) at 95^{o}C. Nonspecific antibody binding was blocked with 3% normal horse serum. Primary antibody was diluted to 1:2,500 (hSV2CpAb) or 1:1,000 (all others). Biotinylated secondary antibody (1:200) signal was enhanced with an avidin-biotin complex (Vector Laboratories) and developed with a 3-3' diaminobenzidine (DAB) reaction for approximately 45 seconds. Fluorescent secondary antibodies were diluted 1:800 and autofluorescence was quenched with a 7-minute incubation in 0.1% Sudan Black solution prior to coverslipping.

Frozen sections. Mice were sacrificed by rapid decapitation or transcardial perfusion. Brains were removed and placed in 4% paraformaldehyde for fixation followed by storage

in 30% sucrose. Brains were sectioned to 40μm. Staining was conducted as described previously (Lohr 2014), except that all washes and dilutions were conducted in PBS with 0.2% Triton X-100. All tissue was imaged using a Zeiss AX10 microscope (brightfield, fluorescent) or an Olympus FV1000-TIRF microscope with a 60X oil-immersion objective (confocal).

Quantification of synapse density:

Confocal micrographs (60X) were taken at a resolution of 3.7 pixels per 1μm. Images were analyzed with ImageJ software. Puncta with a minimum size of 4 pixels and an intensity of at least 20% of average brightness were identified and quantified. Three random sampling sites (212μm^2) from three sections of each animal were analyzed and three animals per genotype were included.

HPLC in striatal tissue:

HPLC for striatal dopamine and DOPAC content was performed as described previously(Lohr 2014). Briefly, unilateral striatal dissections were sonicated in 10X their weight of 0.1M perchloric acid and pelleted at 10,000 X G for 10 minutes. Supernatants were filtered at 0.2μm. Dopamine and DOPAC were detected using an MD-150 × 3.2 mm C18 column. The mobile phase consisted of 1.5 mM 1-octanesulfonic acid sodium, 75mM NaH_2PO_4, 0.025% triethylamine, and 8% acetonitrile at pH 2.9. A 20 μl of sample was injected.

Immunoprecipitation of SV2C and α-synuclein:

Co-immunoprecipitation experiments were performed using the Pierce Co-Immunoprecipitation Kit (Thermo Scientific) according to manufacturer's protocols. mSV2CpAb was cross-linked to agarose beads. WT animals were sacrificed by rapid decapitation and a bilateral striatal dissection was performed followed by homogenization and centrifugation to achieve a crude synaptosomal preparation. Samples were treated with IP Lysis/Wash buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol) incubated with antibody-bound resin overnight at 4˚C and protein complexes were eluted with low-pH elution buffer.

Locomotor activity:

Animals were individually placed in locomotor activity monitoring boxes two hours prior to onset of dark (waking) period. Beam-breaks detected by accompanying software were recorded as a measure of total ambulations for a 24-hour observation period (Lohr 2014).

Gait Analysis:

Animals were trained to walk along a clean paper path to their home cage. On testing day, forepaws were dipped in water-soluble ink and animals were prompted to transverse the paper. Stride length was measured as the total toe-to-toe distance across an average of 3 steps (Tillerson 2002).

Rotorod:

Animals were trained to balance on a slowly rotating rod. On test day, animals were placed on the rod which accelerated to 12RPM over 10 seconds. Latency to fall, speed of rotation at the time of fall, and total distance traveled was recorded and averaged over three testing trials (Tillerson 2002).

Fast-scan cyclic voltammetry in striatal slices:

Fast-scan cyclic voltammetry was performed as described previously (Lohr 2014, 2016; Stout 2016) in 300μm striatal slices bathed in 30°C oxygenated aCSF. Stimulations consisted of a 700μA 4ms monopolar pulse, either single pulse (baseline release) or five at 100Hz (high-intensity stimulation). Four to five recordings (single pulse) were taken at each of four dorsolateral striatal sites with a 5-minute inter-stimulation rest period followed by one 5-pulse, 100Hz stimulation recording. Electrode sensitivity was calibrated to known dopamine standards using a flow-cell injection system. Maximum dopamine release was averaged across sites and kinetic constants were calculated using nonlinear regression analysis of dopamine release and uptake. *Acute nicotine application*: aCSF bath was replaced with increasing concentrations of nicotine in aCSF: 10nM, 50nM, 100nM and 500nM for 30 minutes each. Five recordings (single pulse) were taken in each concentration, followed by a one 5-pulse, 100Hz stimulation recording taken in 500nM nicotine.

***Statistics*:**

All data were analyzed in GraphPad Prism. Differences between genotypes or treatments were compared by two-tailed t-tests. Densitometric analyses were performed with Image Lab software. All errors shown are SEM, and significance was set to $p < 0.05$.

RESULTS

Distribution of SV2C in mouse basal ganglia.

To analyze expression and localization of SV2C, we designed two polyclonal antibodies raised in rabbits against amino acids 97-114 of (1) mouse SV2C (mSV2C; antibody = mSV2CpAb) and (2) human SV2C (hSV2C; antibody = hSV2CpAb). To confirm the specificity of the hSV2CpAb, we performed immunoblots on lysates of transfected HEK293 cells overexpressing either SV2A, SV2B, or SV2C. The hSV2CpAb immunoreactivity was present in SV2C-transfected cell lysate and not in SV2A- or SV2B-transfected cell lysate. Preincubating the hSV2CpAb with the immunizing peptide ("antigen-blocking") blot ablates immunoreactivity of the antibody on the same blot (Fig. 3-1A). We performed shRNA knockdown of SV2C in Neuro-2a (N2a) cells, which are mouse-derived and endogenously express SV2C but not SV2A or SV2B (Dong 2006). Two sequences of shRNA targeting SV2C mRNA were designed, along with a scrambled shRNA sequence. SV2C shRNA robustly reduced normal SV2C immunoreactivity in N2a cell lysates (Fig. 3-1C).

Staining patterns observed with our mSV2CpAb were consistent with reports using other SV2C antibodies (Janz 1999b; Dardou 2011). SV2A and SV2B are strongly

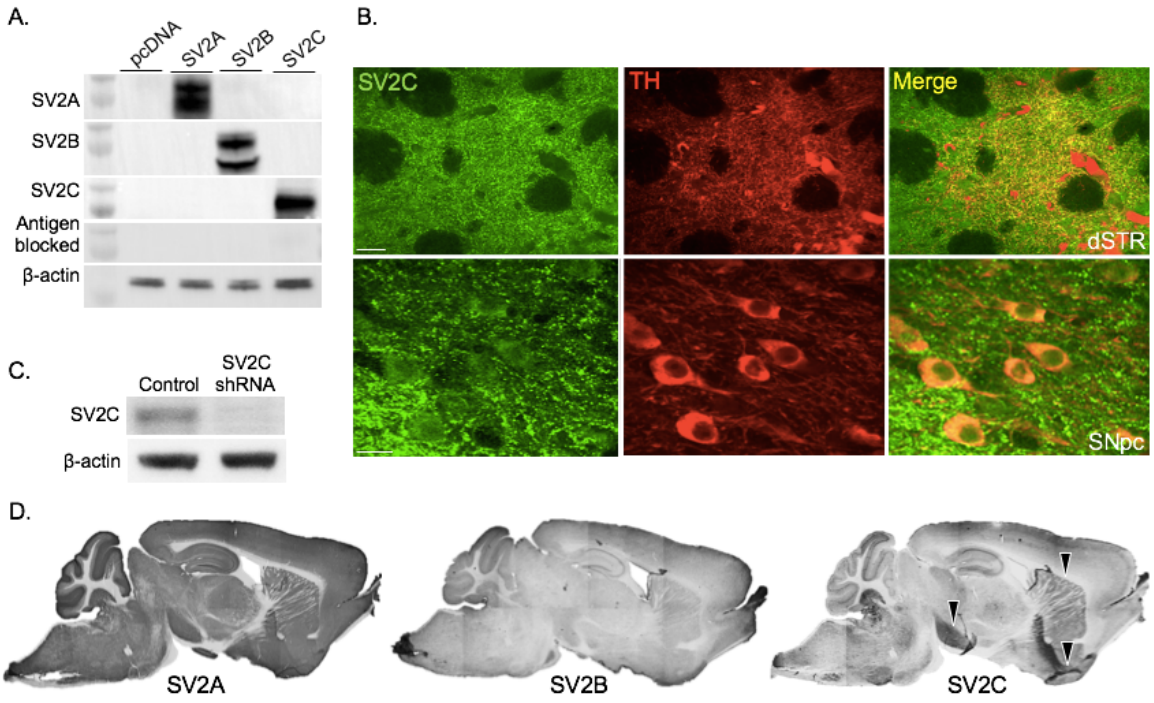

Figure 3-1. Polyclonal SV2C antibody characterization. We generated polyclonal SV2C antibodies: (A) hSV2C antibody recognizes SV2C but does not recognize SV2A or SV2B in transfected HEK293 cells. Pre-incubating the hSV2C antibody with the immunizing peptide ("antigen-blocking") eliminates antibody reactivity. (B) SV2C is highly colocalized with dopaminergic marker tyrosine hydroxylase (TH) in the substantia nigra pars compacta (SNc) and dorsal striatum (dSTR) of mouse. (C) Knocking down endogenous SV2C in Neuro-2a cells (which have no endogenous SV2A or SV2B) using shRNA reduces mSV2C antibody reactivity. (D) Sagittal 3-3' diaminobenzidine-(DAB) stained sections showing ubiquitous SV2A and SV2B expression compared to SV2C expression enriched in the basal ganglia using the mSV2C antibody. Black arrows indicate areas of highest SV2C staining in the mouse brain: midbrain, dSTR, and ventral pallidum. Sagittal sections are composite images of a series of micrographs taken of each section. *Scale bar = 20um*

expressed throughout the mouse brain, whereas SV2C is preferentially expressed in limited nuclei (Fig. 3-1D). We identified strong SV2C immunoreactivity in the ventral pallidum (VP), as well as in cell bodies in the midbrain (substantia nigra pars compacta, SNpc; ventral tegmental area, VTA). SV2C-positive fibers were additionally observed in the substantia nigra pars reticulata (SNpr), dorsal striatum (dSTR), nucleus accumbens (NAc), and globus pallidus (GP). SV2C was highly colocalized with dopaminergic marker tyrosine hydroxylase (TH) in the dSTR and substantia nigra (Fig. 3-1B).

SV2C expression in models of dopamine degeneration.

Next, we explored SV2C expression in mouse models of dopamine cell loss. Each model recapitulates a distinct pathogenic process of cell loss associated with PD, allowing us to evaluate potential involvement of SV2C in various mechanisms of PD-related degeneration.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxication in mice: In order to investigate whether SV2C expression is altered following a lesion to the nigrostriatal tract, we administered three different doses of MPTP to animals to model various stages of degeneration. A 2x15 mg/kg dose, which typically induces a mild loss of striatal dopamine terminals, resulted in a 52% loss of striatal TH immunoreactivity, as measured by immunoblotting (Saline: 10700±996AU, MPTP: 5010±330AU, $p<0.01$). A dose of 4x15mg/kg, which results in an intermediate loss of both dopamine terminals and SNpc cell bodies, induced a 68% loss of striatal TH. A 5x20 mg/kg intoxication, a dose that typically results in significant SNpc cell loss, ablated 86% of striatal TH (Saline: 6310±104AU, MPTP: 916±91.3AU, $p<0.0001$). Concordant with dopamine terminal loss,

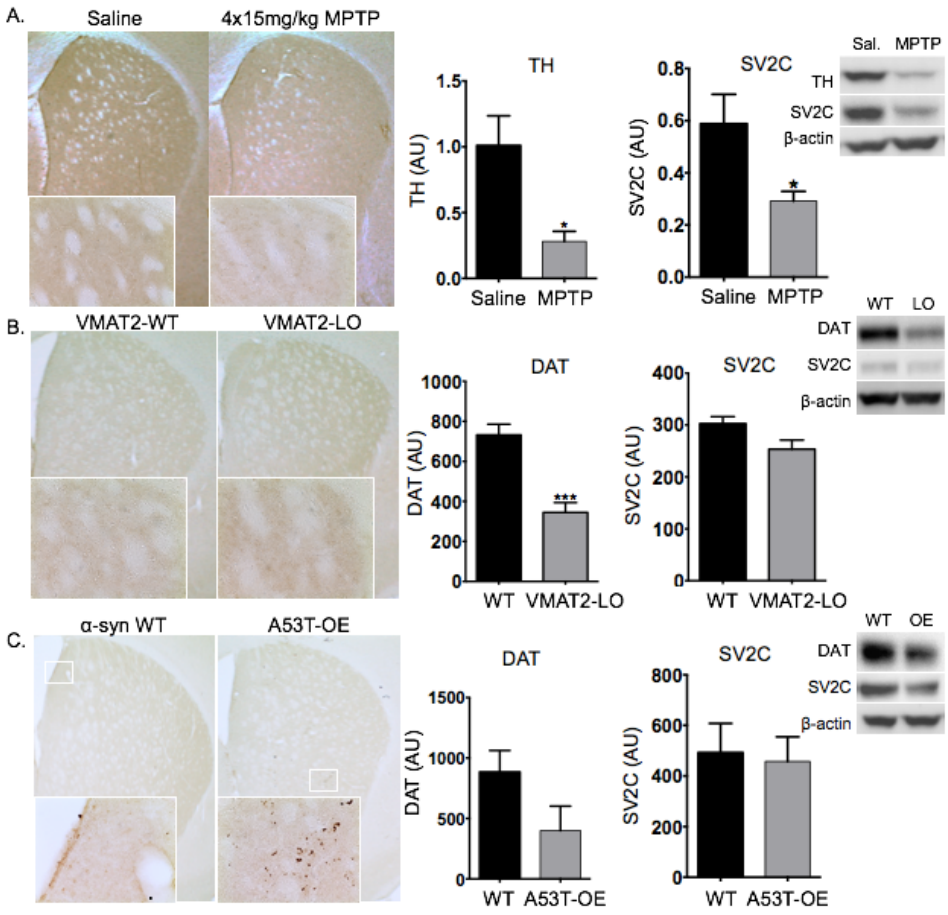

Figure 3-2. SV2C expression in mouse models of PD. (A) Striatal TH is 68% reduced after a 4x15mg/kg dose of MPTP (n=6, $p<0.01$). Striatal SV2C is reduced after MPTP ($p<0.05$). Representative immunohistochemical staining after MPTP reveals that striatal SV2C expression patterns are unchanged. (B) Striatal SV2C levels are slightly, but not significantly, reduced in aged VMAT2-LO animals. SV2C expression patterns remain unaltered, as shown by representative immunohistochemistry in the dorsal striatum. (C) An increase of SV2C-positive punctate staining is observed in the striatum A53T-OE mice as compared to WT mice, particularly in the periventricular mediodorsal striatum. Clusters of punctate SV2C are found distributed sparsely in the striatum of A53T-OE mice (*insets)*. Micrographs were taken at 2.5X and 40X magnification.

striatal SV2C levels were reduced after MPTP (n=6, $p<0.05$ for the 4x15mg/kg paradigm, Fig. 3-2A). SV2C expression patterns appear unaltered after MPTP at any dose.

VMAT2-LO model of PD: To determine whether long-term dysregulation of dopamine storage results in altered expression of SV2C, we evaluated SV2C in animals with a 95% reduction in VMAT2 expression (VMAT2-LO). VMAT2-LO mice have reduced vesicular storage of dopamine, progressive loss of dopamine cells, and motor and non-motor impairments (Caudle 2007; Taylor 2009, 2014). As expected, in aged (20-24 month; n=3) VMAT2-LO mice, we observed a 53% loss of striatal dopamine transporter (DAT, Fig. 3-2B). This terminal loss is consistent with an observed slight, but statistically insignificant, reduction in striatal SV2C, with no change in expression pattern.

A53T α-synuclein overexpression in mice: To determine if α-synuclein disruption alters SV2C, we explored SV2C expression in mice overexpressing PD-associated A53T α-synuclein (A53T-OE) under control of the *Pitx3* promoter, limiting overexpression to midbrain dopamine neurons. These mice display reduced dopamine release, progressive dopamine terminal loss and subsequent motor impairment by 12 months of age (Lin 2012). Dopaminergic overexpression of A53T α-synuclein in aged animals (12mo; n=4) resulted in an increase in punctate SV2C staining observed in the striatum as compared to WT littermates (Fig. 3-2C). This punctate staining was particularly strong in the periventricular region of the dorsomedial striatum and sparse with dense clusters of puncta throughout the dorsolateral striatum.

Genetic deletion of SV2C reduces striatal dopamine content without overt changes in related proteins.

To determine a potential role of SV2C in dopamine-related neurochemistry and behavior, we generated a line of SV2C-KO mice (Fig. 3-3A-C). We performed HPLC in dorsal striatal tissue of SV2C-KO and WT animals to determine if genetic deletion of SV2C altered dopamine tone or metabolism. SV2C-KO animals had a 28% reduction in striatal dopamine content (WT: 65.12±3.5ng/mg protein, KO: 46.76±4.7ng/mg protein, $p<0.01$, n=7, Fig. 3-3D), a 19% reduction in DOPAC (WT: 11.03±1.4ng/mg protein, KO: 8.94±1.1ng/mg protein, n=7, p=0.26, Fig. 3-3E), a dopamine metabolite. This reduction in dopamine content does not appear to be the result of reduced TH, the rate-limiting enzyme in dopamine synthesis, and it does not result in up- or down-regulated DAT as assessed by immunoblot. Further, SV2C deletion does not appear to induce compensatory changes in levels of either SV2A or SV2B, or in total dopamine synapse number in the dorsal striatum as measured by synaptophysin expression (Fig. 3-3B) and synapse density analysis (Fig. 3-3F).

An interaction between SV2C and α-synuclein

Following the observation of disrupted SV2C in A53T-OE mice, we performed immunoblotting and immunoprecipitations to investigate further interaction between SV2C and α-synuclein. SV2C-KO animals had slightly reduced monomeric (~15kD) α-synuclein (69.1±6.17% of WT, n=4-6, p=0.06) and significantly increased high molecular weight (multimeric, ~90kD) α-synuclein expression (3020±561% of WT, n=4-6, $p<0.01$, Fig. 3-4A). In striatal preparations from tissue of WT C57BL/6 mice, α-synuclein co-

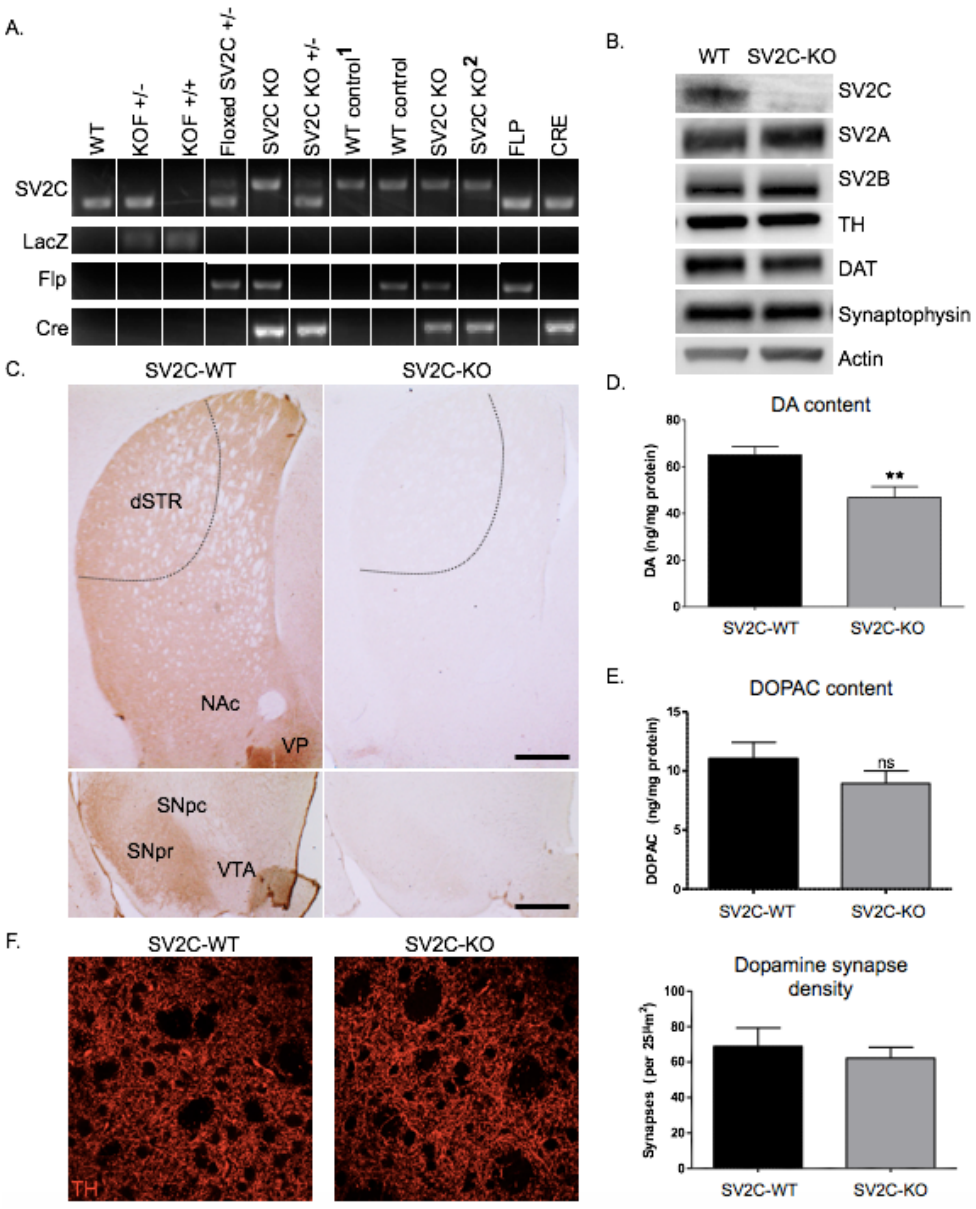

Figure 3-3. Neurochemical characterization of SV2C-KO mice. (A) PCR genotyping of SV2C-KO mice generated using the EUCOMM "knockout-first" allele. This construct allowed for generation of several useful lines of mice: a preliminary knockout animal with a cassette creating a frt-flanked gene trap inserted into the second exon (KOF), a line with a floxed exon following a cross of KOF animals with a Flp-recombinase+ line, and finally SV2C-KO mice following a cross with a nestin-driven Cre-recombinase+ line. Animals used for experiments are denoted by a superscript 1 (WT control) and 2 (SV2C-KO). Lanes in the original gel showing duplicate genotypes were removed from final image for ease of comparison. (B) SV2C-KO does not result in altered

expression of SV2A, SV2B, tyrosine hydroxylase (TH), dopamine transporter (DAT), or synaptophysin in striatum. (C) Genetic deletion of SV2C ablates mSV2C antibody reactivity in the striatum (dSTR) and midbrain. Dotted line delineates dorsolateral striatum where electrochemical recordings were taken (see Fig. 6-7). (D-E) SV2C-KO results in a 28% reduction of dopamine content in the dorsal striatum ($n=7$, $p<0.01$) and a 19% reduction in the dopamine metabolite DOPAC. (F) SV2C-KO does not result in a reduction in dopaminergic synaptic density in the dorsal striatum. *Scale bars = 500µm.*

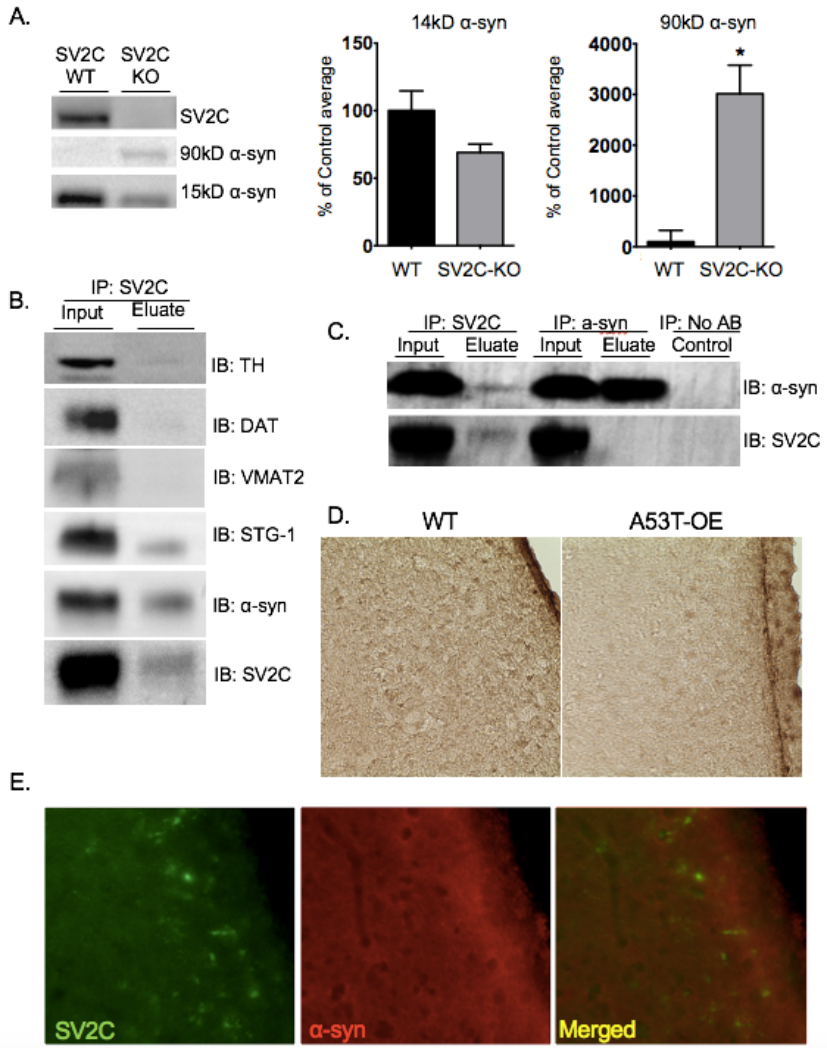

Figure 3-4. **An association between SV2C and α-synuclein.** (A) Midbrain homogenates from SV2C-KO animals have a 31% decrease in monomeric (15kD) α-synuclein (n=4-6, p=0.06) and a 30-fold increase in multimeric (90kD) α-synuclein (n=4-6, p<0.01). (B) Alpha-synuclein co-immunoprecipitates with SV2C in WT mouse striatal homogenates. TH, a cytosolic protein, and DAT, a membrane-associated protein, were included as negative controls. VMAT2 was included as a vesicular protein control. Synaptotagmin-1, which binds to SV2C, was used as a positive control. (C) Further confirmation of α-synuclein and SV2C co-IP with a negative control of non-antibody bound IP column. SV2C-positive puncta do not have similar patterns of expression as SV2A (D) or α-synuclein (E).

immunoprecipitated with SV2C. As expected, neither TH nor DAT co-immunoprecipitated with SV2C (negative control). Synaptotagmin-1, which binds to SV2C (Schivell 2005; Yao 2010), did co-immunoprecipitate (positive control). VMAT2 did not coimmunoprecipitate with SV2C, indicating that we immunoprecipitated protein complexes rather than intact vesicles. Further, using a control IP column with no bound antibody, we confirmed the specificity of the interaction between α-synuclein and mSV2CpAb-bounds resin (Fig. 3-4C). We did not detect SV2C immunoreactivity in an α-synuclein immunoprecipitation. Finally, to address the specificity of SV2C disruption in A53T-OE mice, we performed additional immunohistochemistry of related synaptic proteins. SV2C-positive puncta did not reflect similar patterns of α-synuclein aggregation (Fig. 3-4D). SV2A expression was not similarly disrupted (Fig. 3-4E).

SV2C-KO animals have mild motor deficits.

To determine if SV2C-KO results in altered motor behavior consistent with their decreased dopamine content, we assessed motor function in a variety of tests. We performed a 24-hour locomotor activity assay, gait analysis, and a rotorod test and found that SV2C-KO was associated with mild motor deficits. SV2C-KO animals exhibited reduced locomotor activity, with a 23% reduction in total movement over 24 hours (WT: 38900±2770 ambulations, KO: 30100±2200 ambulations, n=7-8, $p<0.05$). Additionally, SV2C-KO mice displayed a shorter stride length (WT: 7.42±0.17cm, KO: 6.87±0.22cm, n=22-24, $p<0.05$). SV2C-KO animals showed no differences on latency to fall during the rotorod test (WT: 18.3±3.01sec, KO: 20.4±3.36sec, n=7-8, $p=0.52$) (Fig. 5). Additionally,

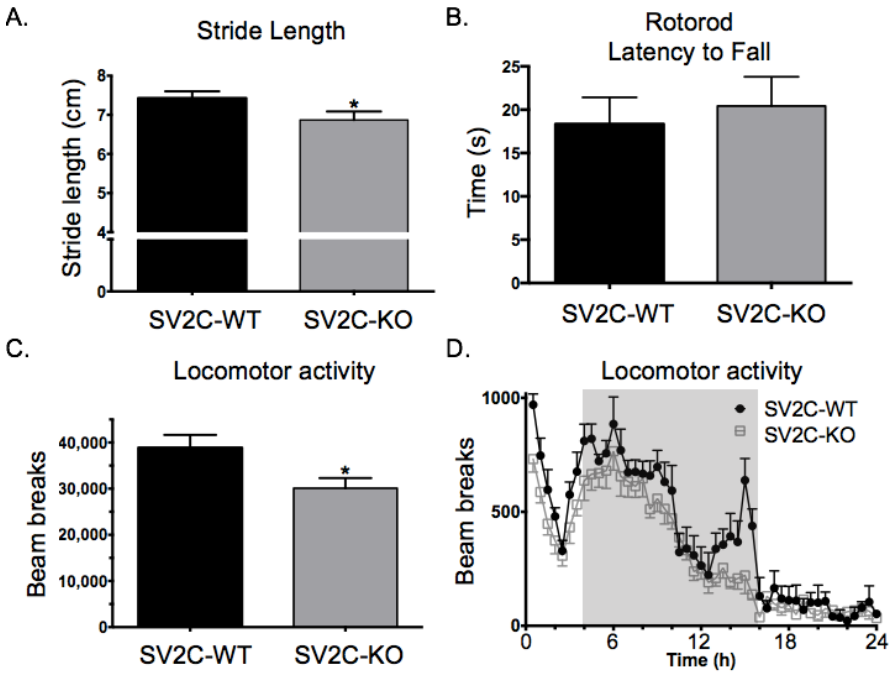

Figure 3-5. Altered motor behavior of SV2C-KO mice. (A) SV2C-KO animals have about a 10% reduction in stride length as measured by a gait analysis assay ($p<0.05$). (B) Genetic deletion of SV2C does not result in impairment on the rotorod test. (C) SV2C-KO animals display a 23% reduction in total ambulations in a 24-hour locomotor activity monitoring ($n=7-8$, $p<0.05$). (D) This reduction in locomotor activity is apparently driven by an ablation of a circadian peak in activity at the end of the active period. Shaded region in (D) indicates dark phase.

SV2C-KO animals had a lower average body size (WT: 19.06±0.91g, KO: 17.24±0.99g, n=53, $p<0.05$).

Genetic deletion of SV2C results in reduced striatal dopamine release.

In order to determine if the observed behavioral deficits of SV2C-KO mice were associated with reduced striatal dopamine release, we performed *ex vivo* fast-scan cyclic voltammetry (FSCV) in the dorsolateral striatum of SV2C-KO animals (see Fig. 3-3B for delineation of dorsolateral striatum). Genetic deletion of SV2C led to a 32% decrease in dopamine release as compared to WT animals (WT: 5.47±0.35μM, KO: 3.71±0.57μM, $p<0.05$, n=9, Fig. 3-6). DAT-mediated dopamine clearance rate was enhanced in SV2C-KO animals as evidenced by reduced tau (WT: 0.39±0.03sec, KO: 0.29±0.02sec, $p<0.05$).

SV2C mediates a neurochemical effect of nicotine.

To assess a potential functional interaction between SV2C and nicotine, we performed FSCV in the presence of 500nM nicotine. This concentration of nicotine typically dampens dopamine release elicited by a single pulse stimulation while enhancing dopamine release elicited by a high intensity (e.g., five pulses at 100Hz) stimulation (Rice and Cragg 2004; Zhang 2004). Genetic deletion of SV2C ablated this effect. In WT tissue, 500nM nicotine enhanced dopamine release elicited by five pulses at 100Hz to 153% of baseline; in SV2C-KO tissue 500nM nicotine *reduced* dopamine release elicited by five pulses at 100Hz to 48% of baseline ($p<0.01$). These data are presented as current traces (Fig. 3-7A-B) and peak dopamine release as a percentage of baseline release (Fig. 3-7C).

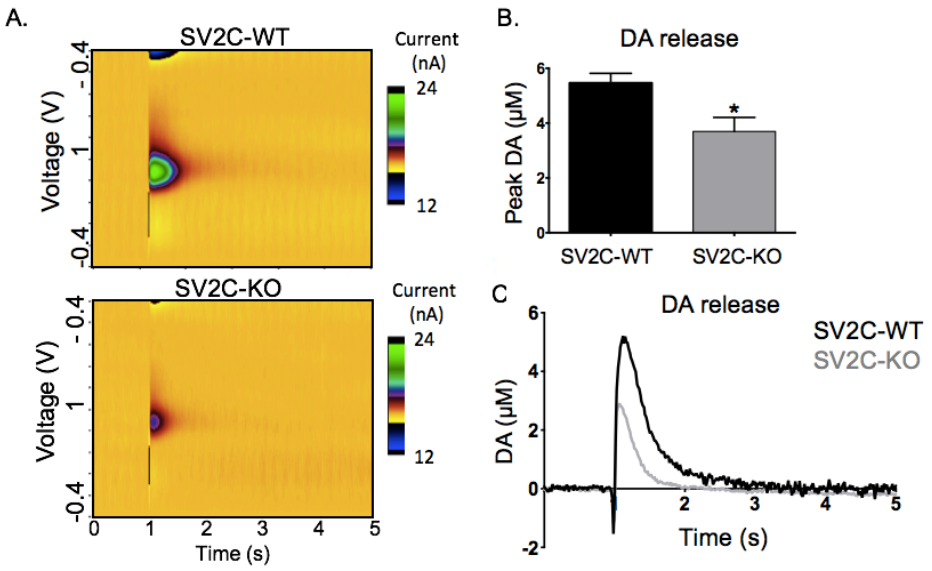

Figure 3-6. Electrochemical measurement of stimulated dopamine release in SV2C-KO. Using fast-scan cyclic voltammetry (FSCV), we measured dopamine release stimulated by a single electrical pulse in the dorsal striatum. Genetically ablating SV2C reduces dopamine release by 32% as shown by (A) a representative color blot and (B) quantified over all recording sites in each animal (n=9, p=0.01) and (C) represented by respective current traces.

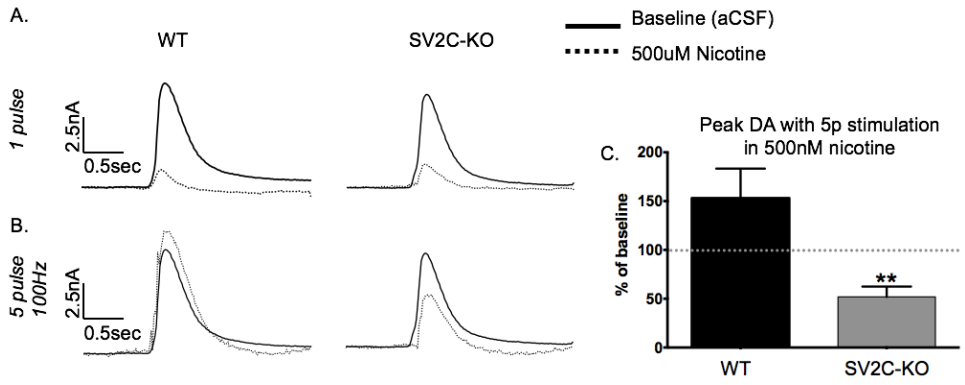

Figure 3-7. Altered neurochemical effect of nicotine after genetic ablation of SV2C as measured by fast-scan cyclic voltammetry. (A) Current traces showing dopamine release at baseline and in the presence of 500nM nicotine. Nicotine normally reduces dopamine release elicited by a one-pulse stimulation to about 10% of baseline in both WT and SV2C-KO striatum. (B)*:* Five-pulse stimulations in the presence of nicotine normally increase dopamine release over baseline release from a 1-pulse stimulation, but this effect is not seen in SV2C-KO animals. (C) Dopamine release elicited by a five-pulse stimulation in the presence of nicotine represented as percent of baseline ($p<0.01$).

SV2C expression in human control and disease cases.

To explore a potential involvement of SV2C in human PD, we obtained human striatum and midbrain samples from tissue banks at Emory University and the University of Washington. Control and disease cases were matched for age (72.4±3.9 vs. 71.7±2.3 years, respectively) and sex (71% vs. 68% male, respectively). Four PD cases, three comorbid dementia with Lewy Bodies (DLB)/PD cases, seven Alzheimer disease (AD) cases, three multiple system atrophy (MSA, including one with comorbid DLB and one with comorbid olivopontocerebellar atrophy (OPC)), two progressive supranuclear palsy (PSP) cases, and seven age-matched controls were examined (Table 3-1). MSA and PSP were chosen for their clinical and pathological similarities to PD, and AD was chosen as a non-basal ganglia neurodegenerative disease.

We performed SV2C immunohistochemistry in substantia nigra and striatum (Fig. 3-8). We observed SV2C-positive staining in the SNpc and in the SNpr, as well as in terminal regions and cell bodies in the caudate nucleus and putamen. In PD, an unexpected and striking alteration in SV2C expression was revealed. We observed abnormal punctate SV2C-positive staining in the SNpc, and a similar pathology throughout the dorsal striatum, including the putamen (Fig. 3-8A). This disruption in SV2C expression was found in each PD case, though it was less severe in the three cases with neuropathology consistent with comorbid DLB. Striatal SV2C staining was largely unaltered in cases of AD, PSP, and MSA (Fig. 3-8B), suggesting a disruption in SV2C may be a unique feature of PD. Finally, expression of SV2A, α-synuclein and synaptophysin do not show a similar pattern of expression in PD (Fig. 3-8C-E). SV2C puntca are ubiquitin-positive (Fig. 3-8F).

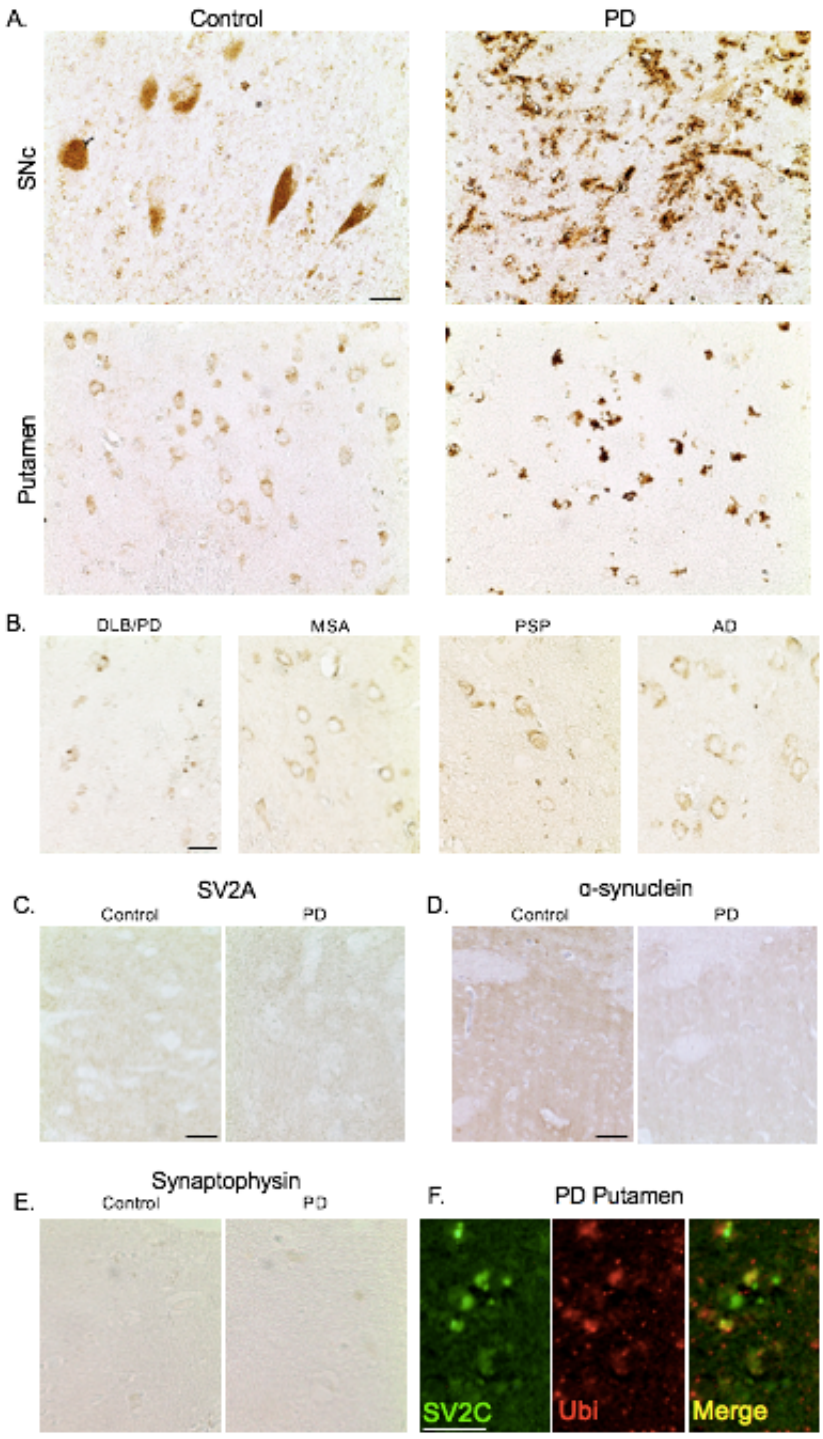

Figure 3-8. SV2C expression is specifically disrupted in PD basal ganglia. Representative micrographs demonstrate SV2C staining in human SNpc and putamen. (A) In PD, SV2C expression is significantly disrupted in the SNpc and putamen. SV2C-positive puncta are distributed throughout the SNpc and putamen in PD but not in age-matched controls. (B) Representative

micrographs indicate that SV2C staining is relatively normal in the putamen of representative DLB/PD, MSA, PSP and AD cases. (C) SV2A is not disrupted in PD putamen. (D) SV2C puncta do not reflect putamen α-synuclein disruption. (E) SV2C-puncta also do not represent a more general disruption of synaptic vesicle proteins, as synaptophysin expression is preserved in PD. *Scale bars = 20μm.*

Table 3-1.
Descriptions of human control and disease cases

Case	Neuropath. Diagnosis	Sex	Age (years)	Illness duration (years)	SV2C pathology†
			Controls		
1	--	M	72	--	None
2	--	F	80	--	None
3	--	M	78	--	None
4	--	F	88	--	None
5	--	M	61	--	None
6	--	M	69	--	None
7‡	--	M	59	--	++
			Parkinson disease		
8	PD	M	78	32	STR: +++
9	PD	M	64	12	STR: +++
10	PD	M	81	10	STR: ++/ SN: +++
11	PD	M	67	7	STR:++/ SN:++
12	DLB/PD	M	68	21	STR: ++
13	DLB/PD	F	62	4	STR: +
14	DLB/PD	M	72	11	STR: + / SN: +
			Alzheimer disease		
15	AD	M	63	Unk	None
16‡	AD	M	75	Unk	STR: ++
17	AD	F	81	Unk	None
18	AD	M	50	Unk	None
19	AD	F	87	Unk	None
20	AD	F	59	Unk	None
21	AD	M	79	Unk	None
			Multiple system atrophy / Progressive supranuclear palsy		
22	MSA	M	61	Unk	None
23	MSA/OPC	F	80	Unk	None
24	MSA/DLB	M	78	Unk	None
25	PSP	M	72	Unk	None
26	PSP	F	86	Unk	None

Table 3-1. Descriptions of human control and disease cases.

† SV2C disruption classification: "+" = SV2C-positive cell bodies plus sparse SV2C-positive puncta. "++" = Abundant SV2C-positive puncta with some SV2C-positive cell bodies. "+++" = SV2C immunoreactivity virtually entirely punctate.

‡ Cases 7 and 16 had moderate SV2C disruption, suggesting that SV2C expression disruption may not be exclusive to PD; though, comorbidity with subclinical PD may be a possibility in these cases.

STR=striatum; SN = substantia nigra

DISCUSSION

The present data reveal that SV2C mediates dopamine dynamics and is disrupted in PD. We demonstrate that SV2C and α-synuclein interact, which suggests an important involvement in disease pathogenesis. Further, we show that SV2C regulates dopamine release and dopamine content, as well as a neurochemical effect of nicotine, providing a molecular link to recent GWAS data identifying SV2C as a mediator of nicotine neuroprotection (40). Results from these experiments establish a novel role for SV2C and a basis for the potential functional consequence of a disruption of SV2C in PD and nicotine neuroprotection.

SV2C expression in models of PD.

As expected, striatal SV2C expression was slightly decreased following significant loss of dopaminergic terminals in mouse models of PD (Fig. 3-2). Unexpectedly, SV2C-positive puncta were observed in the striatum of A53T-OE animals. We did not observe similar punctate staining after MPTP lesion or in control animals: we modeled varying degrees of dopamine cell loss with multiple MPTP paradigms, including a moderate dose (4x15mg/kg) that closely matches the dopaminergic loss observed in A53T α-synuclein OE mice. This indicates that the observed disruption in SV2C is related to a distinct pathogenic mechanism that is not recapitulated by mere nigrostriatal dopaminergic degeneration. Unlike the A53T-OE model, MPTP administration in mice does not typically induce α-synuclein disruption (Shimoji 2005), which further indicates that a link between

α-synuclein and SV2C dysregulation may be particularly important. It is possible that SV2C pathology is not observed after MPTP intoxication due to the relatively acute nature of the lesion; however, we did not observe SV2C deposition in a genetic model, the VMAT2-LO mice (Caudle 2007), which suggests that even a life-long pathogenic mechanism leading to dopamine cell loss is not sufficient to spur similar SV2C disruption.

Genetic deletion of SV2C results in reduced dopamine and motor deficits.

To further explore any functional consequences of disrupted SV2C on the dopamine system in humans or animal models, we first needed to characterize involvement of the protein in basal ganglia functioning. SV2 proteins appear to promote vesicular function in various ways, by coordinating vesicular mobilization (Iezzi 2005; Xu 2001), by interacting with synaptotagmin-1 to facilitate calcium-stimulated exocytosis (Chang 2009; Janz 1999a; Lazzell 2004; Schivell 2005; Wan 2010; Yao 2010), or perhaps by stabilizing intravesicular transmitter (Reigada 2003; Vautrin 2009), though functional evidence has not supported this last hypothesis (Crowder 1999; Custer 2006; Janz 1999a). Though only SV2A has been strongly implicated in epilepsy, recent evidence has hinted at a potential link between SV2C, dopamine and PD (Dardou 2013c; Hill-Burns 2012; Altmann 2016). Our neurochemical data directly indicate that SV2C plays a particularly important role in the dopaminergic nigrostriatal pathway. Here, we demonstrate that genetic deletion of SV2C results in a significant reduction in total dopamine content in the dorsal striatum; this dampened dopamine tone does not appear to result from aberrant dopamine metabolism, as the ratio of dopamine to its metabolites is unaltered (Fig. 3-3). Pursuant to the observed reduction in total dopamine, genetic deletion of SV2C results in impaired

release of dopamine as measured by fast-scan cyclic voltammetry (Fig. 3-6). The mechanism underlying this observed reduction in dopamine content and release is unclear. It does not appear to be due to reduced dopamine synthesis or reuptake, as levels of TH (the rate-limiting enzyme of dopamine synthesis) and DAT (the protein which removes dopamine from the synaptic cleft and returns it to the presynaptic terminal) (Fig. 3-3). In fact, DAT function appears to be increased as measured by the uptake constant *tau*, perhaps as a mechanism to partially compensate for reduced vesicular dopamine release. As noted above, SV2 proteins likely have multiple functions within the synapse, and striatal SV2A and SV2B do not appear to increase in response to SV2C-KO (Fig. 3-3). Accordingly, an absence of SV2C could have significant effects on dopamine synapse function, either through a diminished readily releasable pool of vesicles, impaired calcium-induced endocytosis of vesicles, disrupted vesicular trafficking or perhaps impaired storage or retention of vesicular dopamine. Future studies will elucidate the mechanisms responsible for the observed reduction in dopamine tone and release following genetic deletion of SV2C.

Following the observation of reduced nigrostriatal dopamine, we accordingly demonstrate that SV2C-KO animals have deficits in dopamine-related motor behavior, including reduced stride length and reduced locomotor activity (Fig. 3-5). The reduction in locomotor activity observed in the SV2C-KO animals is primarily driven by apparent disruptions in circadian-mediated spikes of activity at the end of the wakeful period. This phenotype is consistent with other models of reduced dopamine vesicle function, such as the VMAT2-LO mice that exhibit motor impairment, reduced locomotor activity, altered circadian rhythm and smaller body size (Caudle 2007; Taylor 2009). Notably, SV2C-KO

animals are not impaired on the rotorod test, a test which our lab has previously identified as insensitive in detecting motor deficits after moderate dopamine loss (Tillerson 2002). Importantly, these deficits do not appear to be reflective of deficits in other dopaminergic proteins, or reduced synapse density. Indeed, the the only significant alteration in protein expression was α-synuclein (Fig. 3-4). Together, this behavioral and neurochemical data underscore that SV2C is involved in basal ganglia function and provides support for a potential disease relevance for SV2C in PD.

SV2C mediates a neurochemical effect of nicotine.

Nicotine application normally results in a "high-pass filter" effect in which dopamine release is reduced upon low-intensity stimulations but enhanced upon high-intensity stimulations in the presence of 500nM nicotine (Rice and Cragg 2004; Zhang 2004). Nicotine is thus thought to increase effects of salient inputs while dampening effects of transient dopamine release. Genetic deletion of SV2C ablates this effect of acute nicotine such that high-intensity stimulations elicit less dopamine than baseline release (Fig. 3-7). This may indicate a functional interaction between SV2C and nicotine. The precise relationship between SV2C and nicotine neuroprotection remains unknown, but these data indicate that SV2C-KO animals may have altered neurochemical, behavioral, or neuroprotective response to nicotine. These data may hold relevance for recent GWAS data identifying SV2C as a genetic mediator of neuroprotective effects of smoking (Hill-Burns 2012).

A link between SV2C expression and α-synuclein disruption.

Another link between SV2C and PD highlighted by our data is an association between SV2C and α-synuclein (Fig. 3-4). We demonstrate that α-synuclein co-immunoprecipitates with SV2C from striatal homogenates, indicating a physical and perhaps functional interaction. We were unable to detect SV2C in an α-synuclein pull-down, which may be due to the relative abundance of α-synuclein compared to SV2C, the difficulty in immunoprecipitating untagged native proteins from animal tissues, the inability of our α-synuclein antibody to immunoprecipitate α-synuclein oligomers, or the particular conditions of our IP conditions. Nonetheless, an interaction between SV2C and α-synuclein may be the basis for our observed mutual disruption of the two proteins in our mouse models. SV2C-KO animals have increased expression of high-molecular weight α-synuclein with a commensurate decreased in low-molecular weight α-synuclein. This is notable, as the ratio of monomeric to multimeric α-synuclein is altered in PD and is likely important in the induction of α-synuclein aggregation and toxicity (Brown 2010; Dettmer 2015; Roberts 2015b). Monomeric α-synuclein is thought to be the least toxic α-synuclein species, whereas aberrant soluble oligomers cause cellular toxicity by a variety of mechanisms (Choi 2013; Danzer 2007; Pieri 2012; Roberts 2015a; Waxman 2009). Our findings are supported by previous evidence indicating that SV2C and α-synuclein gene transcripts are normally highly correlated, but that this relationship is abolished in PD (Rhinn 2012). These data suggest an association between SV2C and α-synuclein, and that disruptions in α-synuclein or SV2C modify this interaction.

SV2C disruption in Parkinson disease basal ganglia.

Finally, we directly connect SV2C to human PD by demonstrating altered SV2C expression in PD basal ganglia (Fig. 3-8). In control human basal ganglia, SV2C is distributed throughout the basal ganglia in dopaminergic regions of the SNpc and striatum. This pattern of expression was largely unaltered in control cases and in other neurodegenerative diseases affecting the basal ganglia, such as MSA, PSP, and DLB, as well as in non-basal ganglia-related AD. However, SV2C expression alone was consistently altered in PD: SV2C staining was almost entirely punctate in the nigrostriatal tract, revealing intra- and extracellular inclusions of SV2C. These data expose a novel pathologic feature not previously identified in PD. Deposition of SV2C does not appear to be reflective of more general synaptic vesicle perturbation, as neither SV2A nor synaptophysin show a similar pattern of expression. SV2C inclusions also do not show a similar pattern of deposition as α-synuclein-positive Lewy bodies, as Lewy bodies are not typically found in the striatum of PD cases. Furthermore, SV2C puncta are also ubiquitin-positive, which may indicate a pathological accumulation of protein. It is important to note that this pathology appears to be chiefly restricted to PD. While basal ganglia pathologies are characteristic of PD, MSA, PSP and DLB, out of these, SV2C disruption was consistently evident in PD, and is less severe in PD with neuropathology consistent with comorbid DLB. Though MSA, PSP and DLB are all classified as Parkinson Plus Syndromes, they differ clinically, etiologically and histopathologically from PD and are not effectively treated by dopamine replacement therapeutics (reviewed in (Mitra 2003)). The lack of SV2C pathology in PSP, DLB and MSA further distinguish them from PD and suggests that SV2C disruption is related to the concurrent α-synuclein disruption and

dopaminergic degeneration characteristic of PD. It is unknown if similar SV2C disruption is present in forms of PD lacking Lewy body pathology, such as cases stemming from certain LRRK2 mutations (Gaig 2007). SV2C expression was largely unaffected in AD, further emphasizing that SV2C disruption likely is not merely related to a neurodegenerative disease state. As with other neurodegeneration-related protein disruptions, more research is required to fully characterize the alteration in SV2C expression in PD; however, these data directly connect SV2C protein to PD for the first time and strengthen previous data implicating SV2C as a genetic mediator of PD risk in certain populations.

A role for SV2C and PD pathogenesis

The cause and implication of SV2C deposition in the basal ganglia are unclear. As our mouse data suggest, SV2C is particularly important pre-synaptically in regulating dopamine release and in maintaining dopamine tone. Disruptions in SV2C may negatively affect dopaminergic vesicular function, as well as neuron integrity and neurotransmission, thereby contributing to human disease progression. As such, modulating the function of SV2C in order to enhance or restore its ability to preserve proper basal ganglia neurotransmission may be a promising avenue for therapeutics. Indeed, SV2C is a feasible target for pharmacotherapy: SV2C binds botulinum neurotoxin A (Mahrhold 2006; Dong 2006), and its close family member, SV2A, is the specific target for the antiepileptic drug levetiracetam (Lynch 2004). Though beyond the scope of this work, experiments in our laboratory are also underway to delineate the relative importance of SV2C in other neuronal populations. As SV2C is also localized to striatal GABAergic cells, manipulating

SV2C function may have functional and therapeutic implications for both dopamine and GABA neurotransmitter systems.

Finally, the severity and specificity of SV2C disruption in PD is striking. It suggests a novel pathologic feature unique to PD, which further distinguishes it from other basal ganglia neurodegenerative disorders. SV2C is localized to the basal ganglia, is associated with variable PD risk in smokers, promotes proper dopamine homeostasis and motor function, and is disrupted in PD. Taken together, these data establish a role for SV2C in nigrostriatal dopamine neurotransmission and identify it as a potential contributor to PD pathogenesis.

IV. Chapter 4. Genetic ablation of SV2C results in enhanced vulnerability to neurodegeneration

In final preparation for submission as a manuscript:

Amy R. Dunn, Kristen A. Stout, Michelle A. Johnson, Emily J. Winokur, Carlie A. Hoffman, Gary W. Miller. Genetic ablation of SV2C in mice results in enhanced vulnerability to MPTP intoxication. *In preparation*.

ABSTRACT

1-methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a highly selective dopaminergic toxicant. MPTP is converted to the toxic metabolite 1-methyl-4-phenylpyridinium (MPP+) in glia. MPP+ is then taken up by dopaminergic cells via the dopamine transporter (DAT), where it acts as a mitochondrial complex 1 inhibitor. In mice, moderate doses of MPTP cause damage to striatal dopamine terminals, while higher doses lead to a loss of dopamine cell bodies in the substantia nigra pars compacta (SNc) and motor impairment. MPP+ is also a substrate for the vesicular monoamine transporter 2 (VMAT2), which transports monoamines and related compounds from the cytosol into vesicles. Enhancing the ability of synaptic vesicles to store toxicants such as MPP+ confers resistance to dopaminergic toxicants. Impairing the ability of dopamine vesicles to store toxicants results in heightened vulnerability to cell damage. Identifying novel vesicular mediators of toxicant vulnerability is an important area of study, as enhancing vesicular function may be a promising strategy to prevent degeneration in neurological diseases such as Parkinson's disease (PD). The synaptic vesicle glycoprotein 2C (SV2C) has recently been shown to regulate dopamine handling and release, and genetic ablation of SV2C leads to impaired dopamine homeostasis and a variety of PD-relevant phenotypes. Here, we administered multiple doses of MPTP to WT mice and mice lacking SV2C (SV2C-KO). We found that SV2C-KO mice were significantly more vulnerable to MPTP intoxication, with enhanced loss of dopaminergic terminal markers in the striatum and exacerbated dopamine cell death in the SNc. Overall, these data identify SV2C as a novel mediator of dopamine cell vulnerability to degeneration.

INTRODUCTION

Dopaminergic cells are uniquely vulnerable to neurodegeneration. Byproducts of dopamine metabolism and dopamine auto-oxidization lead to an increase of intracellular oxidative species and free radicals, which in turn leads to disruption in proteins, lipids and nucleic acids, and eventual cell death (Ben-Shachar 1995; Goldstein 2013; Jenner 2003; Mosharov 2009; Sulzer 2000b). However, dopamine may be sequestered from the cytosol by synaptic vesicles, thereby reducing oxidative damage (Guillot 2009). This ability of synaptic vesicles to store and sequester dopamine is critical to dopamine cell health. Impaired vesicular function is a feature of Parkinson's disease (PD) (Pifl 2014), and genetic polymorphisms conferring enhanced or reduced vesicular function can either decrease or increase a person's risk for PD, respectively (Brighina 2013; Glatt 2006; Rilstone 2013).

In addition to their role in storing dopamine from the cytosol, dopaminergic vesicles also play an important role in mitigating the effects of exogenous toxicants such as 1-methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is metabolized into its toxic derivative, 1-methyl-4-phenylpyridinium (MPP+) in glia. MPP+ is selectively taken up into dopaminergic terminals via the dopamine transporter (DAT), where it functions as a mitochondrial complex I inhibitor (Przedborski 2000; Richardson et al. 2007). MPP+ may also be sequestered from the cytosol into synaptic vesicles, isolating the molecule from its site of action. Animals with impaired vesicular function have enhanced vulnerability to dopaminergic toxicants such as MPTP and methamphetamine (METH) (Alter 2013; Caudle 2007; Fon 1997; Gainetdinov 1998; Guillot 2008b; Lohr 2016; Mooslehner 2001; Taylor 2009, 2014). Enhancing vesicular function by overexpressing the vesicular

monoamine transporter 2 (VMAT2) is neuroprotective against both MPTP and METH (Guillot 2008a; Lohr 2014, 2015). Interestingly, VMAT2 was originally cloned for its function as a protein mediator of MPTP toxicity (Edwards 1993), highlighting the importance of the vesicle's role in neuronal vulnerability.

Identifying novel vesicular mediators of neuroprotection is important in the development of disease-modifying therapies. The synaptic vesicle glycoprotein 2C (SV2C) is a potential target for research and therapeutics. SV2C is in the SV2 family of proteins, which are 12-transmembrane proteins ubiquitously expressed on synaptic vesicles (Buckley 1985; Feany 1992; Gingrich 1992; Janz 1999b). SV2C is unique in its limited expression within the brain, with the highest levels of expression in the basal ganglia (Dardou D 2010; Dunn 2017; Janz 1999b). It has been posited that this pattern of expression hints at particular relevance of SV2C to disorders of the basal ganglia, such as PD. In fact, polymorphisms upstream of the SV2C gene mediate PD risk in smokers, as well as PD patient response to L-DOPA (Altmann 2016; Hill-Burns 2012). Further, SV2C expression has been found to be disrupted in postmortem PD basal ganglia. In mice, SV2C regulates dopamine release. Knocking out SV2C (SV2C-KO) results in a variety of PD-like features, such as impaired motor function, reduced dopamine, and disrupted alpha-synuclein (Dunn 2017). These data all point to potential involvement of SV2C in dopamine cell function and PD pathogenesis.

To test if SV2C mediates dopamine vesicle function and vulnerability to degeneration, we treated wildtype (WT) and SV2C-KO animals with 1-methy-4-phenyl-1,2,3,6-tetrahydropyridine using multiple dosing paradigms. We found that genetic ablation of SV2C results in enhanced neurodegeneration following MPTP in the striatum

and the substantia nigra by immunohistochemistry, immunoblotting and stereological cell counting. These results suggest that SV2C mediates the ability of the vesicle to sequester toxicants, and disrupted SV2C could lead to enhanced vulnerability to degeneration.

MATERIALS & METHODS

Animals:

Male mice (5-7 mo) were used for all MPTP experiments. Animals were kept on a 12/12 light/dark cycle and given food and water *ad libitum*. SV2C-KO mice were created as described previously (Dunn 2017). Briefly, animals were generated using the EUCOMM "knockout first allele" construct. These animals contained a *LacZ*/neomycin resistance cassette flanked by *FRT* sites inserted into the *Sv2c* gene. Animals were crossed with a line globally expressing Flp-recombinase to excise the cassette, resulting in a line of mice containing a floxed exon 2 of the *Sv2c* gene. These mice were then crossed with a line containing a nestin-driven Cre-recombinase in order to achieve a pan-neuronal knockout of SV2C.

***MPTP injections*:**

MPTP (Sigma or MedChemExpress) was administered according to a 5 x 20mg/kg dose paradigm. Mice were weighed prior to MPTP administration and injected (s.c.) with 20mg/kg MPTP or (freebase) or an equivalent volume of saline (control) once per day for five days, with an intra-injection interval of 24hr. The lesion was allowed to stabilize for 21 days following the final injection. Mice were sacrificed by rapid decapitation. Brains

were removed, dissected, and flash frozen (for immunoblot) or post fixed in 4% (w/v) paraformaldehyde (for immunohistochemistry).

***Stride length*:**

Gait analysis was performed as described previously (Dunn 2017; Tillerson 2002). Briefly, animals were trained to walk along a plain white pathway to their home cage. On test day, forepaws were dipped in water-soluble ink and animals were prompted to walk along the path. Stride length (toe-to-toe distance) was measured and averaged across three steps for each animal. Animals were tested one day prior to MPTP administration, and on day 20 following the final MPTP injection.

Immunoblotting:

Immunoblots were performed as described previously (Dunn, 2017). Briefly, unilateral striatal dissections were homogenized and underwent differential centrifugation to achieve a crude synaptosomal protein preparation. 20µg of protein was run through an SDS-PAGE gel and transferred to a PVDF membrane. Nonspecific antibody binding was blocked with a 7.5% nonfat dry milk solution, and the membrane was incubated in primary antibody overnight at 4C with gentle agitation. Membranes were then incubated in HRP-conjugated secondary antibody for 1 hour at room temperature. Protein was visualized using chemiluminescence (Thermo) and a BioRad UV imager. Protein was quantified using ImageLab software and normalized to an actin loading control.

***Immunohistochemistry*:**

Immunohistochemistry was performed as described previously (Dunn, 2017). Briefly, brains were sectioned to 40μm. Sections underwent antigen retrieval (70ºC citrate buffer (Biogenix) for 1 hour) and endogenous peroxidase was quenched with 10% hydrogen peroxide. Nonspecific antibody binding was blocked with 10% normal horse serum in PBS with 0.2% Triton X-100. Tissue was incubated in primary antibody overnight at 4ºC with gentle agitation. Sections were then incubated in biotinylated secondary antibody at room temperature for 1 hour. Signal was enhanced with avidin-biotin complex (Vector) and visualized with 3-3'-diaminobenzidine (DAB). The DAB reaction was stopped with PBS. Sections were mounted to slides and stained with cresyl violet (0.1% w/v aqueous, Poly Scientific). Sections were destained with 0.1% acetic acid in 95% ethanol, followed by dehydration in ethanol and lipid-clearing in xylenes.

***Unbiased stereological cell counting*:**

Stereological cell counting was performed as described previously using StereoInvestigator software (Lohr 2014, 2015). Every fourth SNpc section was counted using the optical fractionator method. Counting frames of 50um X 50um on a 120um X 120um counting grid were sampled. Cell counts were weighted based on manually measured section thickness. Only counts with a Gunderson coefficient of error (m=0) of <0.10 were included for analysis.

***Antibodies*:**

Rabbit anti-TH and mouse anti-DAT antibodies were purchased from Millipore and used at a dilution of 1:1,000 on immunoblotting and immunohistochemistry. Polyclonal SV2C

antiserum was generated for our laboratory by Covance custom antibody services as described previously and used at a dilution of 1:2,500 (Dunn, 2017). Briefly, immunizing peptides corresponding to amino acids 97-114 of SV2C (mouse SV2C: STNQGKDSIVSVGQPKG; human SV2C: SMNQAKDSIVSVGQPKG) were mcKMH-conjugated and injected into rabbits. Mouse anti-actin was purchased from Sigma and used at a dilution of 1:5,000.

RESULTS

Enhanced neurotoxicity in SV2C-KO mice after MPTP.

We administered two different MPTP doses to separate cohorts of mice. First, we administered a 5x20mg/kg dose of MPTP (Sigma) consisting of five injections (s.c.) over five days with an inter-injection interval of 24 hours (Experiment #1). We observed a greater than 90% loss of striatal DAT immunoreactivity in both WT and SV2C-KO animals following MPTP intoxication (WT: 92.4% loss ± 0.012; KO: 91.4% loss ± 0.040; $P = 0.84$) (Fig. 4-1). We observed a significantly greater loss of TH expression in SV2C-KO animals compared to WT controls (WT: 19.5% loss ± 0.05; KO: 39.0% loss ± 0.07; $P = 0.029$) (Fig. 4-1).

Next, we administered a 5x20mg/kg dose of MPTP obtained from an alternative source (MedChemExpress; Experiment #2). Following this intoxication, we again observed a significantly increased dopaminergic lesion in SV2C-KO mice. There was a significantly greater loss of both striatal DAT (WT: 59.11% loss ± 0.03; KO: 79.62% loss ± 0.08; $P = 0.047$) and striatal TH (WT: 43.9% loss ± 0.035; KO: 71.64% loss ± 0.048; *P*

= 0.003) in SV2C-KO animals compared to WT littermates (Fig. 4-2). Striatal and midbrain TH staining were reduced after MPTP in both WT and SV2C-KO animals, though SV2C-KO animals had visibly greater depletion of TH staining in the striatum and midbrain (Fig. 4-3).

To determine if there was also an exacerbation of dopaminergic cell body loss following MPTP, we performed stereological cell counting. Baseline dopaminergic cell count was equivalent between SV2C-KO and WT animals (WT: 5953 cells ± 980; KO: 5212 cells ± 342, $P = 0.52$). Significant cell body loss was not observed in WT animals following MedChemExpress MPTP administration in this case, though SV2C-KO animals had a 59% loss of TH+ cells in the SNc (Fig. 4-4).

Stride length analysis:

In Experiment #2, we also evaluated the effect of MPTP on motor function using a gait analysis assay. We did not observe a significant impairment of motor function by ANOVA in any genotype or treatment combination (Fig. 4-5).

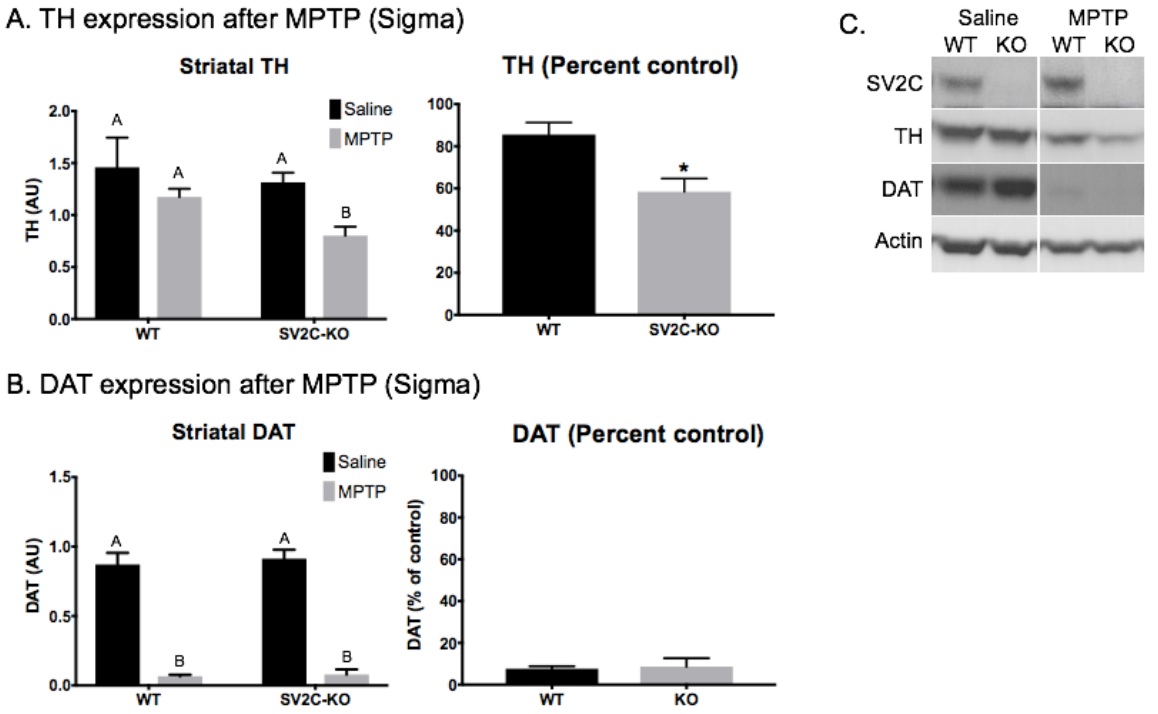

Figure 4-1. Dopamine terminals lesion in SV2C-KO animals following MPTP (Sigma, Experiment #1). (A) TH expression as measured by semi-quantitative immunoblot. At baseline, SV2C-KO and WT animals have comparable levels of dopaminergic marker TH. Following intoxication with MPTP (Sigma), TH is reduced in both WT and SV2C-KO animals, though TH loss is significantly exacerbated in SV2C-KO as compared to WT animals. (B) DAT expression is ablated in both WT and SV2C-KO animals following this dose of MPTP. (C) Representative immunoblots of SV2C, TH, and DAT in saline- and MPTP-treated striata. *N = 3-4*

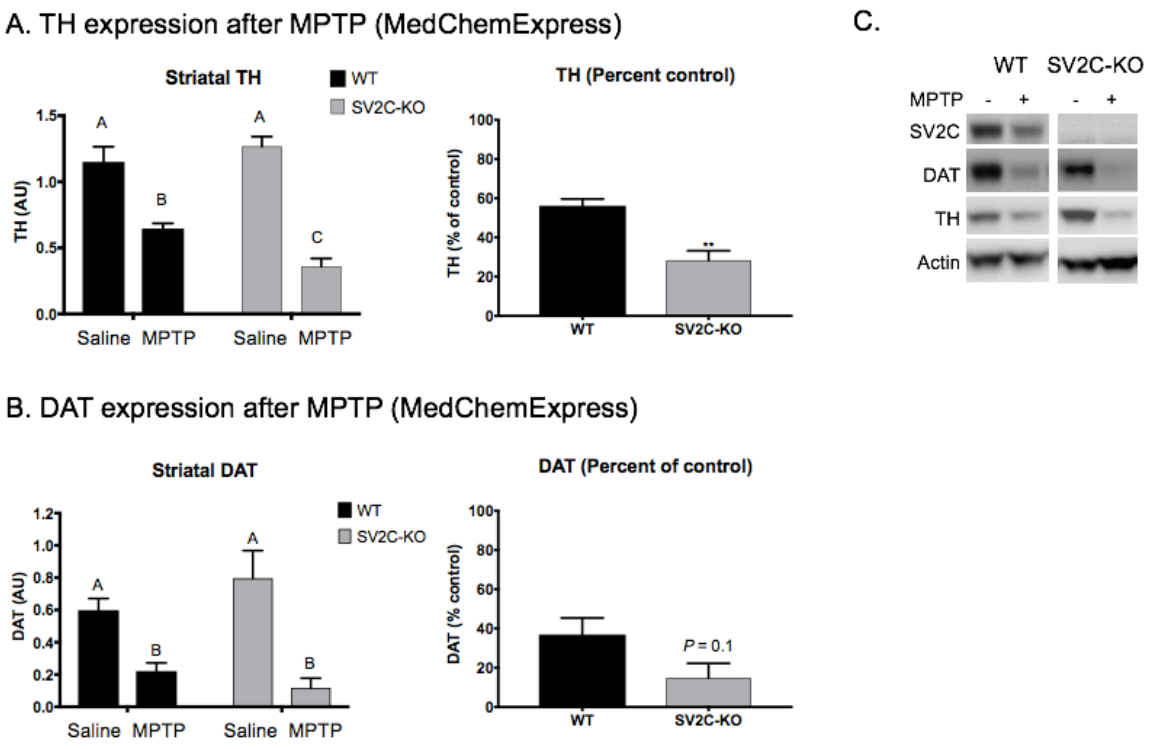

Figure 4-2. Dopamine terminals lesion in SV2C-KO animals following MPTP (MedChemExpress, Experiment #2). (A) Quantification of TH immunoreactivity in western blots of striatal homogenates reveals a significantly increased loss of striatal TH in SV2C-KO animals as compared to WT animals following MPTP intoxication. (B) Striatal DAT loss trends toward being exacerbated in SV2C-KO animals following MPTP as compared to WT controls, though this does not reach statistical significance. (C) These effects are visualized with representative immunoblots. *N=3-5; Bars with the same letters are not statistically different. Bars with different letters are statistically different by at least $P < 0.05$.*

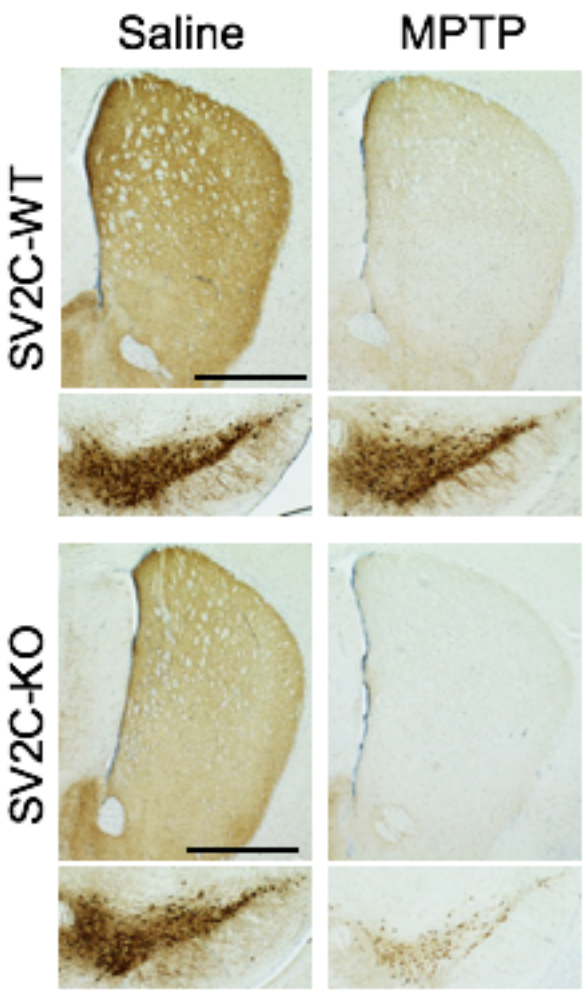

Figure 4-3. Enhanced loss of TH staining following MPTP in SV2C-KO animals. Representative TH immunohistochemistry of the striatum and midbrain of SV2C-KO and WT animals treated with saline or MPTP (MedChemExpress) demonstrates that the lesion to the basal ganglia is enhanced in both the dorsal striatum and SNc of SV2C-KO animals as compared to controls. *Scale bar = 1mm*

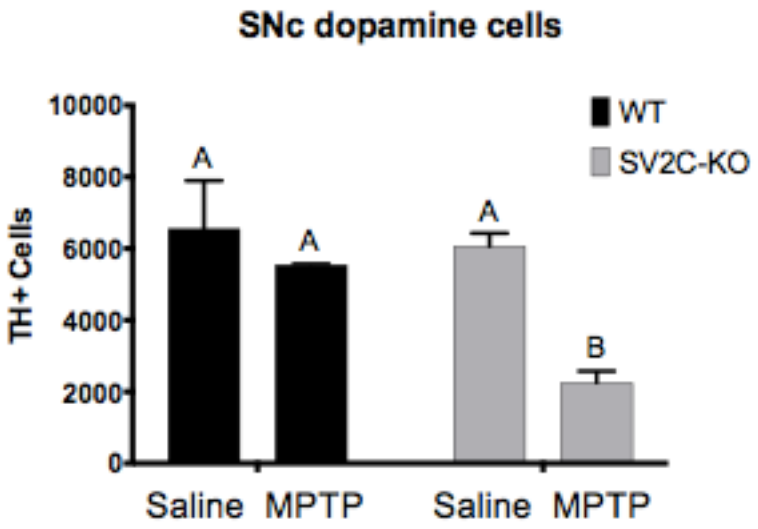

Figure 4-4. MPTP induces cell loss in SV2C-KO, but not WT, SNc following a mild dose of MPTP. Quantification of intact dopaminergic cells of the SNc using unbiased stereological cell counting confirms a significant loss of dopaminergic nigral cells in SV2C-KO, but not WT animals following MPTP. Bars with the same letter are not statistically different. *Bars with different letters are statistically different at a significance level of at least* α *= 0.05. N = 2-5*

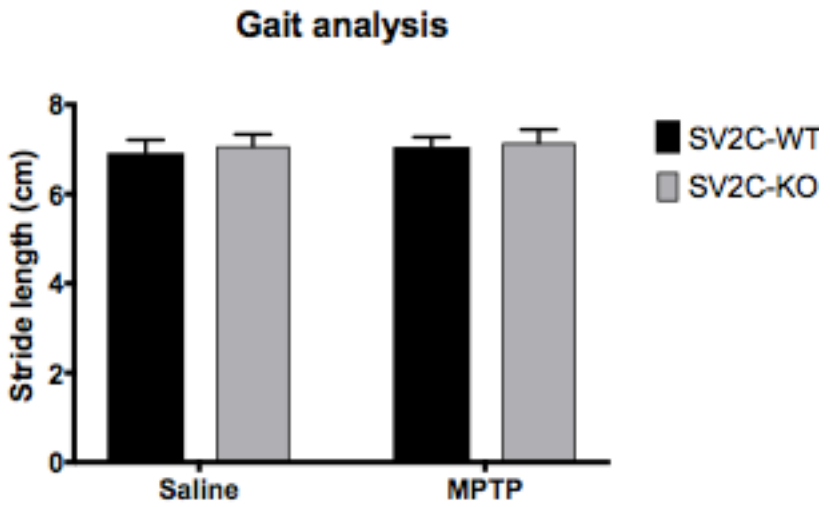

Figure 4-5. Gait analysis following MPTP administration. The mild MPTP lesion did not induce motor impairment in SV2C-WT animals or SV2C-KO animals, as evidenced by no alteration in stride length three weeks following MPTP administration. *N = 3-5*

DISCUSSION

It has been well-established that MPTP toxicity can be regulated by dopaminergic synaptic vesicles (Alter 2013; Bernstein 2014; Caudle 2007; Gainetdinov 1998; Guillot 2009; Przedborski 2000; Taylor, Caudle, and Miller 2011). When dopamine vesicles have a greater capacity to store dopamine and sequester toxicants from the cytosol, neurons are resistant to degeneration, and when this process is impaired, neurons are vulnerable to enhanced cellular damage and death. This has been demonstrated repeatedly by our laboratory in models of varying VMAT2 expression. In fact, VMAT2 was originally identified for its role in sequestering the active metabolite of MPTP, MPP+, and protecting against MPTP toxicity (Edwards 1993). Here, we demonstrate that another vesicular protein, SV2C, is able to regulate the toxic effects of MPTP.

SV2C regulates vulnerability to MPTP intoxication.

The present data reveal that SV2C-KO mice are more vulnerable to degeneration in multiple paradigms of MPTP intoxication and outcome measures. In Experiment #1, we administered MPTP (Sigma) to achieve a dramatic ablation of striatal dopamine terminals (Fig. 4-1). Because DAT immunoreactivity was almost entirely diminished in both WT and SV2C-KO animals, we were unable to determine if dopaminergic terminals were differentially vulnerable to damage in either genotype using DAT expression quantification (Fig. 4-1, bottom panels). Instead, we evaluated striatal TH expression, which is typically more preserved following MPTP (see Lohr 2016). In fact, we observed only about a 20% reduction in TH expression in WT animals, compared to a 40% reduction in SV2C-KO

animals (Fig. 4-1). With MPTP from MedChemExpress in Experiment #2, we achieved a less severe dopaminergic lesion overall, as evidenced by a relative preservation of DAT expression in the striatum and no detectable loss of dopaminergic cells in the SNc in WT animals (Fig. 4-2). Specifically, we observed a reduction of about 60% of striatal DAT expression in WT animals, compared to an approximate 85% loss of DAT in SV2C-KO animals. Interestingly, even though this dose of MPTP is not sufficient to induce cell loss in WT animals, we see about a 60% cell loss in SV2C-KO animals (Fig. 4-4). This administration paradigm of MPTP is relatively mild in WT animals, but induces a damage to the dopamine system comparable to a much more significant dose in SV2C-KO animals. It is important to note that the same protocol was used to administer the same dose of freebase MPTP in both Experiment #1 and in Experiment #2, with the difference being in the evident potency of the MPTP between the two suppliers (Sigma vs. MedChemExpress), which resulted in severe and mild lesions, respectively. The exact mechanism for this observed discrepancy in potency is unknown.

SV2C and vesicular function.

Several lines of evidence suggest that SV2 proteins may be capable of facilitating the storage of intravesicular molecules, including neurotransmitters. The presence of an intraluminal matrix made up of proteoglycans can be visualized by electron microscopy. Various vesicular proteins are glycosylated, but it is thought that the bulk of the intravesicular matrix is composed of the heavily glycosylated intraluminal loop on SV2 proteins. This proteoglycan matrix is capable of directly adsorbing ATP as visualized by atomic force microscopy, and is hypothesized to regulate the release of transmitter

molecules into the synaptic cleft upon endocytosis (de Toledo 1993; Harlow 2013; Reigada 2003; Vautrin 2009). Electrophysiological data have not supported this hypothesis, in that neurons from animals lacking SV2A and SV2B do not have altered quantal size (Crowder 1999; Custer 2006; Janz 1999a). However, the functions of each of the SV2 family members do not appear to be redundant and they may have distinct specializations that are not yet apparent. For example, SV2C is the most highly glycosylated of the SV2 proteins, with five predicted glycosylation sites rather than the three glycan groups found on SV2A and SV2B. This may indicate a more important role for SV2C in contributing to the intraluminal matrix. Additionally, this may reflect distinct vesicular dynamics for various neurotransmitters. For example, intravesicular concentration of GABA (>200mM), acetylcholine (~260mM) and monoamines (~270mM) are generally higher than intravesicular concentrations of glutamate (60-120mM) (Bruns 2000; Hammond 2015; Scimemi 2009; Sulzer 2000a; Webster 2001). Together with our data and others indicating that SV2s are differentially expressed in certain transmitter systems, it is possible that the structure differences between SV2C and SV2A and SV2B may reflect slightly different functions and specializations for particular neurotransmitters.

In addition to a potential role in facilitating uptake of dopamine or MPP+, the proteoglycan matrix could be promoting the storage of these molecules and preventing leakage from the vesicle. Leakage of molecules (dopamine or, in this case, MPP+) from the vesicle to the cytosol has been hypothesized as a contributor to dopamine cell vulnerability to degeneration, and it is unclear which vesicular components may contribute to vesicular leak (Alter 2013; Eisenhofer 2004). It is possible that the SV2C functions in

part to facilitate storage and/or prevent leakage of transmitter and other molecules being sequestered from the cytosol of dopaminergic neurons.

SV2C and neurodegeneration

Here, we present data that showing that SV2C is protective against chemically-induced neurodegeneration. There are several possibilities as to how SV2C may contribute to neuronal resilience. First, these data may indicate that SV2C directly mediates the ability of the vesicle to store and sequester MPP+ from the cytosol. If dopaminergic vesicles lacking SV2C are functioning at a lower capacity, impairment of vesicular function as a result of SV2C-KO may result in higher cytosolic concentrations of MPP+, where the molecule is free to act as a mitochondrial complex 1 inhibitor. Alternatively, genetic deletion of SV2C may contribute to other mechanisms which would indirectly result in more cellular vulnerability to exogenous toxic insults. For example, SV2C-KO mice have disrupted alpha-synuclein expression. Alpha-synuclein disruption, and increased alpha-synuclein oligomers in particular, is independently capable of enhancing vulnerability to degeneration through various mechanisms (Choi 2013; Danzer 2007; Pieri 2012; Roberts 2015a; Waxman 2009). Future experiments will clarify the molecular mechanism behind the protective nature of SV2C in dopaminergic degeneration. To more fully understand the neuroprotective functions of SV2C, it will be important to evaluate the ability of overexpression of SV2C to protect against MPTP- or alpha-synuclein mediated degeneration.

SV2C and Parkinson's disease

We have previously reported a significant disruption of SV2C in the basal ganglia of some cases of PD (Dunn, 2017). The functional implications of such a disruption are unclear without fully understanding the role of SV2C within the nigrostriatal dopamine system, although it is likely that such a disruption results in impaired protein function. Considering SV2C-KO animals as a potential model of "disrupted" SV2C, we may conclude that the present data also suggest that the disruption in SV2C observed in PD may contribute to the pathogenesis of the disease. If the disruption to SV2C expression in human basal ganglia results in a dysfunctional protein, it is possible that this may heighten vulnerability to degeneration as seen in mice. Thus, targeting SV2C may be a promising strategy to promote vesicular function and prevent disease progression.

Overall, these data further support the emergence of SV2C as a relevant player in dopamine neuron and vesicle function. We establish SV2C as a mediator of MPTP toxicity, and suggest that SV2C function and expression is inversely correlated with vulnerability to degeneration. These data point to a role for SV2C in mediating chemically-induced dopaminergic degeneration, and future studies will investigate whether this neuroprotective function of SV2C may extend to additional models of cell death. Additionally, these data may suggest that enhanced SV2C could be neuroprotective, which could have significant implications for the development of PD therapeutics.

V. Chapter 5. The effect of SV2C-KO in non-dopaminergic transmitter systems

ABSTRACT

In the previous four chapters, I have focused on the role of SV2C in the dopaminergic nigrostriatal pathway. However, SV2C is present in a variety of cell types within the basal ganglia and may also play an important role in other transmitter systems. Specifically, the disruption to SV2C in the GABAergic medium spiny neurons (MSNs) in human PD and the strong expression of SV2C in the GABAergic globus pallidus, ventral pallidum, and substantia nigra reticulata, as well as the possible functional interaction between SV2C and the cholinergic system suggest that SV2C may be functioning throughout the basal ganglia to promote proper vesicular function and neurotransmission. Some of the dopamine-centric data presented previously may be translatable to other transmitter systems, and understanding the role of SV2C in these cells will be particularly important in our interpretation of future experiments. Here, I present data evaluating the role of SV2C in GABA and cholinergic cells. In particular, I show unperturbed GABAergic and cholinergic protein expression, similar effects of GABA receptor agonists and antagonists on dopaminergic tone in SV2C WT and KO animals, preserved high-pass filtering effects of specific a nicotinic agonist on dopamine release in SV2C WT and KO animals, similar consumption of nicotine between SV2C WT and KO animals, and preserved GABA vesicle capacity for GABA storage. Taken as a whole, these data do not reveal significant disruptions in basal ganglia GABAergic or cholinergic systems following genetic ablation of SV2C, though these investigations are only scratching the surface of potential alterations. Future experiments may identify disturbances to non-dopaminergic transmitter

systems, though it may be possible that SV2C is uniquely important in dopamine neurons and SV2A and SV2B may compensate for potential deficits in other cell types.

INTRODUCTION

Synaptic vesicles are central to the rapid neurotransmission of chemical messengers within the nervous system. The basic process of vesicle-mediated neurotransmission is similar across transmitter systems, though the molecular mechanisms underlying each step of the process have not been fully described. The synaptic vesicle glycoprotein 2 (SV2) family of proteins contribute to synaptic transmission throughout the nervous system, although their precise molecular function has not been identified (Bajjalieh 1994; Feany 1992; Gingrich 1992; Janz 1999b; Mendoza-Torreblanca et al. 2013). SV2A is ubiquitously expressed in all neurons, though the restricted distribution of SV2C suggests a particular specialization of SV2C's function to the basal ganglia (Dardou D 2010; Janz 1999b). Heretofore in this dissertation, we have focused on the role of SV2C in nigrostriatal dopamine transmission and function. Indeed, SV2C is strongly expressed in dopaminergic neurons and does appear to be important in this pathway, with SV2C-KO animals showing disrupted dopamine homeostasis, impaired motor function, and enhanced vulnerability to nigrostriatal degeneration (Dardou 2013a; Dunn 2017). In addition to disrupting nigrostriatal dopamine, genetic ablation of SV2C also disrupts dopamine in the mesolimbic dopamine pathway and alters reward behavior (unpublished observations).

However, we have also observed strong SV2C expression in non-dopaminergic regions of the basal ganglia. For example, SV2C is highly expressed in GABAergic regions of the dorsal and ventral striatum (globus pallidus, GP; and ventral pallidum, VP), as well as the highly GABAergic substantia nigra pars reticulata (SNr). We and others have identified extensive colocalization between SV2C and markers of GABA such as the

vesicular GABA transporter (vGAT) (Dardou 2013a; Dunn 2017). SV2C is more abundant on GABAergic vesicles than on glutamatergic vesicles (Gronborg et al. 2010). Further, we have identified a significant disruption in SV2C in these GABAergic regions of the basal ganglia in human PD.

Additionally, SV2C colocalizes with markers of cholinergic cells within the basal ganglia such as the vesicular acetylcholine transporter (vAchT) (Dardou D 2010). The possible functional interaction between SV2C and acetylcholine has been highlighted by human data characterizing SV2C as a genetic mediator of PD risk in smokers (Hill-Burns 2012), as well as in previous mouse data from our lab indicating that SV2C-KO animals have altered neurochemical response to nicotine (Dunn 2017), a potent cholinergic agonist. Though SV2C is only present in a minority of cholinergic striatal interneruons, it is possible that SV2C plays a significant impact on acetylcholine function in the basal ganglia.

Striatal neurotransmission is complex. Several transmitter systems converge to modulate inhibitory and excitatory tone on medium spiny neurons (MSNs), the major striatal output neuron type. Dopaminergic inputs from the SNc into the dorsal striatum may have inhibitory or excitatory effects, depending on the particular dopamine receptor subtype housed on the target MSN. Dopaminergic tone in the striatum is modulated cholinergic and GABAergic inputs to the substantia nigra. Striatal output is additionally modulated by cholinergic interneurons and glutamatergic inputs, Acetylcholine and dopamine have a balanced relationship, where acetylcholine firing is dampened in response to high dopamine tone (reviewed in (Bonsi 2011)). Cholinergic agonists such as nicotine dramatically reduce dopaminergic release in the striatum as a result of nicotinic receptor desensitization, and may act as a “high pass filter”, allowing for enhanced dopamine

transmission only upon a salient, high-intensity stimulation (Koranda 2013; Rice and Cragg 2004).

Here, we explore the effect of genetic ablation of SV2C on basal ganglia cholinergic and GABAergic pathways. First, I explore whether varenicline, a nicotinic receptor antagonist, is able to produce a high-pass filter effect on dopamine release in SV2C-KO animals. We also explored expression of acetylcholine pathway markers in SV2C-KO animals. Next, we evaluated potential alterations to basal ganglia GABA function by altering GABA tone pharmacologically and observing its effect on dopamine release. Given the hypothesized role of SV2s in enhancing vesicular storage of transmitter, we also evaluated whether SV2C-KO animals display a reduced capacity for striatal GABA vesicles to store transmitter. Finally, we measured norepinephrine levels with HPLC in SV2C-KO versus WT animals. I did not observe any differences between SV2C-KO and WT animals within their cholinergic and GABAergic basal ganglia pathways, though future experiments may identify possible other postsynaptic or presynaptic effects not identified here.

MATERIALS & METHODS

Animals:

Male WT and SV2C-KO mice (4-6 mo) were used for all experiments. Animals were kept on a 12/12 light/dark cycle and given food and water *ad libitum* and used in accordance with NIH guidelines and Emory IACUC-approved protocols. SV2C-KO mice were generated as described previously (Dunn 2017).

***Immunoblotting*:**

Immunoblots were performed as described previously (Dunn, 2017). Briefly, unilateral striatal dissections were homogenized and underwent differential centrifugation to achieve a crude synaptosomal protein preparation. 20ug of protein was run through an SDS-PAGE gel and transferred to a PVDF membrane. Nonspecific antibody binding was blocked with a 7.5% nonfat dry milk solution, and the membrane was incubated in primary antibody overnight at 4ºC with gentle agitation. Membranes were then incubated in HRP-conjugated secondary antibody for 1 hour at room temperature. Protein was visualized using chemiluminescence (Thermo) and a BioRad UV imager. Protein was quantified using ImageLab software and normalized to an actin loading control.

***Immunohistochemistry*:**

Immunohistochemistry was performed as described previously (Dunn, 2017). Briefly, brains were sectioned to 40um. Sections underwent antigen retrieval (70ºC citrate buffer (Biogenix) for 1 hour) and endogenous peroxidase was quenched with 10% hydrogen peroxide. Non-specific antibody binding was blocked with 10% normal horse serum in PBS with 0.2% Triton X-100. Tissue was incubated in primary antibody overnight at 4ºC with gentle agitation. Sections were then incubated in biotinylated or fluorescent secondary antibody at room temperature for 1 hour. Fluroescently tagged sections were mounted to slides and coverslipped with hard-set mounting medium (Vector). For biotinylated antibodies, signal was enhanced with avidin-biotin complex (Vector) and visualized with

3-3'-diaminobenzidine (DAB). The DAB reaction was stopped with PBS. Sections were mounted to slides, dehydrated in ethanol, and cleared with xylenes.

***Antibodies*:**

Rabbit anti-vGAT antibody was purchased from Synaptic Systems and used at a dilution of 1:1,000 on immunoblotting and immunohistochemistry. Polyclonal SV2C antiserum was generated as described previously and used at a dilution of 1:2,500. Mouse anti-actin was purchased from Sigma and used at a dilution of 1:5,000. Mouse anti-vAchT was purchased though Novus Biologicals and used at a dilution of 1:1,000. Mouse anti-ChAT antibody was purchased through Chemicon and used at a dilution of 1:1,000.

***Fast scan cyclic voltammetry*:**

Fast-scan cyclic voltammetry was performed as described previously (Lohr 2014, 2016; Stout 2016) in 300μm striatal slices bathed in 30°C oxygenated aCSF. Stimulations consisted of a 700μA 4ms monopolar pulse, either single pulse (baseline release) or five at 100Hz (high-intensity stimulation). Four to five recordings (single pulse) were taken at each of four dorsolateral striatal sites with a 5-minute inter-stimulation rest period followed by one 5-pulse, 100Hz stimulation recording. Electrode sensitivity was calibrated to known dopamine standards using a flow-cell injection system. Maximum dopamine release was averaged across sites and kinetic constants were calculated using nonlinear regression analysis of dopamine release and uptake.

<u>Drug application:</u>

aCSF bath was replaced with either bicuculline (Sigma, 10uM), muscimol (Sigma, 20uM) or varenicline (100uM). Concentrations were determined based on previous literature (Yan 1999, Aragona 2008) and a preliminary dose-response test prior to the experiment. Slices were bathed in each drug for 10 minutes prior to stimulation.

[^{3}H]-GABA vesicular uptake:

[^{3}H]-GABA uptake was performed similarly as previously described for [^{3}H]-dopamine uptake (Lohr, 2014). The cytoplasmic vesicular fraction was prepared from homogenized bilateral striata from WT and SV2C-KO mice via differential centrifugation. Vesicles were incubated with GABA (10 µM GABA with 0.5% ^{3}H-GABA tracer) for five minutes at 30˚C and harvested with a vacuum manifold (Brandel) on GF/F filter paper (Whatman). Specific GABA uptake was determined by subtracting counts from reactions lacking ATP. Reactions were normalized to total protein (Pierce).

Coimmunoprecipitations:

Coimmunoprecipitation experiments were performed using the Pierce Co-Immunoprecipitation Kit (Thermo) according to manufacturer's protocols. mSV2CpAb was cross-linked to agarose beads. WT animals were sacrificed by decapitation and a bilateral striatal dissection was performed. Striata were homogenized. A crude synaptosomal preparation was achieved with differential centrifugation. Samples were treated with IP Lysis/Wash buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol) incubated with antibody-bound resin overnight at 4˚C and protein complexes were eluted with low-pH elution buffer.

High-performance liquid chromatography:

HPLC for striatal norepinephrine content was performed as described previously (Lohr 2014; Dunn 2017). Briefly, unilateral striatal dissections were sonicated in 10X their weight of 0.1M perchloric acid and pelleted at 10,000 X G for 10 minutes. Supernatants were filtered at 0.2μm. Norepinephrine was detected using an MD-150 × 3.2 mm C18 column. The mobile phase consisted of 1.5 mM 1-octanesulfonic acid sodium, 75mM NaH_2PO_4, 0.025% triethylamine, and 8% acetonitrile at pH 2.9. A 20 μl of sample was injected.

Statistics:

All data were analyzed using GraphPad Prism. Differences between genotype were evaluated using t-tests. Significance was set to $P < 0.05$. Error bars indicate SEM.

RESULTS

Unaltered GABA markers in SV2C-KO animals

We performed immunoblotting to evaluate the relative expression of striatal VGAT in SV2C-KO and WT animals. We found no difference in expression level of VGAT in SV2C-KO animals compared to their WT littermates (WT: 464±66.1 AU, KO: 472±57.8 AU, $P = 0.93$, $N = 10$-12). Similarly, we did not see differences in pattern of expression of VGAT within the striatum (Fig. 5-1).

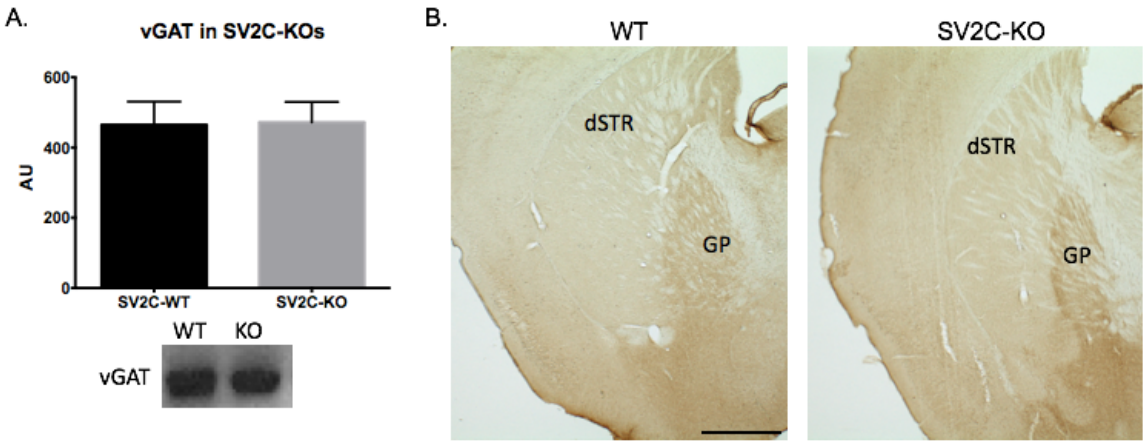

Figure 5-1. VGAT expression in SV2C-KO animals. (A) Levels of VGAT expression are unchanged in SV2C-KO animals (P = 0.93, N = 10-12) as analyzed by immunoblot from homogenized gross striatal dissections. (B) expression patterns of striatal VGAT are not altered in SV2C-KO animals compared to WT. Error bars indicate SEM. *Scale bar = 1mm*

The effects of altered GABA tone on striatal dopamine release

GABA-A agonists such as muscimol typically dampen striatal dopamine release, as they increase inhibitory tone on striatal circuitry when administered during *in vivo* FSCV (Aragona 2008). Conversely, GABA-A antagonists such as bicuculline reduce inhibitory tone and have the effect of enhancing extrasynaptic striatal dopamine when directly delivered to the nucleus accumbens during microdialysis (Yan 1999). By pharmacologically manipulating the effect of GABA on presynaptic dopamine release, we may be able to interrogate the relative contribution of GABA on dopamine output in SV2C-KO versus WT animals. As expected, SV2C-KO animals had reduced baseline striatal dopamine release, though this difference did not reach statistical significance due to the low *N* (WT: 7.67±2.5uM DA, KO: 2.45±1.4uM DA; N = 2-3, P = 0.2, Fig. 5-2A). Bicuculline enhanced dopamine release in both WT and SV2C-KO animals (WT: 149% of baseline, N = 3, P = 0.09 by paired t-test; KO: 151% of baseline, N = 2, P = 0.2 by paired t-test). Muscimol did not affect striatal dopamine release (WT: 115% of baseline, N = 3, P = 0.5, KO: 102% of baseline, N = 2, P = 0.8). When normalized to their own baseline release levels, there is no difference in the effect of bicuculline or muscimol in SV2C-KO animals versus WT (effect of genotype by two-way ANOVA, P = 0.75; Fig. 5-2B). These data are preliminary, though no trends toward a difference between genotype was revealed and a full-scale experiment was not pursued.

[^{3}H]-GABA vesicular uptake

To evaluate the hypothesis that SV2C facilitates transmitter uptake, we performed a radioactive GABA uptake assay in isolated striatal vesicles. We found a similar level of vesicular capacity for GABA in SV2C-KO and WT vesicles (WT: 337.7±102 counts, KO: 402.6±117.9 counts, N=2-3, P=0.72, Fig. 5-3A). This suggests that SV2C either does not facilitate vesicular uptake of GABA, or that there is functional compensation by SV2A and SV2B in response to ablated SV2C.

SV2C and VGAT coimmunoprecipitation

To determine if SV2C is present on GABAergic vesicles, we immunoprecipitated SV2C from homogenized striatal dissections and performed immunoblots to identify SV2C binding partners. Interestingly, despite extensive evidence that SV2C regulates dopamine function, VMAT2 did not coimmunoprecipitate with SV2C. VGAT did coimmuno-precipitate with SV2C, suggesting that SV2C may be relevant to GABA neurotransmission despite negative results from other experiments (Fig. 5-3B).

***Cholinergic marker expression in SV2C-KO animals*:**

To evaluate potential alterations to the cholinergic pathways following genetic ablation of SV2C, we performed immunohistochemistry and semi-quantitative immunoblot on homogenized striata from WT and SV2C-KO animals. vAchT expression appeared to be upregulated in some animals, although when quantified over all animals, this trend did not reach statistical significance (Fig. 5-4B). We also performed immunohistochemistry to evaluate any potential alterations in expression pattern of choline acetyltransferase (ChAT) in SV2C-KO animals, and did not observe differences between WT and SV2C-KO.

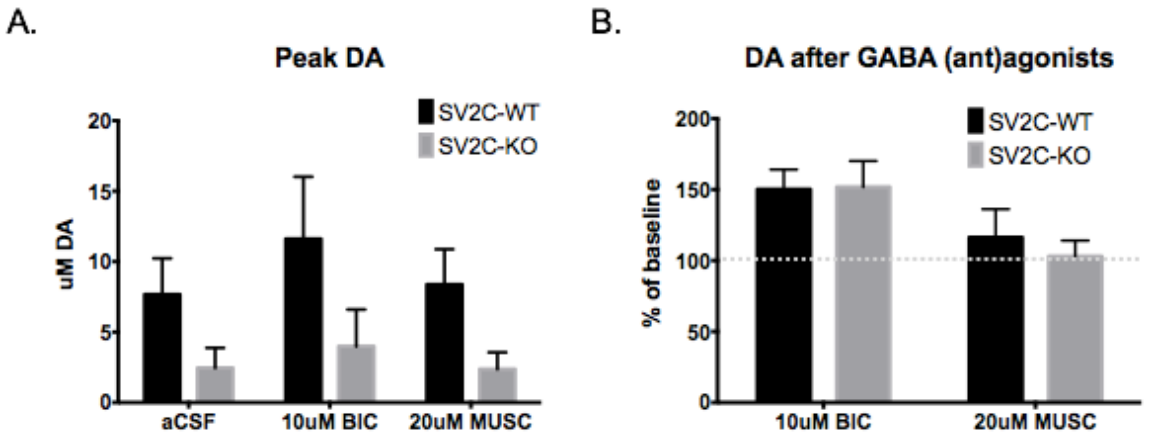

Figure 5-2. Influence of GABA tone on dopamine release in the dorsal striatum of SV2C-KO animals. Ablating GABA tone with 10uM bicuculline (BIC) results in enhanced dopamine release in both WT and SV2C-KO animals. 20uM muscimonl (MUSC) does not affect striatal dopamine release in WT or SV2C-KO animals. N=2-3.

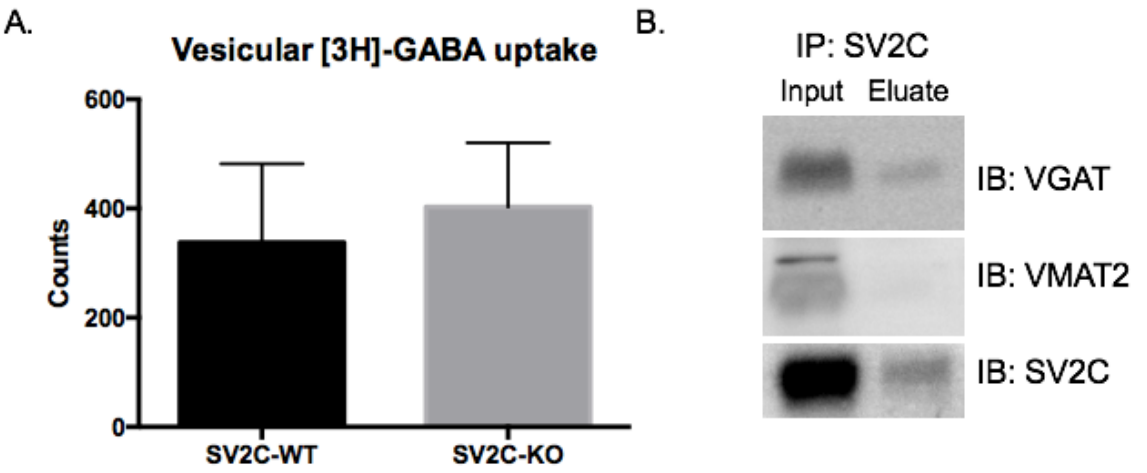

Figure 5-3. Vesicular GABA uptake in SV2C-KO animals. (A) GABAergic vesicles do not have a reduced capacity for GABA in a radioactive GABA uptake assay (WT: 337.7±102 counts, KO: 402.6±117.9 counts, N=2-3, P=0.72). (B) VGAT co-immunoprecipitates with SV2C from WT mouse striatal homogenates. Immunoprecipiatation was repeated twice each with both mSV2CpAb and hSV2CpAb for a total of four times.

***The influence of cholinergic tone on dopamine release in SV2C-KO animals*:**

Acetylcholine and dopamine tone are balanced in the striatum. Cholinergic agonists such as nicotine result in dampened tonic dopamine release. One feature of this relationship, though, is that cholinergic agonists effects, such as nicotine or varenicline, act as a high-pass filter where tonic release is reduced but phasic release is dramatically enhanced (Koranda 2013; Rice and Cragg 2004). In SV2C-KO animals, nicotine fails to produce this effect, suggesting a functional interaction between SV2C and nicotine. To evaluate the relative influence of acetylcholine tone on dopamine release in SV2C WT versus WT animals, we performed fast-scan cyclic voltammetry in the presence of varenicline. Varenicline is an agonist at both β2- and α7-containing nAchR subtypes, which have been identified as mediating the high-pass filter effect as well as some protective effects of nicotine in PD models (Perez 2015; Zhang D 2013). Here, varenicline induced a mild high-pass filter effect in both SV2C-KO and WT animals (Fig. 5-5), with no difference seen between genotypes after normalizing to baseline release. This provides a contrast to the previously-described, altered neurochemical effects of nicotine in SV2C-KO animals.

***Norepinephrine content in SV2C-KO animals*:**

We have previously reported a reduction in striatal dopamine content in SV2C-KO animals as compared to WT littermates. To determine if SV2C-KO affects the content of other monoamines, we performed HPLC to measure norepinephrine content from striatal dissections that also encompassed the BNST. Interestingly, we did not observe a reduction in norepinephrine in SV2C-KO animals, suggesting that the reduction in dopamine is

transmitter-specific and not reflective of broader monoaminergic deficits (Fig. 5-6), though exploring monoamine content in additional brain regions will be valuable.

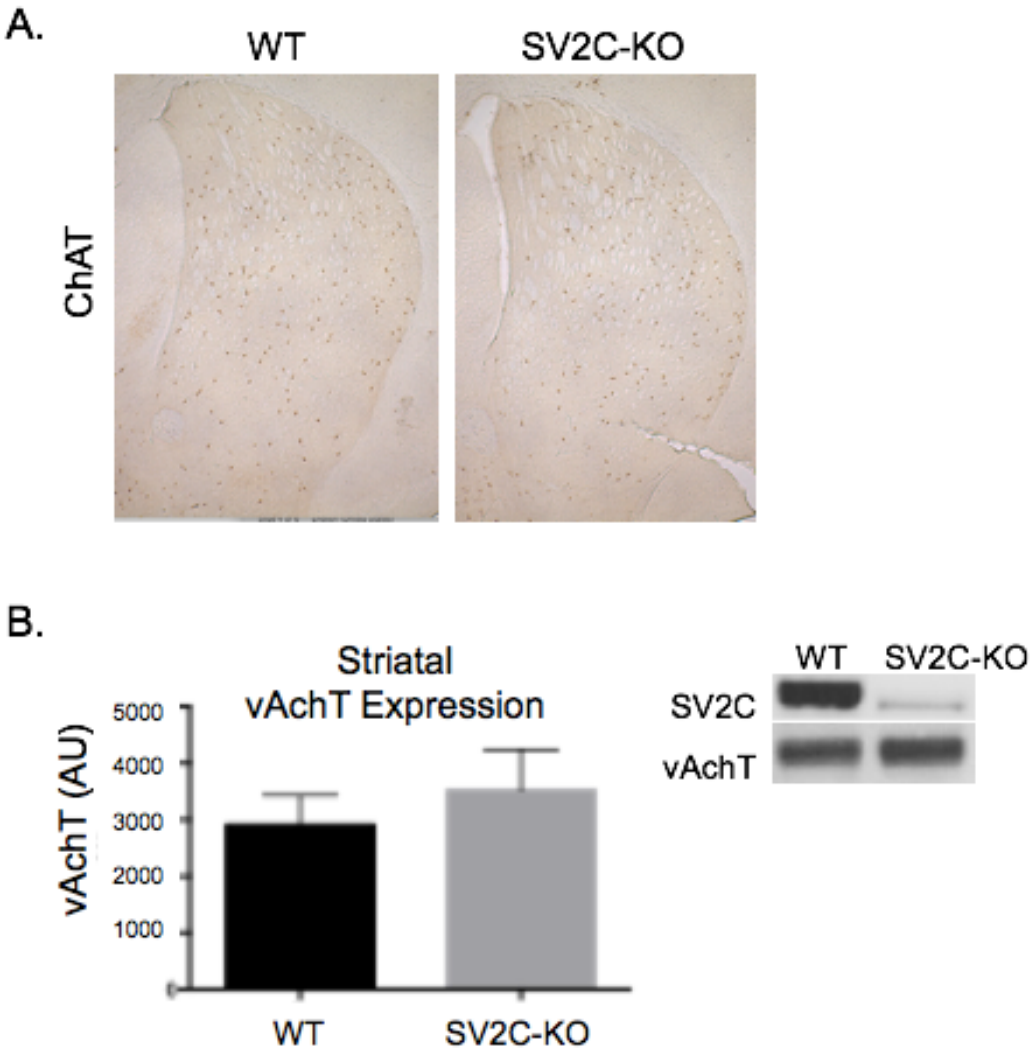

Figure 5-4. Effect of SV2C-KO on striatal cholinergic markers expression. (A) Striatal choline acetyltransferase (ChAT), an enzyme critical to the synthesis of acetylcholine, appears to be expressed similarly in WT and SV2C-KO animals. (B) Striatal vAchT expression levels are slightly, though not significantly, increased in SV2C-KO animals (WT: 2906±305 AU; KO: 3520±414, N = 3, P = 0.3).

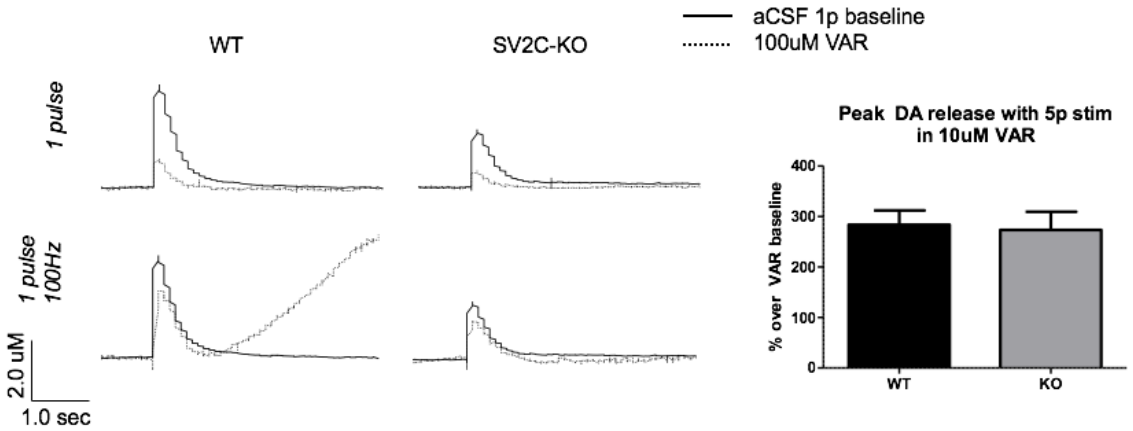

Figure 5-5. High-pass filter effect on striatal dopamine release by varenicline. Acute varenicline application reduces peak dopamine release elicited by a single pulse stimulation. Varenicline application acts as a high-pass filter on dopamine release, where a high-intensity (5pulse, 100Hz) stimulation increases dopamine release in the presence of the drug approximately to baseline (aCSF) levels. This effect, though a dampened high-pass filter effect when compared to that of nicotine, is preserved in SV2C-KO animals.

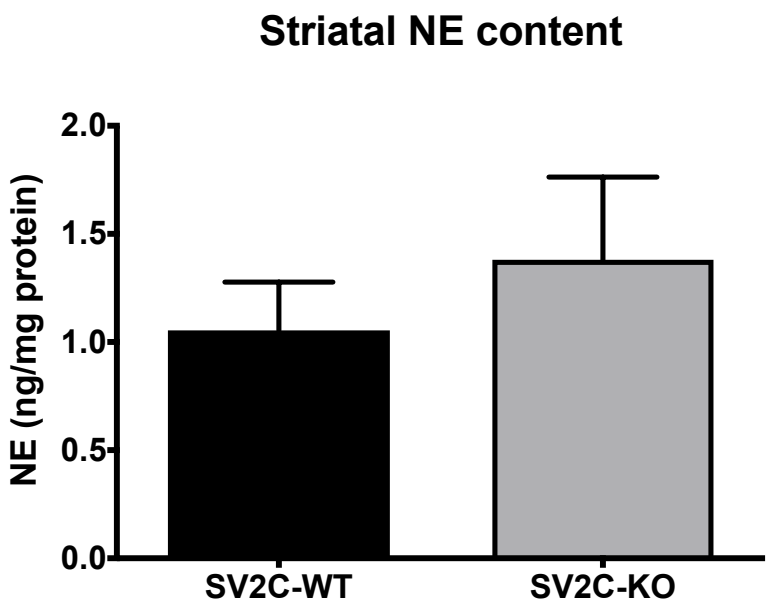

Figure 5-6. Striatal norepinephrine content in SV2C-KO animals. SV2C-KO animals do not have reduced striatal norepinephrine (NE) content compared to WT (WT: 1.05±0.22ng/mg protein, KO: 1.38±0.38ng/mg protein, N = 7, P = 0.47).

DISCUSSION

Given the enrichment of SV2C expression within the basal ganglia, the first reports of its distribution suggested that SV2C may play a particularly relevant role to PD. In fact, our data and others' data has directly linked SV2C to PD and we have previously shown that SV2C regulates various dopamine-associated functions relevant to PD. However, the strong expression of SV2C in highly GABAergic regions, such as the GP, VP and SNr, hint at a likely importance of SV2C in basal ganglia GABA neurons. Additionally, the links between SV2C and the cholinergic pathway suggest that disruptions to SV2C may also affect basal ganglia cholinergic transmission. Here, we evaluate the effects of SV2C-KO in various ways on GABA and acetylcholine neurons.

The effects of genetic ablation of SV2C on GABAergic systems in the striatum.

To evaluate perturbations to striatal GABA following genetic ablation of SV2C, we performed a variety of neurochemical assays. We did not observe alterations to the GABA marker VGAT in either expression level or pattern of expression (Fig. 5-1). Furthermore, pharmacologically manipulating GABA tone did not differentially affect dopamine release in SV2C-KO and WT animals (Fig. 5-2). Finally, we did not see deficits in the vesicular capacity for GABA using a radioactive GABA uptake assay, despite SV2C's localization to GABAergic vesicles (Fig. 5-3). Taken together, these data suggest that SV2C-KO does not result in perturbations to GABA pathways in the basal ganglia. However, further experimentation may reveal alterations to GABA transmission in SV2C-KO animals that

we were unable to identify in the current study. For example, SV2A-KO animals experience significant decreases in inhibitory neuron transmission as a result of altered calcium sensitivity. SV2A-KO also results in impaired vesicular mobility and synaptotagmin trafficking. Electrophysiological evaluation of calcium sensitivity and readily releasable pool size in GABAergic neurons within the basal ganglia could demonstrate alterations to these features in SV2C-KO animals. Furthermore, measuring only the concentration capacity of vesicles for GABA is not a complete evaluation of vesicular function. Additional [3H]-GABA uptake experiments may explore the filling rate and/or leak rate of GABA vesicles at multiple concentrations to more finely characterize striatal GABA vesicle function in SV2C-KO animals.

Interestingly, the finding that vGAT, but not VMAT, co-immunoprecipitates with SV2C further highlights the likelihood that SV2C-KO does disrupt GABA transmission. Identifying disruptions to GABA transmission following SV2C-KO will be particularly interesting, considering the significant alteration to SV2C expression in GABAergic MSNs in the striatum of postmortem PD cases. Results from these future experiments will inform our interpretation of the potential functional consequences of SV2C disruption in PD basal ganglia.

***The effects of SV2C-KO on striatal acetylcholine*:**

To investigate potential alterations to the cholinergic system within the basal ganglia, we evaluated expression of vAchT in SV2C-KO and WT animals. There is a possible trend toward increased vAchT expression in SV2C-KO animals, although this effect does not reach statistical significance when evaluated over several animals (Fig. 5-4). We also

evaluated the effect of a cholinergic agonist, varenicline, on dopamine release in the dorsal striatum. Following previous experiments that revealed altered neurochemical response to nicotine in SV2C-KO animals, we sought to identify whether these effects were nAchR-mediated. Varenicline, like nicotine, induces a high-pass filter on dopamine release(Perez 2015). In our experiment, this effect was not as strong as that of nicotine (Fig. 5-5). Furthermore, we did not see a differential effect of varenicline on SV2C-KO animals as compared to their WT littermates. These data suggest that the altered neurochemical response to nicotine in SV2C-KO animals is nicotine-specific, and is not observed with other nicotinic agonists.

Evidence presented previously in this dissertation supports the idea that SV2C-KO results in significant perturbations to basal ganglia functioning, particularly within the nigrostriatal dopamine pathway. Given the complex nature of basal ganglia circuitry, and considering the integration of various transmitter systems in producing a striatal output, many of these effects could be potentially mediated by alterations in non-dopaminergic transmitter systems. Here, we provide evidence that the previously identified alterations to basal ganglia function are most significantly mediated by disruptions to dopamine input. However, further experimentation is necessary to more fully characterize potential perturbations in non-dopaminergic basal ganglia neurotransmission. It is possible that SV2C functions similarly in non-dopamine neurons as it does in dopamine neurons. This would suggest that SV2C-KO affects GABA and/or acetylcholine transmission in a similar manner to its effects on dopamine cells. The current data call for further investigation into the role of SV2C elsewhere in the basal ganglia.

VI. Discussion, future directions and concluding remarks

The goals of this dissertation were to characterize novel tools for studying SV2C expression and function, reveal the role of SV2C in dopamine neurotransmission and basal ganglia function, identify a possible neuroprotective function of SV2C, and to determine whether SV2C regulates non-dopaminergic basal ganglia neurotransmission.

When we began our investigations into SV2C, there was very little literature on the protein. In a majority of SV2-focused investigations, SV2C was virtually ignored in favor of SV2A. However, what information we did have all pointed towards its potential relevance to PD. This work has revealed a striking importance of SV2C in dopamine function, Parkinson's disease, and neuronal vulnerability to degeneration. We have identified SV2C as a regulator of dopamine transmission and a mediator of dopaminergic vulnerability to degeneration. Additionally, we observed an interaction between SV2C and alpha-synuclein that suggests that disruption in either of these proteins lead to disruption of the other. Further, we have revealed that SV2C expression is disrupted in PD. Our functional data from mouse experiments suggests that this disruption of SV2C in PD could contribute to disease pathogenesis by impairing dopamine transmission, destabilizing alpha-synuclein, and heightening the vulnerability of these dopaminergic neurons to cell death. Ultimately, these data provide a basis for future experiments to evaluate the suitability of SV2C as a potential pharmacotherapeutic target for the treatment of PD or any number of neurological diseases characterized by disrupted dopamine transmission.

The majority of the observations in this dissertation centered on the role of SV2C in dopamine neuron function. These data support a growing body of evidence that SV2C is particularly important in dopaminergic nuclei. For example, SV2C is a mediator of the

efficacy of L-DOPA and dopamine-targeting antipsychotics (Altmann 2016; Ramsey 2013), and SV2C is specifically down-regulated in dopaminergic neurons in PD (Mariani 2016). For its apparent importance in basal ganglia dopaminergic neurons, SV2C may be an ideal drug target for PD or other basal ganglia disorders, in order to focus effects on relevant nuclei while limiting off-target effects. By targeting SV2C, we may be able to restore proper vesicular functioning in order to maintain cell health. Alternatively, an SV2C-targeting compound could be used in conjunction with traditional PD therapeutics such as L-DOPA, in order to improve vesicular handling of the replacement dopamine and extend the treatment window in which it is most effective for patients.

In order to more fully characterize the therapeutic relevance of SV2C, however, future experiments should focus the mechanism of the protein's function. Substantial evidence in the SV2A research community points to an importance of SV2s in regulating calcium sensitivity and synaptotagmin function, as well as a role in proper mobility and organization of synaptic vesicles. Ongoing experiments in the Miller laboratory point to additional roles of SV2C is regulating the storage of vesicular dopamine, preventing vesicular leakage, and maintaining the size of the readily releasable pool of dopaminergic vesicles. Specifically: (1) SV2C overexpression enhances uptake of a fluorescent dopamine analogue, FFN206 in HEK293 cells, and this enhancement appears to be an ability specific to SV2C, as SV2A and SV2B do not confer a similar level of enhancement, and (2) vesicles isolated from SV2C-KO striata are less efficient at storing dopamine long-term, suggesting the leakage of dopamine molecules out of vesicles. We hypothesize that this function is related to the glycosylated intraluminal loop of SV2C and transmitter adsorbtion into the proteoglycan matrix, and have developed mutated SV2C constructs to

further resolve this. Additionally, repeated stimulations deplete the readily releasable pool of vesicles more quickly in SV2C-KO versus WT striata, suggesting deficits in the ability of the readily releasable pool to replenish vesicles.

Delving further into the relationship between SV2C and alpha-synuclein, and the role of SV2C in neuronal vulnerability will also be interesting points of investigation. For example, could pharmacologically stabilizing SV2C prevent alpha-synuclein toxicity? And can overexpression of SV2C be neuroprotective? Additional coimmunoprecipitation studies have suggested that mutated alpha-synuclein differentially associates with SV2C. Investigating the association between SV2C and alpha-synuclein in A53T-OE mice or in human PD versus control brains will be particularly informative. Proposed studies in the Miller lab will also investigate the neuroprotective potential of enhanced SV2C expression in A53T-OE mice and other models of PD. Additionally, we have initiated a collaboration with a medicinal chemist at Emory, Dr. Huw Davies, who has developed putative SV2C-targeting compounds. Testing the effect of these compounds on neuronal toxicity *in vitro* will be a promising first step to identify the therapeutic potential of pharmacological manipulation of SV2C.

This work lays the foundation for what will undoubtedly be a growing field into the study of SV2C in the context of PD. Not only has it given us a greater understanding of SV2C itself, but the results from these experiments will be informative to the field of dopamine synaptic function as a whole, and complementary to existing SV2 literature. It has been quite rewarding to see the evolution of this project from a side project to a main focus of the Miller lab, and I am excited to see where the data take us from here.

VII. *Appendix A: Nicotine neuroprotection in SV2C-KO animals*

Summary

The synaptic vesicle glycoprotein 2C (SV2C) was first introduced to our lab for its role as a genetic mediator of the protective effect of smoking in PD. One of the main questions we had in response to these data was whether SV2C protein mediates nicotine neuroprotection. For these studies, we chronically administered nicotine to SV2C-KO and WT animals prior to MPTP intoxication in a model that has been repeatedly shown to protect against toxicity in a variety of models and species (Huang 2009; Liou et al. 2007; Quik 2001, 2006, 2013) (see Fig. A-2). Unfortunately, due to an unexpectedly reduced potency of a new lot of MPTP purchased from an unfamiliar vendor (MedChemExpress), we did not achieve significant dopaminergic cell body loss following MPTP intoxication in WT animals (Fig. A-3). This limits our ability to interpret the nicotine neuroprotection data. This experiment was repeated in a new cohort of SV2C-KO mice with a previously-tested supply of MPTP (Sigma) to ensure adequate cell loss that may be protected against by nicotine. Nonetheless, the data from the previous nicotine neuroprotection study, as well as preliminary data from the second experiment using Sigma MPTP follow.

In SV2C-KO animals, we did achieve significant cell loss in the SNc with MedChemExpress MPTP and were able to evaluate the potential neuroprotective effects of nicotine on dopaminergic degeneration. There was a trend toward protection against cell loss in the nicotine-treated group, but this trend did not reach statistical significance as it did in pilot experiments with WT animals (Fig. A-1). This suggests that nicotine does *not* significantly protect against cell loss in SV2C-KO animals following MPTP, although it is difficult to interpret these data without the ability to compare to WT littermates. Finally, in our second nicotine neuroprotection experiment, we evaluated striatal dopamine marker

loss following MPTP and chronic administration of either saccharin-sweetened water or nicotine solution. There was a significant difference of TH expression following MPTP and MPTP + Nicotine between genotypes ($p<0.05$).

Nicotine administration:

Chronic nicotine

100ug/mL nicotine (Sigma) was dissolved in 0.2% saccharin (w/v, Sigma) solution. Animals were given nicotine (or 0.2% saccharin) for via their drinking water for 3-4 weeks prior to the first MPTP injection. Nicotine administration continued throughout MPTP intoxication and until animals were sacrificed (Fig. A-2).

Nicotine preference

30ug/mL nicotine (Sigma) was dissolved in 0.2% saccharin (w/v, Sigma) solution. Animals were singly housed and were provided a bottle of nicotine solution and a bottle of 0.2% saccharin solution. Daily water consumption was measured, and the proportion of nicotine solution to total water consumed was reported.

Nicotine neuroprotection

Chronic administration of nicotine prior to dopaminergic lesion is neuroprotective against dopamine cell body loss. This has been observed in several rodent and nonhuman primate models employing both 6-hydroxydopamine and MPTP lesions (Huang 2009; Liou et al. 2007; Quik 2001, 2006, 2013). We administered nicotine via drinking water for three weeks prior to the first injection of MPTP. Because there was no significant loss of cells in WT animals following this administration of MedChemExpress MPTP, we were unable to

observe neuroprotection in WT animals. However, we did observe a significant loss of TH+ SNc cells in SV2C-KO animals following MPTP (Fig. A-3). Chronic administration of nicotine was unable to protect against this lesion. Similarly, when evaluating the striatal lesion resulting from administration of Sigma MPTP in the second experiment, we were able to observe a trend toward nicotine-mediated protection in the WT, but not the SV2C-KO animals (Fig. A-4). Stereological cell counting is the standard for evaluating nicotine-mediated neuroprotection and is ongoing; however, the preliminary results from semi-quantitative western blotting suggest that SV2C genotype mediates the neuroprotective effect of nicotine.

SV2C expression following chronic nicotine administration

Following the observation that SV2C-KO animals have enhanced vulnerability to MPTP toxicity and do not experience the neuroprotective effects of nicotine, we evaluated the effect of chronic nicotine administration on SV2C expression. Chronic nicotine administration did not affect the expression levels of striatal SV2C (WT: 64.1 AU ± 0.09; KO: 52.6 AU ± 0.06; $P = 0.34$) (Fig. A-5).

SV2C and nicotine neuroprotection.

Nicotine has been shown to be neuroprotective in various species and models of PD when chronically administered (Huang 2009; Liou et al. 2007; Quik 2001, 2006, 2013). The mechanisms underlying nicotine-mediated neuroprotection are not well-understood. A genomic wide association study (GWAS) identified polymorphisms at two loci upstream of the SV2C gene that mediate PD risk in smokers, suggesting that SV2C may be involved

in the neuroprotective pathway upon which nicotine is acting (Hill-Burns 2012). Here, we provide evidence that SV2C-KO animals do not experience neuroprotection from chronically administered nicotine, as has been presented in the literature in WT mice. Unfortunately, due to the parameters of the present experiments, we are unable to definitively evaluate the contribution of SV2C to nicotine neuroprotection as the WT animals in our cohort did not show significant neuronal loss in the SNc following MPTP. We were also unable to detect motor impairment as a result of dopamine loss, likely due to the relatively mild lesion resulting from this particular lot of MPTP. Ongoing experiments in our laboratory are investigating various doses of MPTP in SV2C-KO and WT animals with and without nicotine pretreatment to more fully characterize the relationship between SV2C and nicotine neuroprotection. Given that SV2C-KO animals have altered neurochemical response to nicotine, however, it is likely that there is a functional interaction between nicotine and SV2C underlying GWAS data characterizing SV2C as a genetic mediator of PD risk in smokers.

FIGURES.

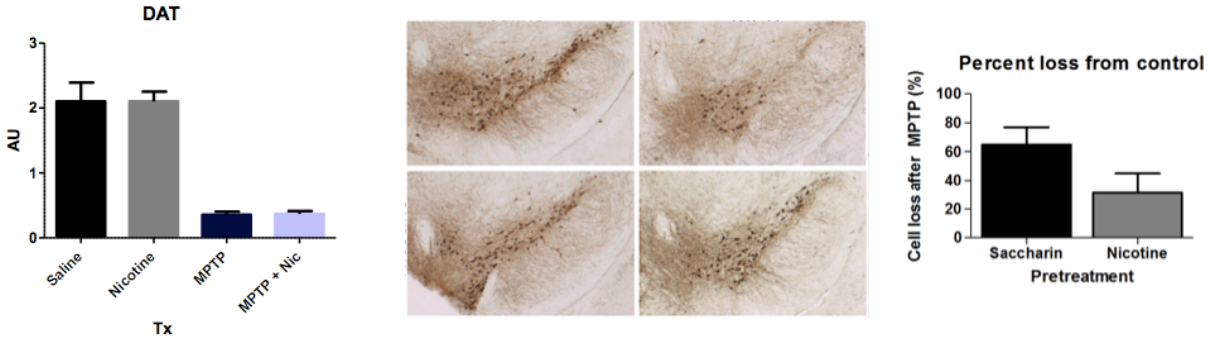

Figure A-1. Nicotine protects against MPTP-induced cell loss in a pilot experiment in WT animals. WT animals treated with MPTP experience an approximate 60% loss of TH+ cell bodies in the SNc. Pretreatment with chronic nicotine protected against about half of this loss, and nicotine-treated animals only lost about 30% of dopaminergic SNpc cells following nicotine intoxication.

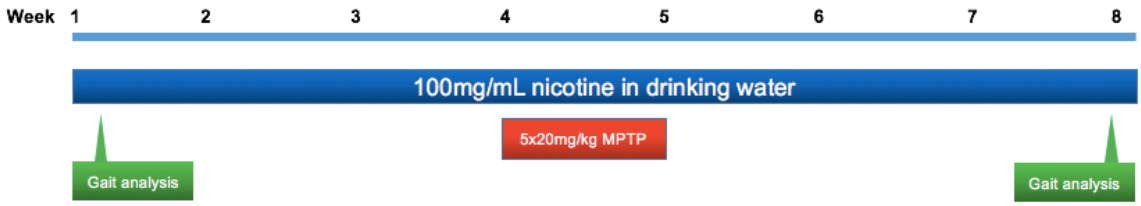

Figure A-2. Diagram of nicotine neuroprotection experimental schedule. Animals are given nicotine via their drinking water for three weeks preceding MPTP. Animals undergo gait analysis to determine baseline motor function. Following one week of MPTP injection, the lesion is allowed to stabilize for three weeks. 20 days following the final MPTP injection, animals once again undergo gait analysis to evaluate any loss of motor function resulting from MPTP treatment. Animals are sacrificed 21 days following the final MPTP injection.

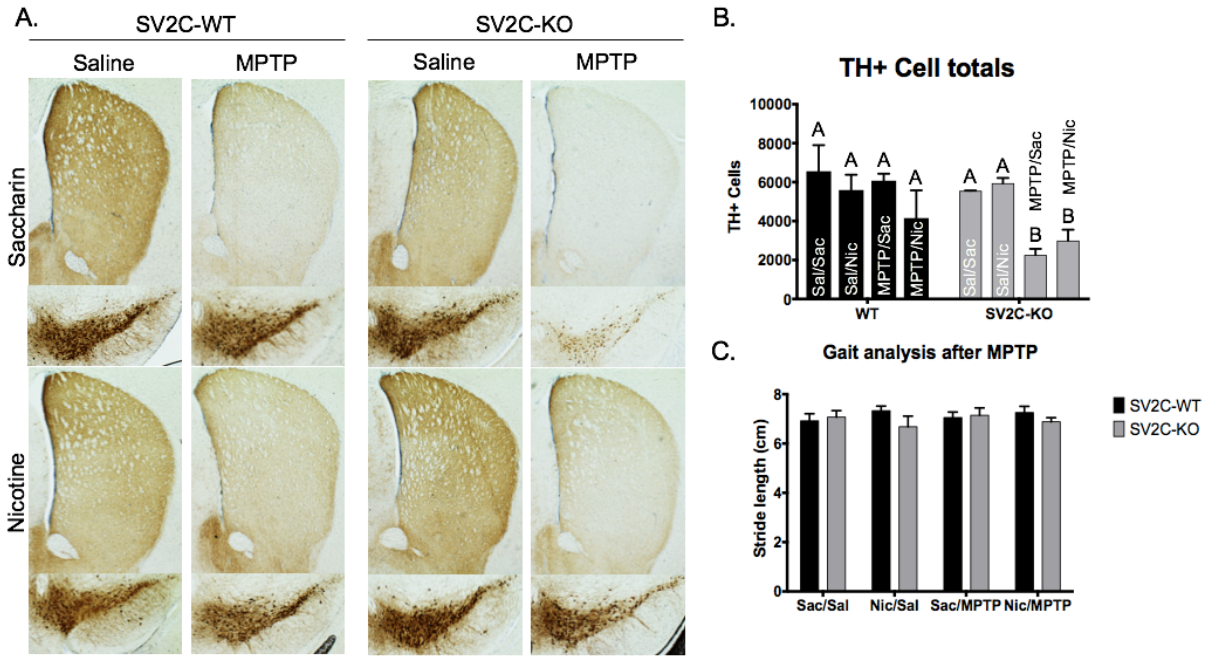

Figure A-3. The effect of chronic nicotine pretreatment on MPTP lesion (MedChemExpress). (A) Representative immunohistochemistry of the striata and midbrain of SV2C-KO and WT animals treated with either nicotine, MPTP or both reveals a possible neuroprotective effect of nicotine on both the WT and SV2C-KO animals. However, when quantified over all animals, (B) there is no statistically significant protection against dopaminergic cell loss in SV2C-KO animals. Because there was no loss of cells following MPTP in the WT animals, we could not evaluate nicotine-mediated neuroprotection in WT animals for comparison. (C) We did not detect alterations in motor function as evaluated by stride length in any experimental group.

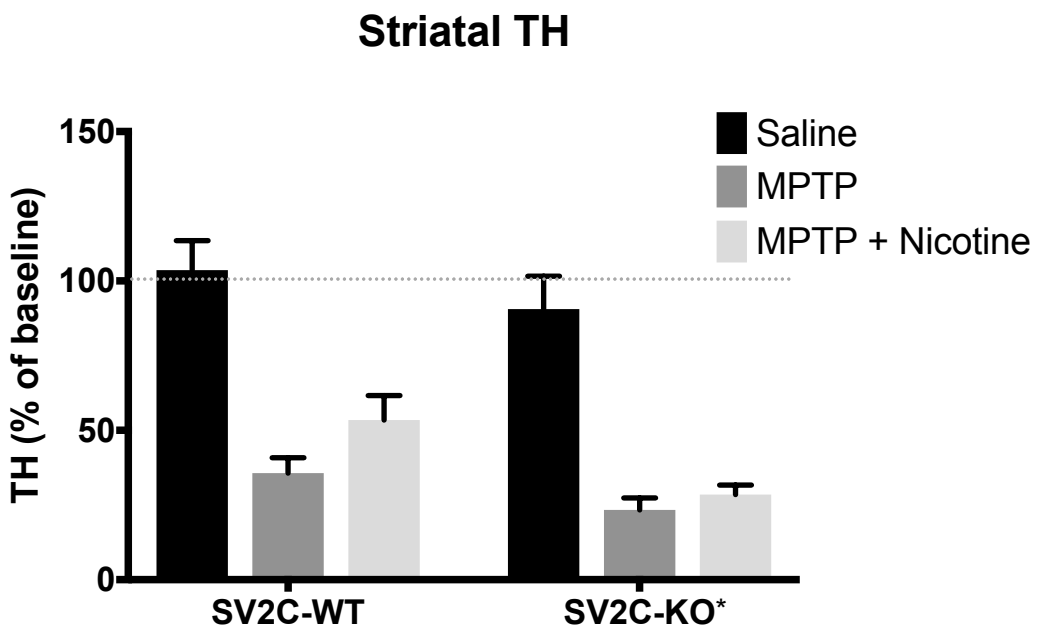

Figure A-4. The effect of chronic nicotine pretreatment on MPTP lesion (Sigma). Semi-quantitative western blot data suggests that WT animals were protected against some striatal MPTP lesion, whereas SV2C-KO animals were not.

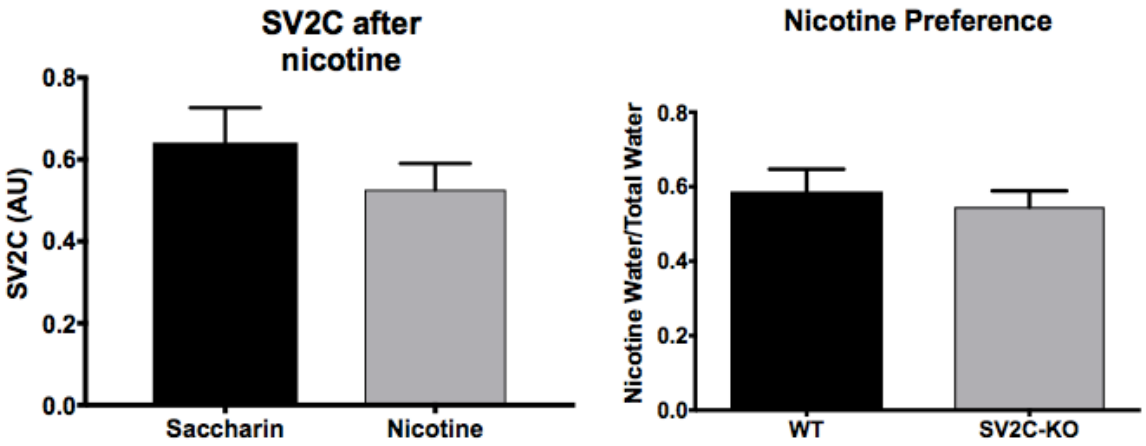

Figure A-5. SV2C expression and nicotine consumption. (A) Striatal SV2C expression levels are unaltered following chronic administration of nicotine. (B) There is no difference in nicotine consumption between WT and SV2C-KO animals in a two-bottle choice test.

VII. ***References***

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A role for the synaptic vesicle glycoprotein 2C (SV2C) in dopamine homeostasis and Parkinson's disease

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