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The synaptic vesicle glycoprotein 2C modulates dopamine release in the ventral striatum and contributes to behaviors associated with reward

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Abstract

The synaptic vesicle is a critical nexus for regulated neurotransmitter release and a number of proteins precisely orchestrate its filling, trafficking, fusion, and endocytosis. Every synaptic vesicle contains the synaptic vesicle glycoprotein 2 (SV2). Three isoforms of SV2 exist: SV2A, B, and C. SV2A and SV2B are expressed ubiquitously through the central nervous system and contribute to normal neurotransmission. SV2C expression is restricted to the basal ganglia, labeling 70% of dopamine neurons. Additionally, SV2C is strongly expressed in the ventral pallidum, the main output of the nucleus accumbens. The contribution of SV2C to the synaptic vesicle cycle has not been well established. I hypothesized that SV2C modulates dopamine release and contributes to behaviors associated with reward. To test this hypothesis, I first optimized voltammetric recording of dopamine release in the ventral pallidum of wildtype mice and demonstrated that it is correlated with reward behavior. I used this technique, in combination with established protocols, to assess reduced dopamine release in the ventral striatum and pallidum of SV2C knockout (SV2C-KO) animals generated in our lab. I interrogated SV2C mechanism using stimulus train depletion and radioactive vesicular uptake. Behavioral metrics support the observed neurochemical deficits; SV2C-KO animals have increased methamphetamine-stimulated locomotor activity and reduced conditioned place preference compared with controls. Interestingly, female mice express 40% more SV2C and show increased reduction in dopamine release and reward behavior than male mice. Given its discrete expression, importance in dopamine neurotransmission, and contribution to reward behavior, SV2C is a promising target for further interrogation towards the development of novel therapeutics for addiction treatment.

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Table of Contents

List of Figures

CHAPTER 1 - Introduction: Rationale, hypothesis, and scope

The brain is a network of complex circuitry that works together to generate voluntary and involuntary behavior. Neurons communicate via electrical signals. However, in most cases, neurons do not directly interact, necessitating the use of neurotransmitters to propagate information at synaptic junctions. Neurotransmitters (e.g. GABA, glutamate, monoamines) are stored in vesicles in the presynaptic neuron. This sequestration serves two purposes: it prepares the neuron for expeditious release upon the receipt of an action potential and it protects the neurotransmitter from breakdown by cytosolic metabolizing enzymes. The process by which vesicles fuse, release their stored neurotransmitter, and are recycled is termed the synaptic vesicle cycle. While much is known about this elegantly orchestrated process, the mechanisms of many key protein participants is still debated.

Three such proteins are members of the synaptic vesicle glycoprotein 2 (SV2) family. SV2 proteins are key regulators of synaptic transmission, though their exact functions are not fully defined (Crowder, 1999). SV2s are 12-transmembrane domain proteins in the major facilitator superfamily localized to the synaptic vesicle (Bajjalieh et al., 1992; Janz et al., 1998). Neither transporter activity nor substrate for any of the 3 members of the SV2 family has been identified; rather, considerable evidence suggests that they are involved in vesicle trafficking and regulated transmitter release. SV2s bind to and assist in trafficking and regulation of the vesicular calcium sensor, synaptotagmin (Schivell, 2005; Yao, 2010). SV2s also have a role in trafficking synaptic vesicles to the readily releasable pool (Nowack et al., 2010) and facilitate calcium-induced fusion of primed vesicles to the synaptic membrane (Chang, 2009). Additionally, intraluminal SV2

glycosylation may enhance transmitter loading and release through a process known as ***chemiosmotic stabilization***, wherein sugar moieties bind intraluminal dopamine, thereby reducing the dopamine gradient, and allowing increased packaging of cytosolic dopamine (Chang, 2009).

Three isoforms of SV2 exist: SV2A, SV2B, and SV2C. Of these, SV2A has been the most extensively studied due to the importance of the protein in epilepsy. Little is known about how SV2 isoforms differ from one another. Expression profiles of the three proteins differ dramatically. SV2A is ubiquitously expressed, labeling nearly every presynaptic terminal in the central nervous system (Bajjalieh, 1994). SV2B is also widely expressed, though slightly more restricted than SV2A (Bajjalieh, 1994). Interestingly, SV2C is highly enriched in the basal ganglia (Janz, 1999). Within the basal ganglia, SV2C is most strongly expressed in the ventral pallidum, a region integral to processing signals from the reward systems in the brain. Given the importance of SV2s to neurotransmission and the isoform specific expression of SV2C, I hypothesized that **SV2C modifies vesicular function and alters neurochemical and behavioral response to methamphetamine (METH).** In the following dissertation, I address this hypothesis systematically. First, I provide a review of the relevant SV2 literature, which provides the basis for the experiments conducted within this work. Second, I demonstrate optimization of fast-scan cyclic voltammetry to record dopamine release in the ventral pallidum, the region that most strongly expresses SV2C. This technique was used to demonstrate the importance of the ventral pallidum to methamphetamine-induced behavioral sensitization in wildtype mice. Third, I investigate the contribution of SV2C

to dopamine release in the nucleus accumbens and ventral pallidum and subsequent downstream alterations in reward related behavior. Finally, I provide interesting observations suggesting sex differences in behavioral reward response compared to wildtype mice.

The nature of reward. A species' survival is entirely dependent on acquiring food, water, and sex. The neural processes that have evolved to reinforce the performance of these pro-survival behaviors, thereby ensuring their repetition, are defined as the reward system (Kelley and Berridge, 2002). Mesocortical dopamine neurons, which project from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and other reward-related structures, are central to reward neurocircuitry. Given the importance of this system to evolutionary success, dopamine systems are found throughout eukarya, from drosophila to humans. Drugs of abuse converge upon this system, resulting in supraphysiological augmentation of normal reward signaling.

Mechanism of METH-induced reward signaling. METH reward is primarily mediated via enhancement of dopamine (DA) release from neurons mesocortical dopamine neurons (Fleckenstein et al., 2007; Nakajima et al., 2004; Ross and Peselow, 2009). METH acts by several mechanisms to enhance DA transmission in these neurons (Sulzer, 2011; Sulzer et al., 2005): 1. Inside DA terminals, METH inhibits the vesicular monoamine transporter 2 (VMAT2), which is responsible for packaging cytosolic monoamines into synaptic vesicles (Guillot, 2008; McFadden et al., 2011). 2. METH decreases cytosolic dopamine metabolism by inhibiting monoamine oxidase and increases dopamine

synthesis by enhancing tyrosine hydroxylase activity (Fibiger and McGeer, 1971). 3. METH alters the physiology of the dopamine transporter (DAT), which normally acts to transport DA from the synapse into the terminal. After utilizing normal DAT transport to enter the neuron, METH changes DAT function to a channel-like state with unidirectional outward dopamine flow (Goodwin et al., 2009; Kahlig et al., 2005). METH-induced terminal changes increase cytosolic dopamine, which drives DAT reversal (Harrod et al., 2001; Kahlig et al., 2005). These changes ultimately result in supraphysiological dopamine release and greatly enhanced postsynaptic signaling. Therapeutics that limit METH-induced dopamine release will likely inhibit the rewarding effects of METH, reduce drug intake, and aid in the addiction recovery process. recovery process.

Modulation of vesicular function as a target for inhibition of METH action. Reduced vesicular function alters response to METH (Damaj et al., 1997; Guillot, 2008). Pharmacological inhibition of VMAT2 reduces METH self-administration, conditioned place preference, and locomotor sensitization suggesting a decrease in reward in response to reduced vesicular function (Dimatelis et al., 2012; Harrod et al., 2001; Nickell et al., 2011). However, VMAT2 expression is not limited to dopamine neurons and numerous adverse effects including severe depression, anxiety, and gastrointestinal disturbances result from VMAT2 inhibition in serotonergic and noradrenergic neurons (Fasano and Bentivoglio, 2009; Jankovic and Clarence-Smith, 2011; Jankovic et al., 1984). A novel vesicular target with more restricted dopaminergic expression may reduce these off target effects while providing a similar anti-addiction therapeutic profile.

Chapter 2 - The synaptic vesicle glycoprotein 2: structure, function, and disease relevance

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An isoform of the synaptic vesicle glycoprotein 2 (SV2) localizes to every neurosecretory vesicle in the human body. Such a ubiquitous presence in the vesicular proteome suggests that SV2 is integrally important to vesicular function. However, no definitive function for any isoform has been identified. Here, we discuss structural, functional, and pathological properties of SV2 proteins as described in the literature.

Structure

SV2 proteins are members of the major facilitator superfamily, a large family of membrane transporters expressed widely throughout bacteria, archaea, and eukarya (Pao et al., 1998). Like other major facilitator superfamily proteins, SV2s have 12 transmembrane domains, with cytosolic N- and C-termini. Additionally, SV2s have a large N-glycosylated intraluminal loop between transmembrane domains 7 and 8 (Figure 2-1, 2). Three isoforms of SV2 exist in vertebrates: SV2A, SV2B, and SV2C. These isoforms share 61-64% sequence homology and 80% structural homology (Bajjalieh, 1994). Two other homologous proteins, SV2-related (SVOP) and SVOP-like (SVOPL) share 20-22% sequence homology with SV2s, though they are not glycosylated. Orthologues of these proteins are present in invertebrates, including *Drosophila melangoster* and *Caenorhabditis elegans*.

Expression

While SV2s are found in every neurosecretory vesicle, individual isoforms vary in their expression profiles. SV2A is the most ubiquitously expressed, labeling every structure in the brain to varying degrees (Bajjalieh, 1994). SV2B expression is more restricted, with strongest expression in the trigeminal and motor nuclei and very little

expression in the globus pallidus, dentate gyrus of the hippocampus, cerebellum, and substantia nigra pars reticulata (Bajjalieh, 1994). SV2C expression is the most limited, localizing to evolutionarily older brain regions, with strong expression throughout the striatum, midbrain, and ventral pallidum and very little expression in the neocortex (Dardou, 2013; Janz, 1999).

At the vesicular level, SV2 is sorted with high precision; each synaptic vesicle contains five copies of the SV2 protein, with very little intravesicular variation (Mutch et al., 2011). Genetic ablation of SV2A results in compensatory upregulation of SV2B/C, presumably maintaining total levels of SV2 in the animal (Crowder, 1999; Xu, 2001). SV2A and SV2B are co-expressed in a large number of neurons and immunoprecipitation of intact vesicles reveals co-labeling of individual vesicles (Bajjalieh, 1994). SV2C co-expression data has not been reported. Co-labeling of expression has been suggested to indicate functional redundancy (Bajjalieh, 1994). However, expression patterns also suggest isoform independent function. While SV2A is found in both excitatory and inhibitory neurons, SV2B is limited to excitatory synapses (Bajjalieh, 1994). SV2C is expressed in 70% of dopaminergic neurons but is also expressed strongly in GABAergic medium spiny neurons of the striatum (Dardou, 2013; Dunn et al., 2016). This expression correlates with lesser expression of SV2A and SV2B in the striatum (Bajjalieh, 1994). Interestingly, SV2A and SV2B are not expressed in cell bodies (Bajjalieh, 1994). SV2C, however, is expressed in TH-positive midbrain dopamine neuron cell bodies (Dunn et al., 2016), suggesting SV2C may play a role in somatodendritic dopamine release. SV2C is also expressed in cell bodies of striatal GABAergic medium spiny neurons (Dunn et al., 2016).

Function

The function of SV2 remains elusive despite excellent experimental questioning. Most of the literature centers on the function of SV2A, which is the molecular target of the anti-epileptic therapeutic levetiracetam (Gillard et al., 2006; Harada et al., 2013; Kaminski et al., 2009; Klitgaard and Verdru, 2007; Lynch, 2004; Nowack, 2011). Over the course of the last twenty years SV2 has been investigated for many potential functions, including: vesicular transport, laminin interactions, calcium sensing, regulating vesicle recruitment, anchoring vesicular structure, and stabilizing vesicular loading.

SV2 as a vesicular transporter. Given their family heritage, it was initially assumed that SV2 functioned as a novel neurotransmitter transporter (Bajjalieh et al., 1992; Feany, 1992; Gingrich et al., 1992; Janz, 1999). However, despite efforts to this end, no direct evidence of SV2-mediated vesicular transport exists. Interestingly, the protein does act as a physical transporter for botulinum and tetanus neurotoxins (Dong et al., 2006; Yeh et al., 2010). Additionally, evidence suggests that SV2 may act as a sugar transporter when localized to the plasmalemmal membrane during vesicle fusion (Madeo, 2014).

One of the most interesting aspects of transmembrane vesicular proteins is their altered interactome through the phases of neurotransmission. During exocytosis luminal proteins become extracellular proteins, completely changing potential binding partners (Figure 2). Botulinum toxins, the most potent neurotoxins known, make use of this aspect of SV2s, binding to luminal domain 4 and hijacking an endocytotic ride into the presynaptic neuron (Chang et al., 2015; Dong et al., 2006; Fu et al., 2009; Mahrhold et al., 2013; Peng et al., 2011; Rummel et al., 2009). Botulinum toxin serotype alters the

affinity of the toxins for specific SV2 isoforms. Likewise, tetanus toxin also gains entry to central neurons via SV2 endocytosis (Yeh et al., 2010).

In addition to neurotoxins, it has been proposed that SV2s may transport sugars (Madeo, 2014). Recombinant human SV2A expressed in a hexose-transporter deficient *Saccharomyces cerevisiae* strain survived exclusively on galactose-containing medium. Further, radioactive galactose transport was levetiracetam- and proton gradient-sensitive, thus characterizing SV2A as a galactose symporter in yeast. Sugar transport function has yet to be demonstrated in higher order models, as the assay is inherently more difficult. Transport activity for SV2 in vertebrates is only apparent during fusion, when SV2 is appropriately localized to transport a substrate from the synaptic cleft into the neuron. Traditional vesicular uptake assays would not capture such a phenomenon, as the protein is inside-out in intact vesicles. Sugar transport coupled to vesicular fusion is a practical idea, potentially allowing increased energy substrate transport during times of high synaptic activity. The idea is reminiscent of acetylcholine transmission, where plasmalemmal choline acetyltransferase localizes to cholinergic synaptic vesicles, allowing increased recycling of transmitter during stimulation (Ferguson and Blakely, 2004).

Potential interactions with the extracellular matrix. In support of this "inside-out hypothesis," evidence suggests that SV2 acts as a laminin-1 receptor(Son et al., 2000). Upon vesicular fusion, the luminal domain of SV2 becomes the extracellular domain and is free to interact with extracellular matrix proteins, such as laminin-1 (Son et al., 2000) (Figure 2-2). This is an activity-dependent interaction, as increased vesicular fusion

results in increased SV2 in the plasmalemmal membrane. While the functional consequences of the interaction are unknown, it is possible that the SV2-laminin interaction slows vesicular recycling, allowing for greater release of neurotransmitter during periods of high signaling, perhaps dictating full versus transient vesicle fusion. Interestingly, pharmacological or genetic inhibition of laminin produces similar synaptic dysfunction to SV2 knockouts, including: lack of calcium sensitivity (Chand et al., 2015), defects in plasticity (Nakagami et al., 2000), and altered synaptic release (Knight et al., 2003).

Regulation of calcium sensitivity. Mice lacking SV2A have decreased calcium-induced neurotransmission in both excitatory and inhibitory neurons (Chang, 2009; Crowder, 1999; Custer et al., 2006). Cultured hippocampal neurons from SV2A/B double mutant mice display a total reduction in synaptic release but, interestingly, display synaptic facilitation during stimulus trains and increased paired-pulse response compared with SV2B mutant neurons, which are not significantly different than wildtype neurons (Chang, 2009; Janz et al., 1999). These increases are calcium-dependent, as treatment with chelator EGTA-AM abolishes the enhanced synaptic activity without affecting the total reduction in stimulated release in SV2A/B double mutants (Chang, 2009). Given its structure, it was originally hypothesized that SV2 functioned as a calcium transporter (Janz et al., 1999). However, this hypothesis has been the subject of debate (Chang, 2009; Iezzi, 2005). Though calcium is required for the observed synaptic facilitation, deletion of SV2A/B does not change synaptic calcium affinity: while total release is reduced there is no change in relative IPSC amplitudes at escalating calcium dose, treatment with

ionophore ionomycin does not produce differential results in double mutants, and EGTA-AM treatment produces identical relative decreases in train release regardless of SV2 presence (Chang, 2009). These complexities led to the hypothesis that SV2 doesn't directly transport calcium but rather renders primed vesicles more calcium responsive (Chang, 2009).

As loss of SV2A is lethal around 3 weeks of age, all experiments in SV2A knockout mice must be conducted in earl postnatal mice. This is problematic as SV2 expression varies through development (Bajjalieh, 1994; Cohen, 2011; Crèvecoeur, 2013) and, thus, the functional consequence of the protein may change over the lifespan of the animal. As such, the consequence of SV2B deletion was interrogated in adult retinal ribbon synapses, where SV2B is the major isoform (von Kriegstein and Schmitz, 2003; Von Kriegstein et al., 1999). Calcium concentration, both resting and stimulus-induced, is elevated two-fold in SV2B-null mature rod bipolar neurons compared to WT (Wan et al., 2010). This effect was concurrent with decreased readily releasable pool size, decreased calcium sensitivity during exocytosis, early facilitation during initial pulses of stimulation trains, and increased time to recovery of membrane capacitance (Wan et al., 2010). Normalizing elevated calcium concentration in neurons from SV2B-null mice rescues aberrant neurotransmission. Conversely, elevating calcium concentration in WT neurons induced similar secretory deficits, suggesting that the phenotype induced by SV2B deletion is due to altered calcium homeostasis (Wan et al., 2010). These data again suggest that SV2 may function as a calcium transporter, sequestering calcium into synaptic vesicles (Wan et al., 2010), though no direct evidence of calcium transport has

been established. Vesicular uptake assays with radiolabeled calcium are necessary to directly address the capacity of SV2 to transport calcium.

The mechanism of calcium sensitivity may also be attributable to the interaction of SV2 with the calcium sensor, synaptotagmin (Bennett et al., 1992; Lazzell, 2004), which is direct, phosphorylation dependent, and sensitive to calcium, with an IC50 of approximately 10 μM (Pyle et al., 2000; Schivell et al., 1996; Schivell, 2005). While all three isoforms bind synaptotagmin-1, the N-terminus of SV2A and SV2C contain a unique site that is functionally important for neurotransmission: injection of N-terminal SV2A and C peptides into the presynaptic neuron inhibits evoked postsynaptic potential amplitude by 20-30% in a calcium-dependent manner (Schivell, 2005). Additionally, evidence suggests that SV2A and synaptotagmin are co-trafficked to vesicles (Yao, 2010). Deletion of SV2A or mutation of its endocytic motif reduces vesicular localization of synaptotagmin (Yao, 2010). The functional relevance of the SV2-synaptotagmin interaction has been debated as lentiviral expression of N-terminal ablated SV2 in cortical neurons of SV2A/B null mice rescues reduced train release and synaptic facilitation. These effects are indistinguishable from rescue with WT SV2, suggesting that the syanptotagmin-1 interaction is dispensable to SV2 function (Chang, 2009). However, the lentiviral constructs used to rescue SV2 deletion were tagged with EGFP (Chang, 2009), which contains a similar endocytotic motif to SV2 (Yao, 2010). Thus the observed rescue may be EGFP-dependent and could artificially mask the importance of SV2 in regulating synaptotagmin localization.

SV2 in vesicle trafficking. As with calcium interactions, the involvement of SV2 in the synaptic vesicle cycle has been the subject of debate. Deletion of SV2A in neurosecretory adrenal chromaffin cells reduces the size of the readily releasable pool by 50% (Xu, 2001). This reduction is correlated with decreased high molecular weight SNARE complex formation, suggesting that SV2 acts prior to fusion (Xu, 2001). Interestingly, the kinetics of vesicle fusion are unaltered, suggesting that the trafficking deficit, and not calcium affinity, dictates the SV2 deletion phenotype. This initial decrease in synaptic release has been demonstrated in retinal neurons (Wan et al., 2010), cortical neurons (Chang, 2009), hippocampal neurons (Custer et al., 2006), striatal slices (Dunn et al., 2016; Stout et al., 2016), the rostral ventral pallidum (Stout et al., 2016), and peripheral sympathetic neurons (Vogl et al., 2015). Additionally, neurons lacking SV2 show delayed recovery of the readily releasable pool following stimulus-induced depletion (Vogl et al., 2015; Wan et al., 2010). It has been postulated that deficits in the readily releasable pool are due to neuronal adaptation to chronically elevated calcium levels in SV2 ablated neurons (Wan et al., 2010).

Anchor of vesicle structure and cycling. Over 80 integral membrane proteins reside on the synaptic vesicle membrane. The transmembrane domains of these proteins are thought to occupy 25% of the overall vesicle membrane surface (Takamori et al., 2006). Additionally, reconstructed images of the frog neuromuscular junction obtained via electron tomography indicate that luminal macromolecules occupy 10% of the vesicle's volume, contacting the luminal membrane at 25 sites (Harlow et al., 2001; Harlow, 2013). This luminal assembly has a bilateral shape consisting of four arms radiating out

from a central focal point and is found, nearly identically, in all synaptic vesicles (Harlow, 2013). The points at which the density contacts the luminal membrane are associated with the macromolecules that regulate fusion to the active zone, and as the vesicle traffics to the active zone its orientation is brought into precise and consistent alignment (Harlow, 2013). Possible transmembrane vesicular protein candidates that contribute to the luminal density include SV2, synaptobrevin, and synaptotagmin (Harlow, 2013). Of these proteins, SV2 the proposed mainstay of luminal protein assembly due to the length of its luminal domain, which is greater than the diameter of the vesicle lumen. Given that five copies of SV2 localize to all synaptic vesicles (Mutch et al., 2011), it is likely that these large intravesicular loops interact to form a backbone that anchors all other transmembrane vesicular proteins. Molecular evidence exists to support this hypothesis: large vesicular protein complexes between SV2 and other vesicular proteins have been identified (Baldwin and Barbieri, 2007; Baldwin and Barbieri, 2009; Bennett et al., 1992) Thus, SV2 may act as the master regulator of vesicle structure, anchoring all other transvesicular proteins into their appropriate orientation to enable efficient association with vesicular fusion machinery.

Stabilizer of neurotransmitter loading. SV2 proteins create a proteoglycan matrix within the lumen of synaptic vesicles (Alvarez de Toledo et al., 1993; Reigada, 2003; Scranton et al., 1993). Though SV2 proteins have predicted molecular weights ranging from 77-83 kDa based on their amino acid structure, glycosylation of luminal asparagine residues increases their size to ~95 kDa for lightly glycosylated and ~250 kDa for heavily glycosylated SV2 proteins (Scranton et al., 1993). This proteoglycan matrix binds 95% of

neurotransmitter and ATP within the vesicle, requiring ionic exchange for release and thereby regulating neurotransmission (Reigada, 2003). This work characterizes an SV2 ortholog found in the electric organ of *D. ommata* fish, which shares 60-62% homology with SV2A/B and 80% homology with SV2C. The importance of the intravesicular proteoglycan matrix to vesicular uptake, storage, and release of neurotransmitter has not been addressed.

Isoform independent function. Genetic deletion of SV2 results in synaptic dysfunction. Interestingly, in several systems loss of SV2A or SV2C results in compensatory upregulation of the other isoform, presumably to maintain total SV2 levels (Iezzi, 2005; Xu, 2001). Compensatory upregulation is insufficient to rescue aberrant neurotransmission, suggesting that SV2A and SV2C are not redundant (Iezzi, 2005; Xu, 2001). Genetic deletion of SV2B does not appear to have the same regulatory effect on SV2 expression, as loss of SV2B in the retina of mice does not alter SV2A expression and reduces total SV2 expression in the eye by 50% (Morgans et al., 2009).

SV2s in neurological disease

SV2s have been implicated in several neurological diseases. SV2A, as the molecular target for the antiepileptic drug (AED) levetiracetam (LEV) and its derivatives (e.g. seletracetam and brivaracetam), has been the subject of extensive investigation for its role in epilepsy. More recently, LEV has shown efficacy in treating symptoms and neurological features of mild cognitive impairment (MCI) and Alzheimer's disease (AD). This, combined with evidence of altered SV2A and SV2B expression in AD models, suggests a function for SV2A 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 for the treatment of neurological 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.

SV2A and epilepsy. The efficacy of LEV as an AED was well-known before identification of its molecular target, with FDA approval granted in 1999 for the treatment of partial onset seizures in adults. Serendipitously, the severe seizure phenotype of SV2A-KO mice was published in the same year (Crowder, 1999) though the levetiracetam-SV2A interaction wasn't discovered until 2004 (Lynch, 2004). This discovery 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 in epilepsy (Bennett, 2007; von Rosenstiel, 2007). LEV, along with its derivatives, is the only AED that targets the synaptic vesicle to reduce hyperexcitability and epileptogenesis. The exact role SV2A plays in epilepsy has not been fully described, though various lines of evidence implicate the protein in epilepsy pathology.

Epilepsy patients commonly have reduced SV2A gene and protein expression and this effect is recapitulated in animal models (Feng, 2009; Gorter et al., 2006; Shi, 2015; van Vliet et al., 2009), suggesting that reduced SV2A increases vulnerability for epileptogenesis. Alternatively, SV2A expression may increase during seizure kindling (Ohno et al., 2009). Consistent with this, low frequency stimulation in the hippocampus

in pharmacoresistant spontaneously epileptic rats increased SV2A expression and subsequently decreased seizure frequency (Wang et al., 2014). In one strain of chicken, 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, tumor level of SV2A expression is predictive of LEV-responsiveness, as patients with lower SV2A levels are more likely to be LEV non-responders (de Groot, 2011). Beyond altered protein expression, there is no apparent SV2A polymorphism associated with epilepsy risk or variation in LEV efficacy (Dibbens et al., 2012; Lynch et al., 2009). However, homozygosity for a recessive SV2A mutation results in LEV-nonresponsive epilepsy among a host of other neurological abnormalities (Serajee and Huq, 2015). 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 probablility, 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 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, brivaracetam 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 brivaracetam inhibit SV2A's role in trafficking vesicles to 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 and promote pathogenesis. 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 in epilepsy (Sugata et al., 2011). Finally, 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 AD, regardless of gender, race or ethnicity, has been known for decades (Chin and Scharfman, 2013; Horvath et al., 2016; Sherzai et al., 2014). Abnormal hyperactivity within the hippocampus occurs in the prodromal 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 (Chan et al., 2015; Ping et al., 2015; Siwek et al., 2015; Sola et al., 2015; Ziyatdinova et al., 2016). 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). Further, 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 with comorbid epilepsy (Sola et al., 2015). In particular, LEV reduces the characteristic hippocampal hyperactivity observed in AD and improves memory performance (Bakker et al., 2012). LEV has also been shown to improve cognition in non-demented epilepsy patients (Helmstaedter and Witt, 2010; Wu et al., 2009), as well as in non-epileptic aged mice and some mouse models of AD without seizure activity (Celikyurt et al., 2012; Devi and Ohno, 2013). 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. It has been hypothesized that pathological hippocampal hyperactivity in early stages of the AD prodromal period creates heightened neurodegenerative vulnerability in hippocampal CA3 cells. In fact, asymptomatic people who are at high genetic risk for AD (ApoE4, familial AD mutation carriers) have hyperactive CA3 nuclei. CA3 hyperactivity may interfere with memory formation and retrieval and increase the likelihood of neurodegeneration. Dampening hyperactivity with LEV treatment is effective at improving memory function (Bakker et al., 2012). However, this is not likely the only explanation for the efficacy of LEV in AD, as it would be expected that any AED, not just LEV, would show similar results on cognition. LEV stimulated neuritogenesis and increased synaptic marker expression in an *in vivo* model of late-onset AD, possibly through direct interaction with newly discovered mitochondrial SV2A (Stockburger et al., 2015). This may counter the regional loss of

synapses observed in aMCI/AD progression (Robinson et al., 2014; Sze, 2000), and may be an additional mechanism behind the cognitive improvement seen with LEV treatment.

SV2B may also play a 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 a differential posttranslational modification profile (Heese et al., 2001). Furthermore, SV2B-KO appears to protect against toxicity and cognitive deficits in mice induced by injected 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 (1999), the investigators immediately postulated that it may be important in basal-ganglia functions and PD (Janz, 1999). Indeed, subsequent studies have supported this idea. SV2C was recently identified as a genetic modifier of PD risk in smokers: the minor alleles at multiple loci in the promoter region of the SV2C gene confer a significantly increased risk for PD in smokers, reversing the normally protective effect of nicotine consumption in PD (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, 2013).

More recently, our group has shown a more direct relationship between SV2C 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 et al., 2016). 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 (Alter, 2013). Finally, SV2C's promotion of proper vesicular and neuronal function may explain in part the observation that statins protect against PD, as SV2C expression increases after treatment with statins (Schmitt, 2016).

SV2C in addiction. 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). Knockdown of SV2C expression by stereotaxic lentiviral miRNA injection into the midbrain of adult mice reduced cocaine place preference compared to controls, whereas global SV2C knockout mice show no alteration in preference (Dardou, 2013). Our group recently demonstrated that global SV2C-KO animals have reduced preference for

methamphetamine (METH), in conjunction with reduced locomotor stimulant effect (Stout et al., 2016).

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 the vesicular monoamine transporter 2, which is responsible for packaging monoamines such as dopamine into vesicles, protecting it from metabolism and readying the neuron for appropriate release. Historically, the VMAT2-targeted drug, tetrabenazine has shown promise in treating a number of diseases, but non-desirable effects presumably from concomitant depletion of other monoamines (serotonin, norepinephrine, histamine) has limited the applicability of targeting the nondiscretely expressed protein (Jankovic and Clarence-Smith, 2011; Jankovic et al., 1984; Kenney et al., 2007; Kenney et al., 2006; Leung and Breden, 2011; Paleacu et al., 2004). Recent evidence, however suggests that these effects are actually off-target, the byproduct of interaction of the ß enantiomer with D2 receptors. Isolation of the VMAT2-targeting enantiomer drastically improves both the tolerability and efficacy of the drug in treating tardive dyskinesia (Muller, 2015), suggesting improved outcomes for other disease treatments. Regardless, SV2C represents a novel, unexplored, and discrete therapeutic target for the treatment of addiction.

Conclusions

Synaptic release is an elegantly orchestrated phenomenon, in which dozens of proteins work together in synchrony to accomplish the single goal of neurotransmission. It is clear that SV2 plays a significant role in this process, though the details of its exact role have yet to be unveiled. Further research is needed to fully elucidate the mechanism by which SV2 modulates release. In particular, analysis of temporally and spatially controlled SV2 ablation is needed. Further, interrogation of the role the protein plays in endocytosis, the other side of the vesicle cycle, is vital. The potential of SV2 as an effective therapeutic target is unquestionable. Further application of SV2 targeting drugs is needed, particularly isoform specific therapeutics. Drugs that stabilize neurotransmission are needed for a plethora of disorders beyond epilepsy, including: addiction, neurodegeneration, motor disorders, mood disorders, and schizophrenia.

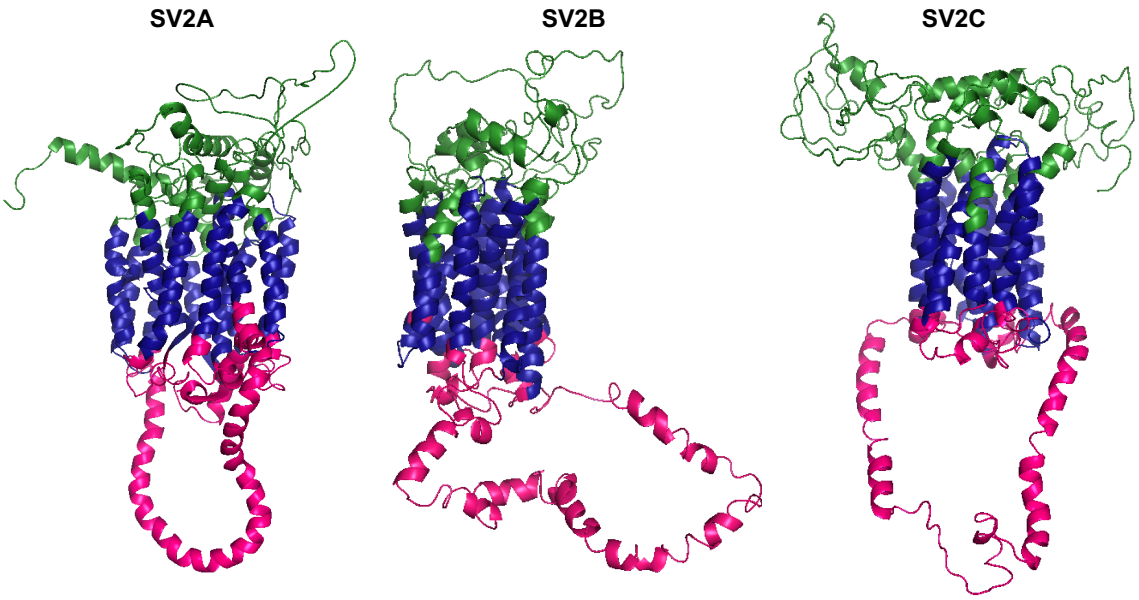
SV2A
SV2B
SV2C

Figure 2- 1. Predictive structures of SV2 isoforms.

Protein modeling for SV2A, SV2B, and SV2C was conducted using MacPyMOL. Cytosolic domains are shown in green, transmembrane domains in blue, and intravesicular/extracellular domains in pink.

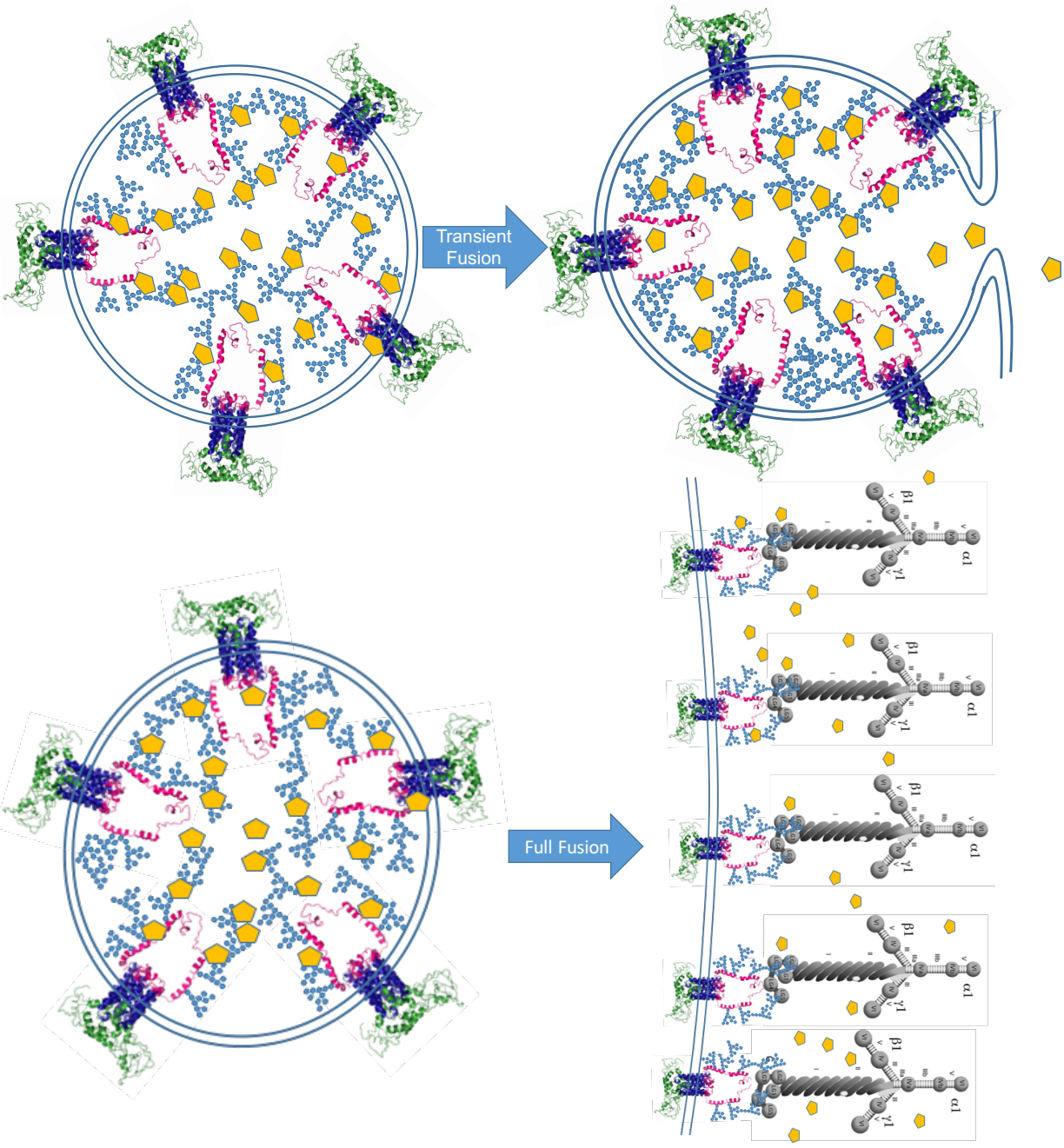
Transient Fusion
Full Fusion

Figure 2- 2. Potential SV2 mechanisms.

Glycosylation of SV2 (light blue) in non-fused vesicles is predicted to bind free neurotransmitter (yellow pentagon) within the vesicular lumen, allowing increased packaging (A, C). Evidence suggests SV2 interacts with the extracellular protein, laminin. This interaction, shown in D, may dictate full (D) verses transient (B) vesicle fusion and may also be important for appropriate endocytotic trafficking

Chapter 3: Selective enhancement of dopamine release in the ventral pallidum of methamphetamine-sensitized mice

This chapter is a reproduction of the following published article:

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Abstract

Drugs of abuse induce sensitization, which is defined as enhanced response to additional drug following a period of withdrawal. Sensitization occurs in both humans and animal models of drug reinforcement and contributes substantially to the addictive nature of drugs of abuse, as it is thought to represent enhanced motivational wanting for drug. The ventral pallidum, a key member of the reward pathway, contributes to behaviors associated with reward, such as sensitization. Dopamine inputs to the ventral pallidum have not been directly characterized. Here we provide anatomical, neurochemical, and behavioral evidence demonstrating that dopamine terminals in the ventral pallidum contribute to reward in mice. We report subregional differences in dopamine release, measured by *ex vivo* fast-scan cyclic voltammetry: rostral ventral pallidum exhibits increased dopamine release and uptake compared with caudal ventral pallidum, which is correlated with tissue expression of dopaminergic proteins. We then subjected mice to a methamphetamine-sensitization protocol to investigate the contribution of dopaminergic projections to the region in reward related behavior. Methamphetamine-sensitized animals displayed a 508% and 307% increase in baseline dopamine release in the rostral and caudal ventral pallidum, respectively. Augmented dopamine release in the rostral ventral pallidum was significantly correlated with sensitized locomotor activity. Moreover, this presynaptic dopaminergic plasticity occurred only in the ventral pallidum, and not the ventral or dorsal striatum, suggesting that dopamine release in the ventral pallidum may be integrally important to drug-induced sensitization.

Key words: dopamine, ventral pallidum, voltammetry, sensitization, methamphetamine

Introduction

Psychostimulant (e.g., cocaine and methamphetamine) abuse is a major public health concern. In 2013, an estimated 2.15 million Americans were recent psychostimulant users, contributing significantly to the estimated $712 billion societal cost of substance abuse[1-4]. One of the most pernicious characteristics of addiction is its persistence: 40-60% of drug users relapse within one year of abstinence (McLellan et al., 2000). Chronic drug use alters brain neurochemistry and these changes do not quickly normalize after drug cessation (Kalivas and Volkow, 2005; Nestler, 2001; Nestler, 2005). Understanding the long lasting neurobiological changes induced by chronic drug use is critical for both the treatment of addiction and the prevention of relapse.

One such persistent neurobiological change caused by drugs of abuse is sensitization, defined by heightened response to additional drug following a period of withdrawal. Sensitization occurs in human (Boileau et al., 2006; Cox et al., 2009) and animal (Shuster et al., 1975; Wallach and Gershon, 1971) models following chronic drug exposure. The incentive sensitization hypothesis of addiction posits that sensitized behavior (typically measured in rodents as augmented locomotor behavior) stems from hypersensitization of mesocorticolimbic circuits, resulting in enhanced salience, or motivational wanting, to drugs and drug-related cues (Robinson, 2008). Non-contingent dosing regimens, such as sensitization and conditioned place preference, recapitulate many neurocircuitry alterations induced by response-contingent dosing regimens, such as self-administration and reinstatement (reviewed by Steketee and Kalivas (2011) and Vezina (2004)). Given the similarity in circuitry changes, it has been proposed that these models share similar

construct validity and both recapitulate important aspects of the human condition (Steketee and Kalivas, 2011). Recent studies have identified the ventral pallidum, the major output of the nucleus accumbens, as a mediator of sensitization (Chen et al., 2001; Johnson and Napier, 2000; McDaid et al., 2005; McDaid et al., 2006a; McDaid et al., 2006b; McDaid et al., 2007; Mickiewicz et al., 2009; Napier and Istre, 2008; Rokosik et al., 2013).

The ventral pallidum was originally described as the ventral extension of the globus pallidus; while this description partially defines the anatomy of the ventral pallidum, the subcommisural structure extends far more rostral than its globus counterpart, reaching to the most rostral portions of the striatum (Figure 1, 2). The ventral pallidum forms reciprocal feedback loops with the major structures involved in reward signaling, including the nucleus accumbens (NAc), ventral tegmental area (VTA), substantia nigra, lateral hypothalamus, thalamus, amygdala, and others (Groenewegen et al., 1993; Kalivas et al., 1993; Lavin and Grace, 1998). In turn, the ventral pallidum projects strongly to the brain stem, including the pedunculopontine tegmentum, acting as a central convergence point for translation of limbic stimuli into motor output (Smith et al., 2009). Additionally, stimulation of the ventral pallidum is capable of directly initiating reward signaling (Gong et al., 1996; Gong et al., 1999; Panagis et al., 1995).

Electrophysiological, neuroanatomical, and behavioral data suggest differential roles for rostral versus caudal ventral pallidum (RVP and CVP, respectively). The CVP, defined here as the ventral pallidum caudal to the fused anterior commissure (+0.14 bregma) (Mahler et al., 2014), contains neurons electrophysiologically similar to neurons in the

globus pallidus. In contrast, RVP neurons, defined as rostral to the fused anterior commissure(Mahler et al., 2014), are more akin to their neighboring neurons in the NAc (Kupchik and Kalivas, 2013; Mogenson et al., 1983; Napier et al., 1991). Further, CVP stimulation is considerably more rewarding than RVP stimulation, though rats will self-administer direct electrical stimulation to both regions (Panagis et al., 1995). Ablation of RVP signaling attenuates cue-induced cocaine reinstatement, whereas CVP inhibition blocks drug-primed reinstatement (Mahler et al., 2014).

Several pieces of evidence identify the ventral pallidum as an important participant in drug-induced sensitization. The ventral pallidum is integrally involved in induction of morphine-sensitization; pharmacological inhibition of mu opioid receptors by microinjection into the ventral pallidum completely abolishes both induction and expression of sensitized behavior (Johnson and Napier, 2000; Mickiewicz et al., 2009). For stimulants, methamphetamine (METH) sensitization alters pCREB and ΔFosB expression in the ventral pallidum and NAc of sensitized rats at 3 days post drug withdrawal, indicative of increased postsynaptic activity. At 14 days post withdrawal rats remain sensitized to METH and activity-dependent changes (upregulation of pCREB and ΔFosB expression) persist only in the ventral pallidum (McDaid et al., 2006a), suggesting that activity in the ventral pallidum may drive sensitized behavioral response to stimulants.

The ventral pallidum receives input from VTA dopaminergic neurons. The role of dopaminergic innervation in subregions of the ventral pallidum has not been fully

described, though our research builds on several key experiments suggesting dopamine in the region plays a key role in reward behavior. First, microinjection of stimulants or dopamine agonists/antagonists into the ventral pallidum elicits a motor response and can induce sensitization and place preference (Fletcher et al., 1998; Gong et al., 1996; Gong et al., 1999; Napier and Chrobak, 1992). Further, 6-OHDA lesioning of the ventral pallidum, which preferentially lesions dopamine terminals, blocks cocaine place preference acquisition (Gong et al., 1997). Additionally, amphetamine sensitization in rats increases production of dopamine metabolites, 3,4-dihydroxyphenoylacetic acid and homovanillic acid, in the ventral pallidum (Chen et al., 2001). Though the mechanism of this augmentation has not been established, one likely explanation is increased dopamine release. Given these data, we hypothesized that stimulant sensitization induces presynaptic dopamine plasticity and that such enhanced dopamine release contributes to the long-term behavioral alterations associated with stimulants. To test this hypothesis, we used fast-scan cyclic voltammetry to measure dopamine release in the RVP and the CVP and demonstrate a substantial and selective enhancement of dopamine transmission in the ventral pallidum of METH-sensitized mice.

Results and Discussion

The contribution of dopaminergic inputs in the ventral pallidum to reward behavior is not well established. Here, we present the first recording of dopamine release in the ventral pallidum by FSCV. We utilized the technique to assess differential dopamine neurotransmission in rostral versus caudal ventral pallidum. Additionally, we provide

evidence of selective augmentation of baseline dopamine transmission in the ventral pallidum of sensitized mice.

Dopaminergic neuroanatomy of the ventral pallidum. We performed immunohistochemistry to define dopaminergic neuroanatomy in the ventral pallidum (Figure 1). The synaptic vesicle glycoprotein 2C (SV2C) is robustly expressed throughout the entire ventral pallidum (Dardou, 2013; Janz, 1999), irrespective of subregion, and was used to define the structure. Dopamine terminal markers tyrosine hydroxylase (TH), the dopamine transporter (DAT), and the vesicular monoamine transporter 2 (VMAT2) are expressed in the ventral pallidum. TH expression is robust in the region, particularly in the RVP, though less than in canonically dopamine-rich regions such as the striatum. VMAT2 is sparsely but consistently expressed throughout the ventral pallidum. Of the three dopamine terminal markers, DAT is expressed at the lowest levels, with slightly more expression in RVP than CVP. Given the relative abundance of TH compared to DAT, these terminals likely rely on synthesis of new dopamine for release rather than recycling of neurotransmitter via plasmalemmal uptake.

These results are supported by immunogold electron microscopy studies conducted by Mengual and Pickel, which show that TH and DAT are both expressed in terminal regions and small, unmyelinated axons within the ventral pallidum, though DAT expression is significantly lower than TH expression (Mengual and Pickel, 2002; Mengual and Pickel, 2004). Interestingly, this work also identified graded expression of the proteins in medial and lateral subregions of the ventral palidum, with DAT expressed most strongly in lateral versus medial ventral pallidum (Mengual and Pickel, 2004).

Additionally, TH expression in the medial region does not strongly colocalize with presynaptic dopamine D2 autoreceptors (Mengual et al., 2002). These three observations: low DAT, high TH, and no autoreceptor expression are the hallmark identifiers of a recently identified subpopulation of atypically fast-firing VTA dopamine neurons that project to the medial prefrontal cortex (Lammel et al., 2008). In line with the tissue expression of DAT, voltammetry recordings of dopamine release in the prefrontal cortex reveal substantially decreased dopamine clearance compared to striatal regions (Garris et al., 1993). Low DAT:TH ratio and reduced D2 autoreceptor expression are also hallmarks of dopamine neuron terminals within the ventral pallidum and, as described below, these neurons also have substantially decreased clearance kinetics. Additionally, it has been demonstrated that animals that lack DAT have profound neuronal plasticity due to reduced clearance rate, which potentiates the signal (Jones et al., 1998). Extrapolation of this data implies that regions which express very little DAT but moderate amounts of TH, such as the prefrontal cortex or ventral pallidum, may be at enhanced risk for pathogenic alterations due to augmented signaling produced by drugs of abuse.

Dopamine release in subregions of the ventral pallidum by fast-scan cyclic voltammetry (FSCV). Dopamine release in the ventral pallidum was detected by FSCV. Initial experiments to identify optimal stimulation parameters were conducted in sagittal brain slices, irrespective of subregion. The optimal parameters identified were 60 pulse, 60 Hz, 600 μA, 2 ms, at 10 minute intervals (Figure 3). Though larger current and pulse width stimulation resulted in larger dopamine overflow, these settings resulted in

electrolytic lesioning in a number of brain slices, necessitating reduction of these parameters.

To investigate subregional differences within the ventral pallidum, coronal slices containing the RVP and CVP were carefully chosen to ensure correct identification of the appropriate region (Figure 2, 5A-D). Stimulation elicited an average of 1.04 μM and 0.38 μM dopamine release in the RVP and CVP, respectively ($p=0.011$, RVP: $n=19$, CVP $n=12$, Figure 4C-D, G). Additionally, dopamine clearance, as measured by the rate constant tau, was faster in RVP than CVP (3.19 versus 8.25 seconds, respectively, RVP: $n=19$, CVP $n=12$, Figure 4H), though significantly slower than in dorsal or ventral striatum (Figure 1, 0.59 and 0.75 seconds, respectively, $p=0.002$, one-way ANOVA with Newman-Keuls multiple comparison test). Nomifensine (10 μM), a dopamine and norepinephrine transporter inhibitor, increased release 260.2% in the RVP ($p=0.003$, $n=3$, Figure 6I, one-way ANOVA with Newman-Keuls multiple comparison test) and 23.9% in the CVP ($p=0.003$, $n=3$, Figure 6J, one-way ANOVA with Newman-Keuls multiple comparison test). These data further confirm enhanced DAT expression in RVP compared with CVP, given the 10-fold increase in augmentation in RVP. Neither region demonstrated substantial or significant enhancement of signal in response to α2-adrenergic autoreceptor idazoxan (Figure 6 I-J), which augments norepinephrine release, further confirming the measured analyte was dopamine.

As this is a novel region for FSCV experiments, recording site validity was carefully assessed. One key advantage of slice voltammetry is visualization of the recording site, which ameliorates many of the concerns of electrode placement. Slices were carefully chosen as described in Figure 4A-D, using the anterior commissure,

internal capsule, and lateral olfactory tract (which are readily visible in slices) as reference. Additionally, the recording site in a number of brain slices was electrolytically lesioned using a new electrode (using the stimulation electrode as a reference to maintain site location, n=6). These brain slices were then embedded in a hydrogel solution followed by passive lipid clearing, generating an optically clear and antibody-permeable slice (Chung and Deisseroth, 2013; Chung et al., 2013). In both RVP and CVP brain slices, recording sites did not co-localize strongly with DAT compared with neighboring characteristically dopaminergic structures, NAc and dorsal striatum (STR, Figure 5H). DAT expression was chosen due to the incredibly high specificity of the antibody for dopaminergic regions. Strong and specific antibodies are vital to effective CLARITY staining. Other antibodies which specifically label the ventral pallidum, including SV2C and substance P, generated insufficient resolution to be viable for assessing electrode placement.

The ventral pallidum is a heterogeneous structure; several groups have identified key differences in the neurochemical (Smith et al., 2009), electrical (Kupchik and Kalivas, 2013; Root et al., 2015), and anatomical (Groenewegen et al., 1993; Kalivas et al., 1993; Kupchik and Kalivas, 2013; Mahler et al., 2014; Root et al., 2015) properties of the RVP versus the CVP. Here, we add to the evidence of dichotomy between the structures by characterizing differential dopamine release within the ventral pallidum, with highest dopamine release and uptake in the rostral regions (Figure 4). Increased dopamine tone in the RVP versus the CVP is coupled with enhanced dopamine clearance, evidenced by decreased rate constant, tau (Figure 4H), enhanced effect of plasmalemmal transporter inhibition (Figure 5I,J), and increased tissue expression of DAT (Figure 1).

The potential behavioral importance of differential dopamine release within the ventral pallidum is of particular interest. Microiontophoretic injection of dopamine or dopamine receptor agonists into the ventral pallidum alters firing in about 50% of tested neurons, both increasing and decreasing postsynaptic activity (Napier and Maslowski-Cobuzzi, 1994; Napier and Potter, 1989). Additionally, co-administration of dopamine with GABA or glutamate reduces neuronal firing rate, though potentiation was observed in a subset of recordings (Johnson et al., 1997). Interestingly, this work displayed a rostrocaudal distribution in the neuromodulatory effect of exogenous dopamine administration, with less alteration of GABA and glutamate activity with co-application of dopamine in the rostral subregion. This is not surprising, given the reduction in tau and dopamine transporter level in the region. Assuming an equivalent administration in both regions, dopamine injection in the CVP should have a greater effect, as it persists in the synaptic space substantially longer than in the RVP. Recent work identified that in a rat reinstatement model RVP modulates cue response (Mahler et al., 2014), whereas CVP is more attuned to modulation of hedonic response (Cromwell and Berridge, 1993; Ho and Berridge, 2013; Panagis et al., 1995; Smith and Berridge, 2005; Tindell et al., 2005). Given the enhanced efficacy of dopamine in the CVP, the potential importance of these terminals for modulation of plasticity in response to hedonic stimuli is profound. Likewise, plasticity induced by dopamine release in the RVP may be integral to cue-dependent behavior. Though clearance is faster in rostral than caudal subregions of the ventral pallidum, it is still substantially slower than striatal clearance. Additionally, the RVP releases significantly more dopamine than the CVP, thus dopamine-induced synaptic modulation may be quite profound in the RVP. Finally, the lack of association

with presynaptic autoreceptors makes these neurons prime candidates for presynaptic plasticity, as activation of D2 autoreceptors is thought to reduce dopamine production by inhibition of TH (Lindgren et al., 2001; O'Hara et al., 1996; Pothos et al., 1998), alter VMAT2 expression (Brown et al., 2001), and augment DAT function (Benoit-Marand et al., 2001; Cass and Gerhardt, 1994; Meiergerd et al., 1993), thereby inhibiting dopamine signaling. Further, D2 knockout mice have substantially augmented dopamine release compared to WT controls (Schmitz et al., 2002). Thus dopamine release in the ventral pallidum, which persists for many seconds within the synaptic space and is not regulated by D2-dependent feedback mechanisms, may make the region uniquely vulnerable to modulation by drugs of abuse. Further investigation into how dopamine inputs in the ventral pallidum modulate both pre- and post-synaptic plasticity is key to more fully understanding the mechanistic importance of these projections.

Chronic METH treatment enhances baseline dopamine release in the ventral pallidum. To determine whether sensitization to METH persistently enhances dopamine release in the ventral pallidum, we performed sensitization experiments (Jing et al., 2014). Mice received 2 mg/kg METH or saline intraperitoneally for 7 days. Following a 7-day washout (on day 14), we challenged all animals with 1 mg/kg METH and measured locomotor response. Mice pre-treated with METH exhibited marked sensitization (Supplementary Figure 2, 224% increase compared with saline pre-treated animals, $p<0.0001$, $n=21$, two-tailed t-test). METH-induced behavior (Nikaido et al., 2001), dopamine overflow (Chen et al., 2007), and direct measurement of METH concentration in brain tissue (Zombeck et al., 2009) return to baseline within 4 hours of drug administration. As METH is no longer present within the brains of challenged

animals, baseline neurochemical changes were assessed the following day. On day 15, we extracted brains and performed FSCV. In this drug-free state, sensitized mice displayed a 507% increase in dopamine release in RVP ($p=0.003$, $n=6$, Figure 6A, two-tailed t-test) and a 308% increase in CVP ($p=0.017$, $n=8$, Figure 6B, two-tailed t-test) compared with saline controls. This effect was selective to the ventral pallidum: no augmented release was observed in DSTR, NAc core or shell (Figure 7, Table 1). Interestingly, elevated locomotor activity is directly correlated with the magnitude of dopamine release in the RVP ($r^2=0.645$, $p=0.03$, $n=7$, Figure 6, linear regression analysis). No correlation exists between baseline dopamine release and motor behavior in the CVP ($r^2=0.001$, $p=0.931$, $n=8$, Figure 6, linear regression analysis). Though it is not likely that residual drug is present during FSCV experiments, it is possible that drug challenge produces an acute augmentation of dopamine release in sensitized animals that we are capturing by assessing 24 hours after testing. As all animals (METH sensitized and saline controls) receive the 1 mg/kg METH challenge, this effect would still be due to sensitization. Though beyond the scope of this initial work, additional experiments to assess the time course of augmented release could reveal interesting insights into the role of the ventral pallidum in sensitization.

The subregional difference in motor response is of particular interest, given the heterogeneity of signaling in the two subregions with respect to behavior. The CVP modulates hedonic response (Ho and Berridge, 2013). Direct electrical stimulation of the CVP is highly rewarding, with threshold frequency (a mathematical calculation indicative of the reinforcing efficacy of stimulation) similar to those observed in the regions of highest reward, VTA and dorsal raphe (Panagis et al., 1995). Ablation of CVP signaling

produces sucrose aversion (Cromwell and Berridge, 1993; Smith et al., 2009) and blocks drug primed reinstatement of cocaine seeking (Mahler et al., 2014). This proposed hedonic hotspot led to the theory that the CVP plays a major role in drug "liking". Less is known about the RVP, but it may be more involved in modulation of drug "wanting" as ablation of the region abolishes cue-induced cocaine reinstatement (Mahler et al., 2014; Smith et al., 2009). Interestingly, expression of locomotor sensitization is cue dependent: animals moved to a novel environment following sensitization induction do not express heightened locomotor response on test day (Battisti et al., 1999; Cabib, 1993; Mead et al., 2004; Tirelli and Terry, 1998). Thus, it is logical that augmented dopamine release in the RVP is strongly associated with a cue-dependent behavior like locomotor sensitization.

Mechanistic considerations. The mechanism of augmented dopamine release in the ventral pallidum of sensitized animals is not clear, though long-term plasticity in the region is apparent. In general, discussions of plasticity normally address augmented postsynaptic response to a given stimulus. Postsynaptic plasticity can have two possible causes: enhanced sensitivity of postsynaptic receptors or enhanced presynaptic release. One of the key advantages of voltammetry is the ability to directly examine presynaptic release. Here we show that chronic METH administration enhances presynaptic release in the absence of exogenous drug (Figure 6), suggesting METH induces long-term modulation and enhancement of baseline dopamine signaling. Further, dopamine plasticity only occurs in the ventral pallidum (Figure 7, Table 1) and is tightly correlated with sensitized behavior in rostral subregions (Figure 6). Plasticity in reward circuits

underlies addiction, particularly relapse (Luscher, 2013). A major addiction hypothesis posits that addiction occurs when normally innocuous cues become linked with drug consumption (Robinson, 2008). Reintroduction to such cues, even after years of abstinence, can elicit relapse (Luscher, 2013). It is thought that these cue associations are encoded in the brain via plasticity in reward circuits (Luscher, 2013). Thus, long-term dopamine plasticity in the ventral pallidum, particularly in the RVP, which modulates cue associations (Mahler et al., 2014), likely plays an integral role in addiction.

Additional mechanistic contributions may include neurocircuitry changes to reward pathways in sensitized animals. Chronic stimulant administration induces long-term depression of GABAergic NAc projections to the ventral pallidum (McDaid et al., 2005; Thomas et al., 2001). Additionally, pharmacological GABA receptor inhibition in the ventral pallidum increases locomotor behavior (Kalivas and Duffy, 1990). It follows that depression of GABAergic inputs to the ventral pallidum would contribute to enhanced dopamine tone in the structure, which may contribute to the augmented motor response observed in sensitization. Additionally, the NAc shell is thought to be particularly important in sensitized behavior (Pierce and Kalivas, 1995). Interestingly, the shell projects primarily to the medial subcommissural ventral pallidum (Heimer et al., 1991), which is encompassed in our RVP slice. This may explain both the disparity in augmentation of dopamine tone between the CVP and RVP and the correlation between dopamine release and motor behavior in the RVP of sensitized mice (Figure 6).

The ventral pallidum is a heterogenous structure and the behaviors it contributes to are complex. Here, we define subregional differences in dopamine release, dopamine

clearance, and dopaminergic protein expression in the ventral pallidum. Additionally, we identify subregional differences in response to METH-sensitization in the ventral pallidum. To our knowledge, this is the first report of enhanced baseline dopamine release in any brain region of behaviorally-sensitized mice. These data demonstrate that METH induces presynaptic dopaminergic plasticity and suggest that augmented dopamine release, selectively in the ventral pallidum, mediates locomotor sensitization and may initiate drug seeking motor behavior.

Materials & Methods

Mice: All procedures were carried out in accordance with NIH guidelines and the Institutional Animal Care and Use Committee at Emory University. Male C57BL/6 mice were purchased from Charles River Laboratories. Mice were group housed in a 12-h light cycled room with food and water ad libitum. Behavioral and neurochemical experiments were conducted at 3-6 months of age. Most of the literature cited within the introduction was conducted in rats. These experiments were conducted in mice due to the enhanced genetic tools available in mice, as subsequent research will build upon these preliminary studies, analyzing dopamine release within the ventral pallidum of genetically manipulated mice.

Immunohistochemistry: Mice were perfused transcardially with 4% paraformaldehyde. Brains were removed and processed for frozen sectioning. Slices (40 μm) underwent hot citrate buffer antigen retrieval (Biogenex) and were blocked in 10% normal goat serum or

3% normal horse serum. Polyclonal anti-SV2C serum was isolated from rabbits injected with an N-terminal peptide (amino acids 97-114: STNQGKDSIVSVGQPKG) conjugated to Imject® Maleimide Activated mcKLH (Thermo Scientific) and sera were generated for our laboratory using Covance Custom Immunology Services. Sections were incubated with polyclonal rabbit anti-SV2C serum (Covance, 1:2,500), rat anti-DAT (Millipore MAB369, 1:1,000), mouse anti-TH (Millipore MAB318, 1:1,000), or rabbit anti-VMAT2 (generated in our laboratory (Lohr, 2014), 1:10,000) followed by biotinylated (Jackson ImmunoResearch: goat anti-rabbit biotin 111-065-144, goat anti-mouse biotin 115-065-003, goat anti-rat 112-065-003) secondary antibody. Visualization was performed with a 3,3-diaminobenzidine (DAB) reaction (Vector Laboratories) for biotinylated secondary antibodies. Images were acquired with a NeuroLucida epifluorescent microscope (MicroBrightField).

Fast-scan cyclic voltammetry: FSCV was performed in sagittal and coronal slices as previously described (Lohr, 2014). In brief, animals were rapidly decapitated and the brain sliced at 300 μM in oxygenated, ice-cold artificial cerebral spinal fluid (aCSF [in mM]: NaCl [126], KCl [2.5], NaH_2PO_4 [1.2], $CaCl_2$ [2.4], $MgCl_2$ [1.2], $NaHCO_3$ [25], and glucose [11], pH 7.4) with added 194 mM sucrose using a vibrating tissue slicer. Slices containing the ventral pallidum were identified visually primarily by the shape of the anterior commissure (AC), which is a key advantage of slice voltammetry. The slice rostral of the fully fused AC (Figure 2) was chosen as the RVP slice (Figure 2, 5A,C) and the slice immediately caudal, where the AC begins to break up, chosen as the CVP slice (Figure 2, 5B,D). Slice orientation was maintained throughout the experiment to ensure

that the slice surface recorded from was nearest the fused AC in either the rostral or caudal direction. Brain slices were placed in a slice perfusion chamber and incubated at room temperature in oxygenated aCSF for 30 minutes. The appropriate slice was then transferred to a recording chamber where it was perfused with oxygenated aCSF at 32ºC. Following a 30-minute incubation, a carbon fiber microelectrode was inserted 50-70 μM below the surface of the brain slice and the stimulating electrode placed approximately 250 μM away. DA release was elicited by electrical stimulation (1-60 pulse, 30-60 Hz, 300-700 μA). Optimal stimulation parameters identified for the ventral pallidum were 60 Hz, 60 pulse, 600 μA, and 2 ms pulse width with 10-minute intervals between stimuli (Figure 3). All other regions stimulation parameters were 1 pulse, 700 μA, and 4 ms pulse width at 5-minute intervals. A cyclic voltage ramp (-0.4 to 1.3V) was applied to the carbon fiber microelectrode and resultant background-subtracted current measured. All reported regions were surveyed with 4 recording replicates at 3 independent sites, which were averaged for each animal. Experiments were conducted and analyzed using Demon software (Wake Forest University). Following experimentation, a number of slices were electrolytically lesioned with a new electrode to further confirm recording site. The experimental recording electrodes were calibrated to known dopamine standards using a flow cell.

Electrophoretic tissue clearing: Following FSCV experiments, a number of brain slices underwent CLARITY preparation, as described by Chung and Diesseroth (Chung et al., 2013). Slices were incubated at 4°C in hydrogel monomer solution (40% acrylamide, 0.25% VA-044, 4% PFA in PBS) for several days. Slices were polymerized at 37°C

following nitrogen de-gassing. Polymerized slices were passively cleared for 1 week in clearing buffer (200 mM boric acid, 4% SDS) at 37°C. Slices were then rinsed for 2 days in 0.1% Triton-PBS. For imaging, slices were incubated in buffer (0.1% Triton X-100, 1 M sodium borate, pH 8.5) plus the antibody of interest (1:500) for 2 days at 37°C, rinsed for 1 day in PBST, incubated with secondary antibody (1:100) in PBST+1M sodium borate for 2 days then rinsed for one day. The slices were transferred to 80% glycerol in water, which matches the optical density of the clarified slice, and incubated for 1 day. The slices were imaged with a NeuroLucida epifluorescent microscope (MicroBrightField).

Locomotor sensitization: The day prior to the first day of drug administration, animals were habituated to the locomotor recording chamber. 8-10 week old male mice were injected intraperitoneally with either 2 mg/kg METH or equivalent volume saline control for seven days (Jing et al., 2014). On day 14, all animals received 1 mg/kg METH intraperitoneally. Consecutive beam breaks were recorded for 30 minutes following drug injection in a chamber that measures infrared beam breaks (Photobeam Activity System, San Diego Instruments). On day 15, mice were rapidly decapitated and FSCV recordings taken to measure dopamine release in the VP. As voltammetry experiments are rate limiting, with a maximum of recording from two animals in a day, drug administration was staggered such that all animals received identical dosing regimens.

Drugs: Free-base corrected methamphetamine hydrochloride (Sigma Aldrich, St. Louis, MO, USA) was prepared immediately before injections. Nomifensine (Sigma Aldrich,

St. Louis, MO, USA) and idazoxan (Sigma Aldrich, St. Louis, MO, USA) were made in DMSO at 10 mM then serially diluted in aCSF to designated concentrations.

Statistical analysis: Unless otherwise noted, all data are represented as means with standard error of the mean. Data were analyzed by two-tailed t-tests or ANOVA to determine statistical significance. Statistical analysis was conducted using GraphPad Prism 6 and significance defined as $p<0.05$.

Acknowledgements: We would like to thank Dr. Sara Jones and her laboratory, in particular James Melchior, for their considerable technical assistance, wisdom, and encouragement. We also thank Dr. Donita Robinson for suggesting the presentation of the correlation between enhanced locomotion and dopamine release in the ventral pallidum. Finally, we acknowledge the expert technical assistance of Minagi Ozawa. This work was supported by NIH grants R0ES1023839, P30ES019776, T32ES012870, F31DA037652, F31NS089242, and the Lewis Dickey Memorial Fund.

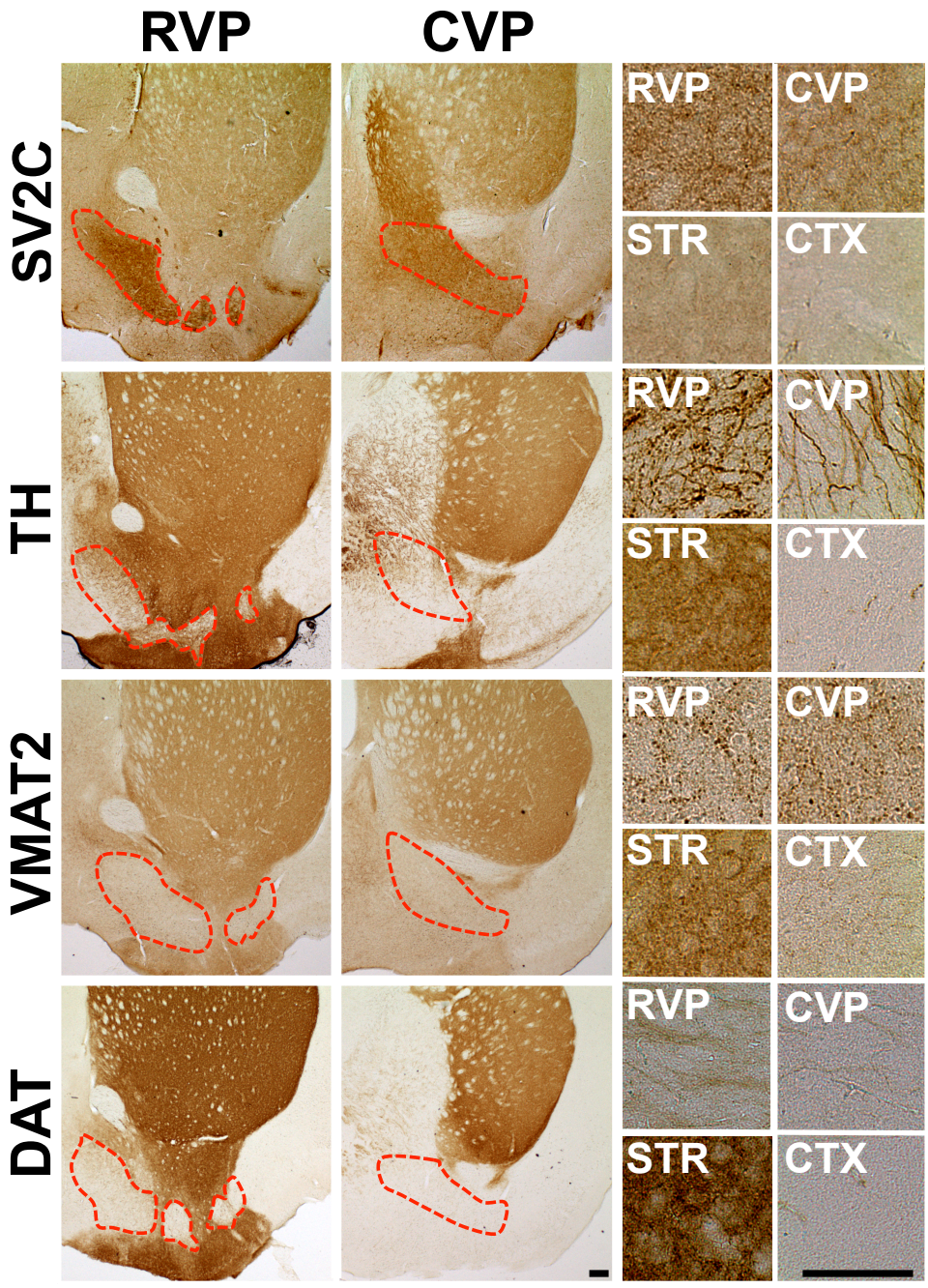

RVP
CVP
SV2C
TH
VMAT2
DAT
RVP
CVP
STR
CTX

Figure 3- 1. Dopamine neuroanatomy in the ventral pallidum.

Immunohistochemistry in coronal (field view) and sagittal (magnified) slices revealed the expression of dopaminergic proteins in the ventral pallidum (dotted red lines). SV2C expression was used to delineate the ventral pallidum. TH, VMAT2, and DAT expression are shown in the RVP, CVP, dorsal striatum (DSTR), and cortex (CTX). Scale bar = 200 μm.

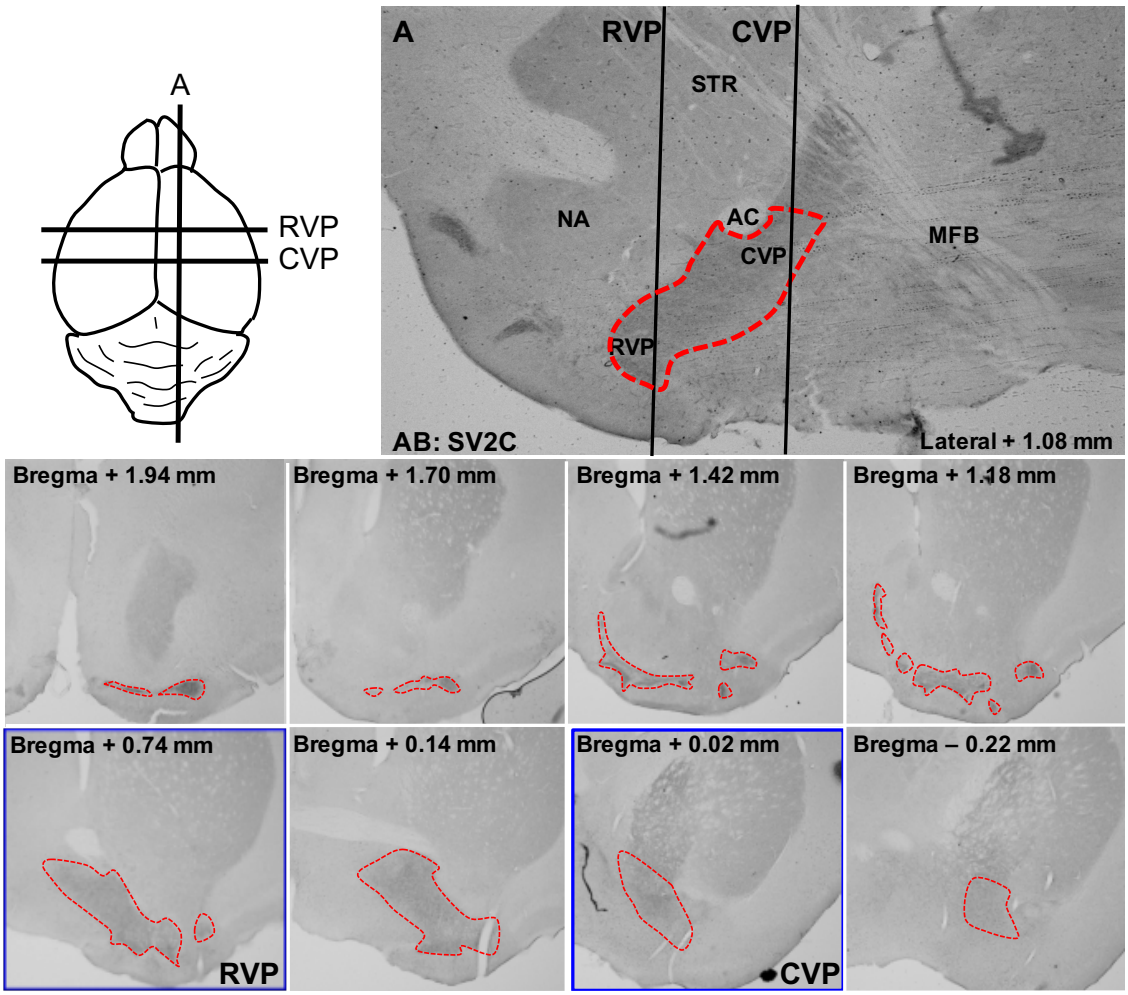

A
RVP
CVP
A
RVP
CVP
STR
NA
AC
CVP
MFB
RVP
AB: SV2C
Lateral + 1.08 mm
Bregma + 1.94 mm
Bregma + 1.70 mm
Bregma + 1.42 mm
Bregma + 1.18 mm
Bregma + 0.74 mm
RVP
Bregma + 0.14 mm
Bregma + 0.02 mm
CVP
Bregma – 0.22 mm

Figure 3- 2. Identification of ventral pallidal slices.

RVP and CVP slices (blue boxes) were chosen as shown in the diagram, using the anterior commissure for reference. The most rostral slices were not measured, given the technical difficulty of targeting ventral pallidum islands in unstained tissue. Additionally, the breadth of the ventral pallidum is shown by synaptic vesicle glycoprotein 2C immunohistochemistry. Dotted lines delineate the border of the ventral pallidum.

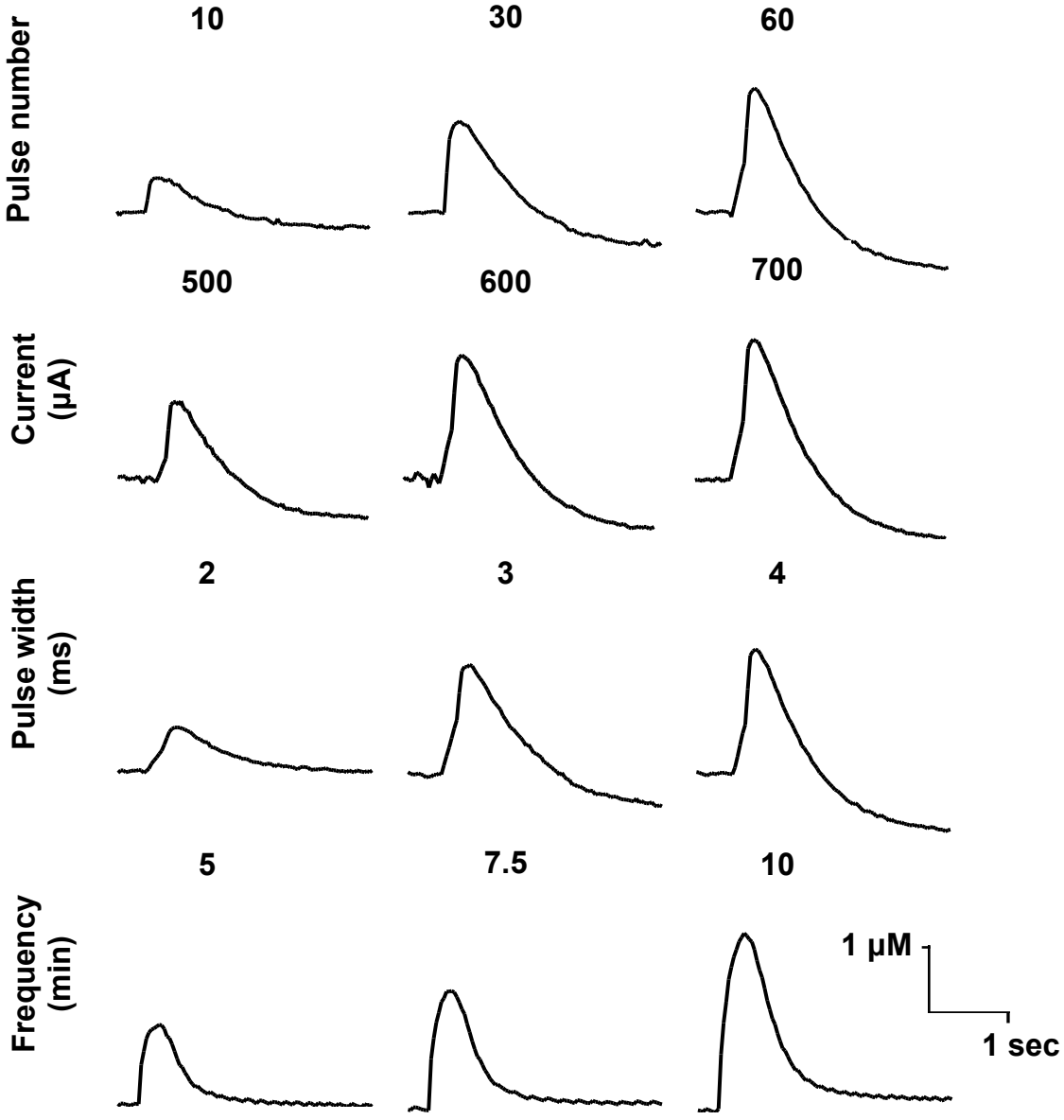

Pulse number
10
30
60
Current (μA)
500
600
700
Pulse width (ms)
2
3
4
Frequency (min)
5
7.5
10
1 μM
1 sec

Figure 3- 3. Optimization of stimulation parameters.

Pulse number (10-60), current (500-700 μA), pulse width (2-4 ms), and collection frequency (5-10 min) were varied systematically and resultant dopamine release measured. Optimal stimulation parameters of 60 pulse, 60 Hz, 600 μA, and 2 ms pulse width at 10 minute intervals produced the most consistent release without lesioning the slice.

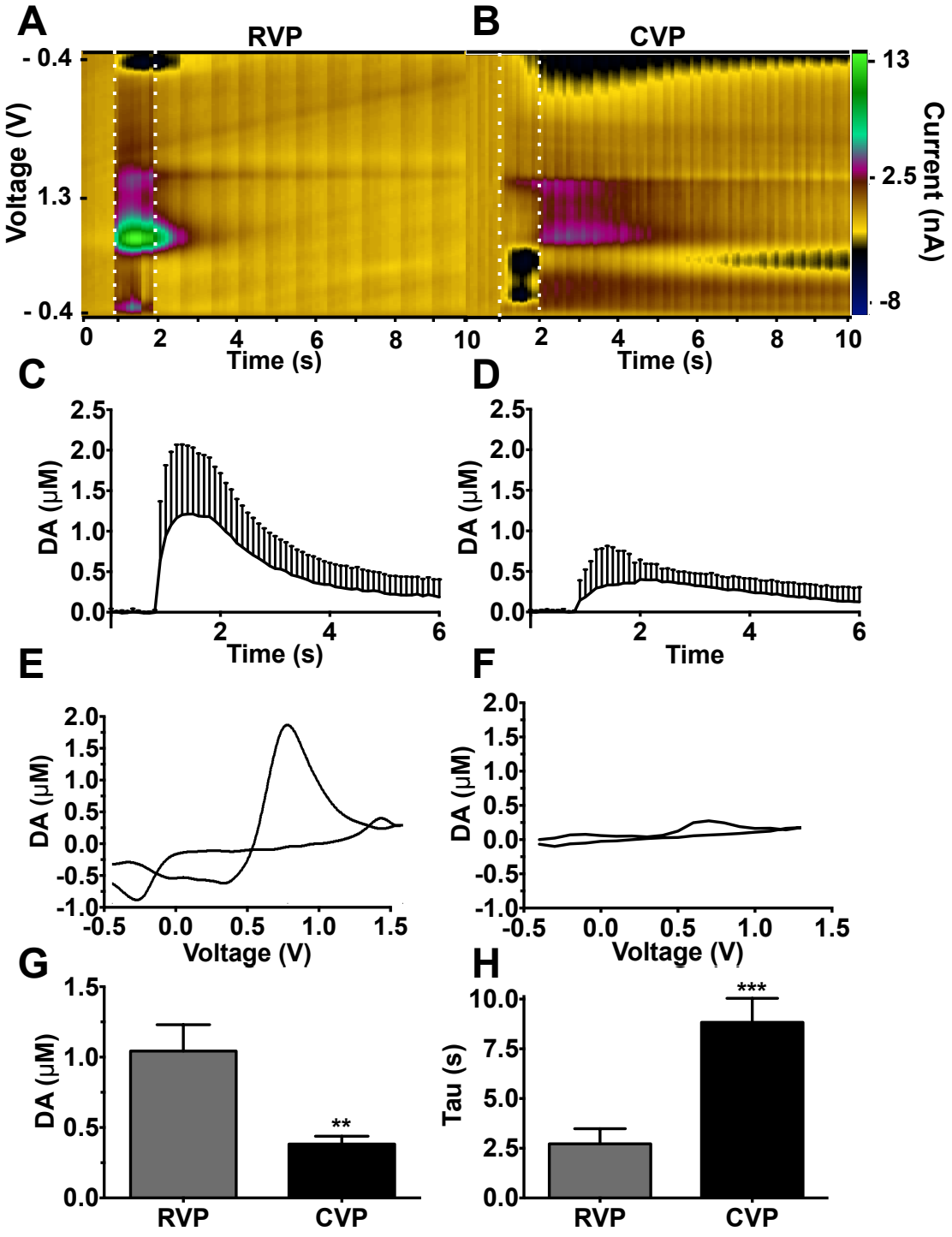

A
RVP
B
CVP
- 0.4
1.3
- 0.4
Voltage (V)
- 13
- 2.5
-8
Current (nA)
0
2
4
6
8
10
Time (s)
C
D
DA (µM)
2.5
2.0
1.5
1.0
0.5
0.0
Time (s)
Time
E
F
-1.0
-0.5
Voltage (V)
G
H
**
***
Tau (s)
10.0
7.5
5.0
2.5
RVP
CVP

Figure 3- 4. Dopamine release in RVP and CVP.

Stimulation of RVP produced significantly greater dopamine release compared with CVP (1.04 μM vs. 0.38 μM, respectively; p=0.0084, n=12, two-tailed t-test). Dopamine clearance is slower in CVP than RVP (p=0.0148, n=4, two-tailed t-test). Representative color plots and voltammagram shown. Current time traces are cumulative. Dotted lines indicate stimulation boundaries.

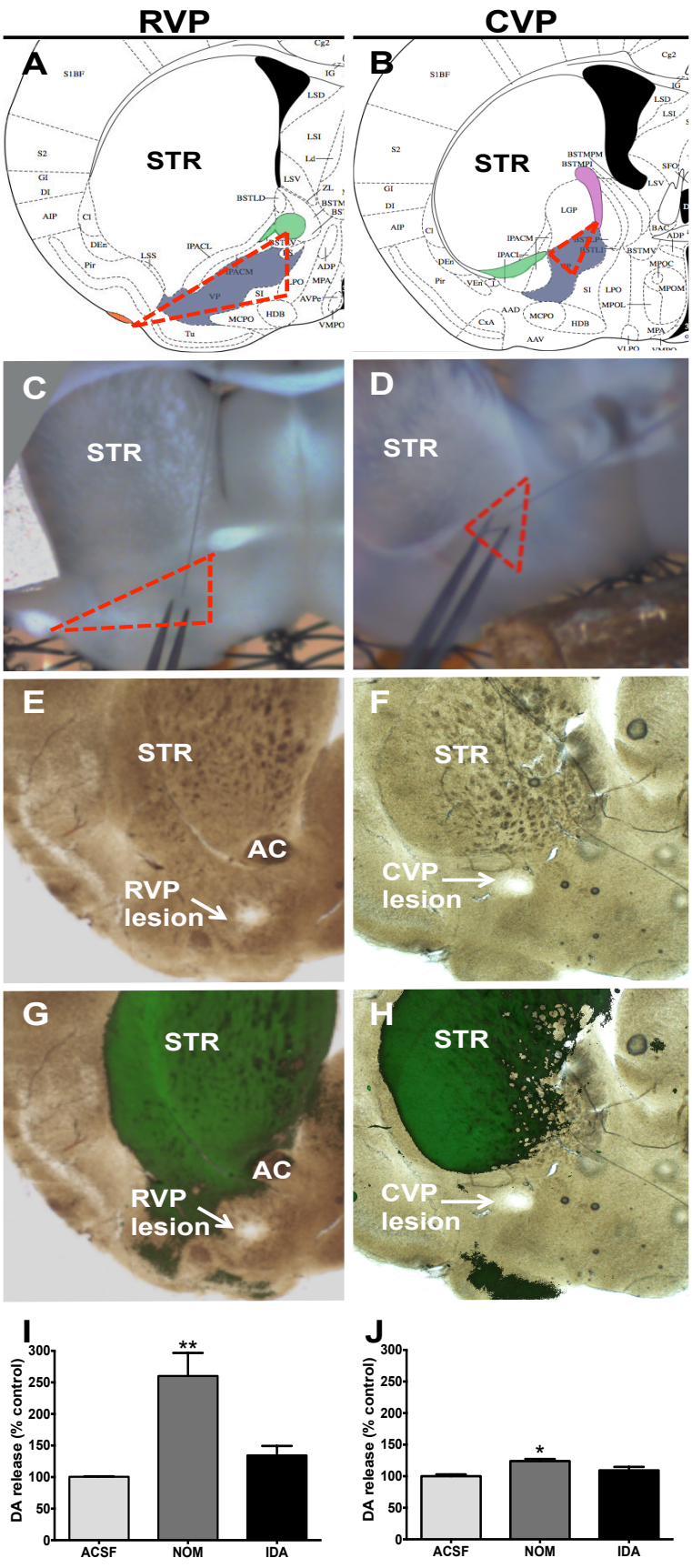

RVP
CVP
A
B
STR
C
D
E
F
G
H
AC
RVP lesion
CVP lesion
I
J
DA release (% control)
ACSF
NOM
IDA
**
*

Figure 3- 5. Confirmation of recording site.

Slices were carefully chosen by visualization of structures in accordance with the Allen Brain Atlas (A-B, VP shown in grey). Representative recording sites demonstrate identification of VP boundaries (dotted red lines) using the anterior commissure (green), lateral olfactory tubercle (orange), and the internal capside (fuschia, A-D). To further confirm several slices were electrolytically lesioned at the recording site and the tissue cleared via CLARITY. RVP and CVP lesions in transmitted light (TL) and DAT immunolabeled (green) representative slices are shown. Lack of significant colocalization with DAT expression is indicative of correct electrode placement. NET and DAT inhibitor nomifensine (10 μM) augmented release in RVP ($p=0.003$, $n=3$, one-way ANOVA with Newman-Keuls multiple comparison test) and CVP ($p=0.003$, $n=3$, one-way ANOVA with Newman-Keuls multiple comparison test). Selective NET inhibition with idazoxan (10 μM) did not significantly increase release.

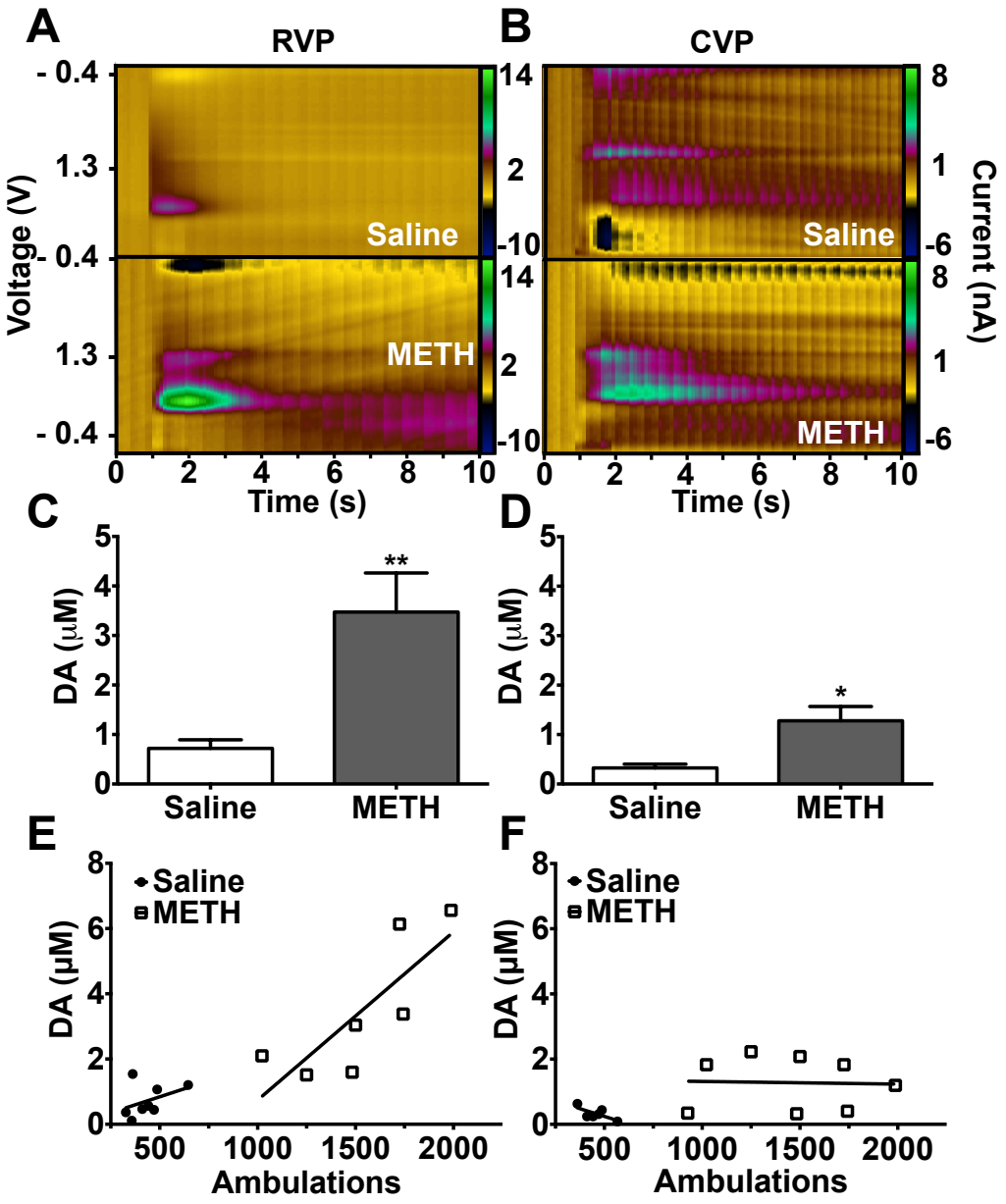

A
RVP
B
CVP
- 0.4
1.3
- 0.4
Voltage (V)
14
2
-10
Saline
METH
8
1
-6
Current (nA)
0
2
4
6
8
10
Time (s)
C
D
DA (μM)
**
*
Saline
METH
E
F
Saline
METH
DA (μM)
500
1000
1500
2000
Ambulations

Figure 3- 6. Chronic METH treatment enhances baseline dopamine release in the ventral pallidum.

Mice were sensitized to METH or saline (2 mg/kg, IP, 7 days). METH pre-treated animals have enhanced baseline DA release. In RVP slices METH pre-treatment augmented baseline dopamine release by 482% ($p=0.003$, $n=6$, two-tailed t-test). In CVP slices, METH pre-treatment increased dopamine release by 393% ($p=0.017$, $n=8$, two-tailed t-test). RVP release is correlated with motor behavior ($r^2=0.6449$, $p=0.0296$, $n=7$, linear regression analysis) whereas there is no correlation between baseline dopamine release and motor behavior in the CVP ($r^2=0.001$, $p=0.931$, $n=8$, linear regression analysis).

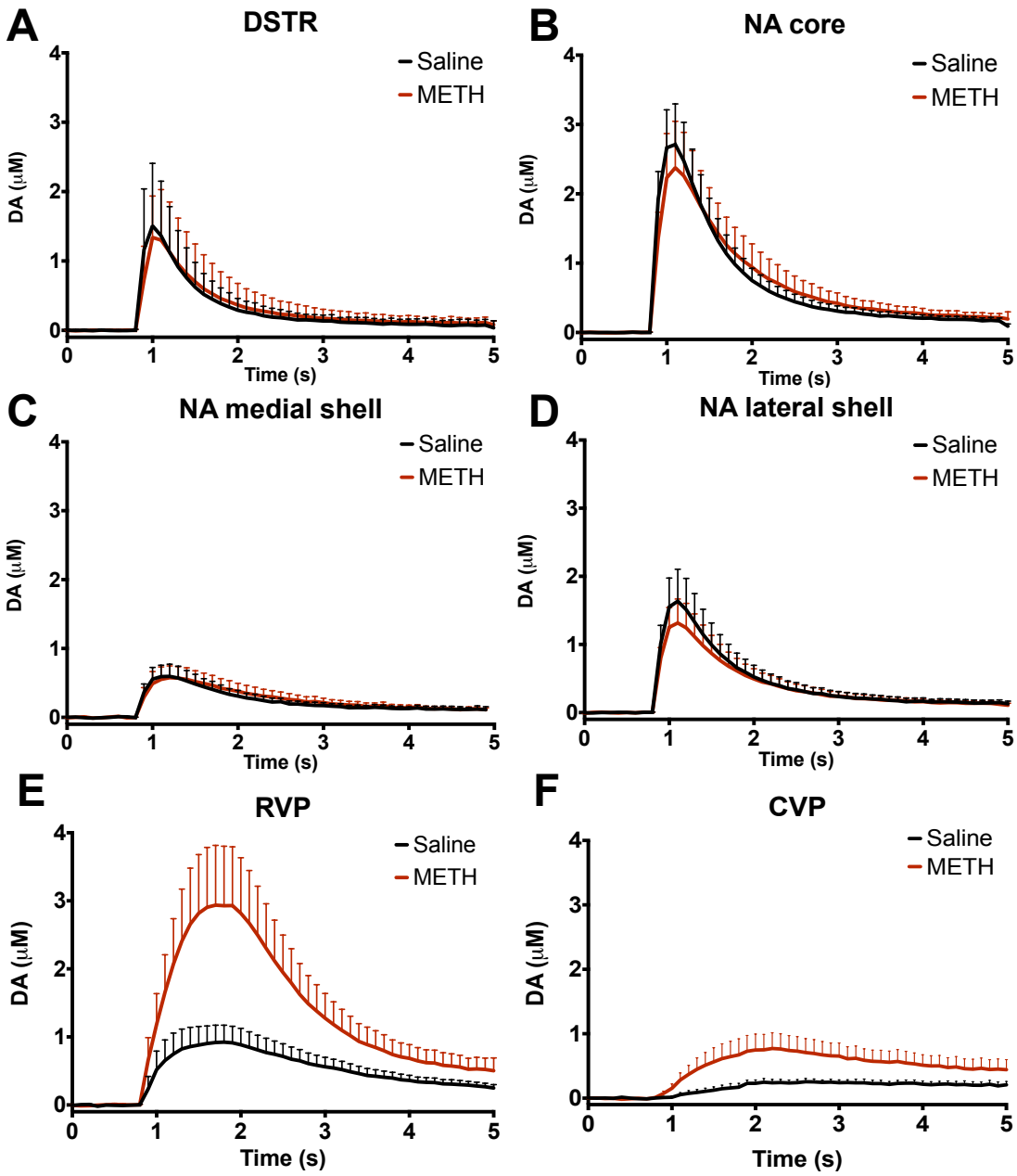

A
DSTR
B
NA core
C
NA medial shell
D
NA lateral shell
E
RVP
F
CVP
Saline
METH
DA (μM)
Time (s)

Figure 3- 7. METH-sensitization preferentially augments dopamine release in the ventral pallidum.

FSCV of dorsal and ventral striatal regions revealed no increase in baseline dopamine release of sensitized animals. Concentration time traces are averaged from 3-8 mice, dotted lines represent standard error of the mean.

	DA release (μM)		
Region	**Saline**	**METH**	**Relative change**
DSTR	**1.38 (0.40)**	**1.54 (0.23)**	**111.5 (p=0.62, n=7)**
NA - core	**2.85 (0.60)**	**3.26 (1.01)**	**114.3 (p=0.73, n=8)**
NA – med. shell	**0.62 (0.19)**	**0.75 (0.23)**	**121.1 (p=0.70, n=3)**
NA – lat. shell	**1.43 (0.39)**	**1.08 (0.14)**	**75.5 (p=0.40, n=7)**
RVP	**0.75 (0.16)**	**3.8 (0.85)**	**506.7 (p=0.002, n=6)**
CVP	**0.38 (0.06)**	**1.17 (0.35)**	**307.9 (p=0.004, n=5)**

Table 3- 1. Peak dopamine release in sensitized animals.

Animals sensitized to METH have significantly augmented peak release in RVP and CVP. No significant enhancement was observed in dorsal striatum or nucleus accumbens (two-tailed t-test). Parentheticals are standard error of the mean.

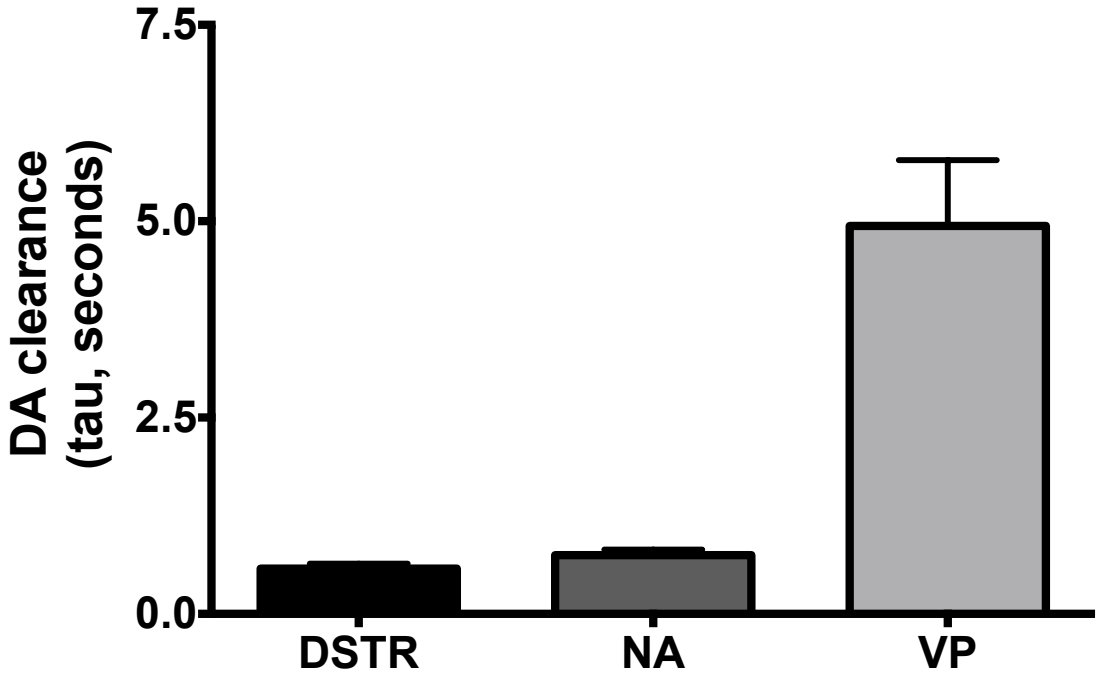

DA clearance
(tau, seconds)
7.5
5.0
2.5
0.0
DSTR
NA
VP

Figure 3-8. Dopamine clearance in striatum and ventral pallidum.

Dopamine clearance is substantially slower in the ventral pallidum compared with dorsal striatum and nucleus accumbens ($p<0.01$, $n=8$, one-way ANOVA with Newman-Keuls multiple comparison text).

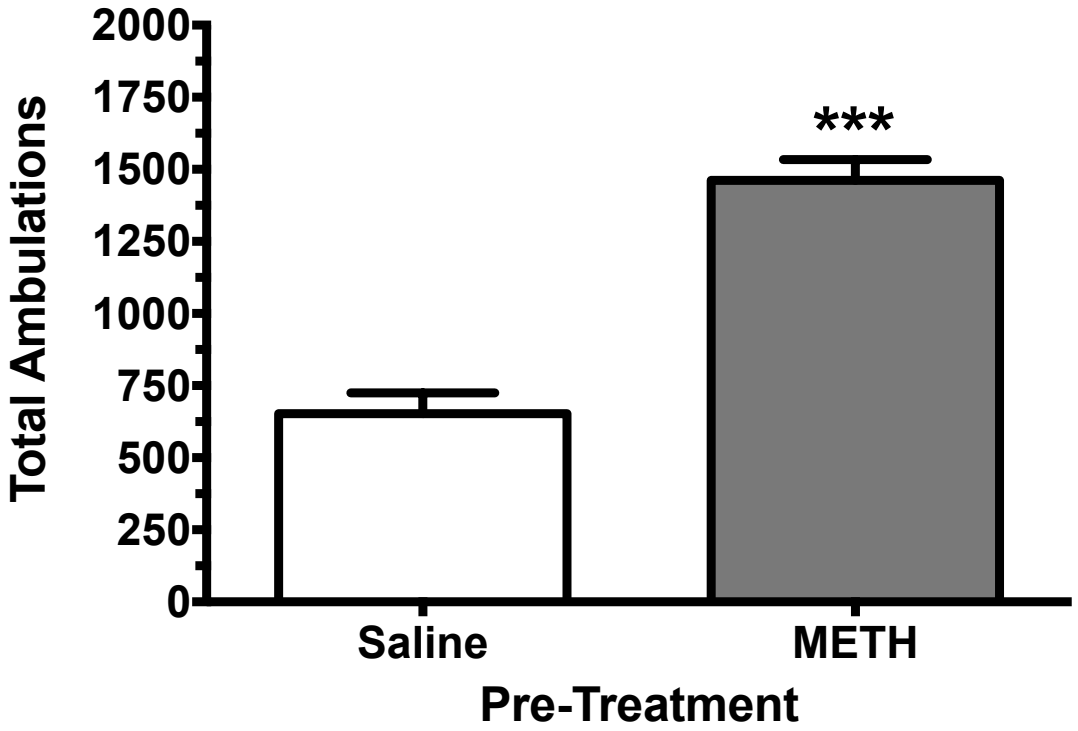

2000
1750
1500
1250
1000
750
500
250
0
Total Ambulations
***
Saline
METH
Pre-Treatment

Figure 3-9. Locomotor sensitization.

Animals were sensitized with daily injections of 2 mg/kg METH or equivalent volume saline. Following a 7-day washout, all animals received 1 mg/kg methamphetamine and ambulations were recorded (beam breaks). METH pre-treated animals exhibit a 224% increase in locomotor behavior compared with saline controls ($p<0.0001$, $n=21$).

Chapter 4: Genetic ablation of synaptic vesicle glycoprotein 2C (SV2C) reduces dopamine neurotransmission and alters methamphetamine-induced behavior

In final preparation for submission as:

Kristen A Stout, Amy R Dunn, Carlie Hoffman, Kenny Igarza, Minzheng Wang, Gary W Miller. Genetic ablation of synaptic vesicle glycoprotein 2C (SV2C) reduces dopamine neurotransmission and alters methamphetamine-induced behavior.

Abstract

The synaptic vesicle glycoprotein 2C (SV2C) localizes to presynaptic vesicles within the basal ganglia. The contribution of this protein to synaptic dopamine release in the ventral striatum and downstream reward-related behavior has not been reported in the literature. To address this question, we generated a line of mice lacking SV2C (SV2C-KO). SV2C-KO mice show reduced stimulated dopamine release compared to littermate controls by fast-scan cyclic voltammetry in the nucleus accumbens. Dopamine release was differentially altered in the ventral pallidum, with reduced dopamine release in the ventral subregions but no difference in caudal ventral pallidum. Additionally, animals lacking SV2C show reduced responsiveness to repetitive or intense stimulation compared to controls. In line with reduced dopamine release, SV2C-KO animals displayed a 35% reduction in total dopamine levels, measured by HPLC. Dopamine neuron integrity in the ventral striatum and pallidum of SV2C-KO mice was preserved. In fact, expression of the dopamine transporter is augmented in KO mice compared with littermate controls. While vesicular storage capacity was unaffected, purified vesicles from SV2C-KO animals had reduced ability to retain stored dopamine. In line with the observed neurochemical deficits, animals lacking SV2C show augmented locomotor response and reduced conditioned place preference to methamphetamine, without other observed behavioral deficits. These results identify SV2C as a regulator of dopamine neurotransmission and a key contributor to the synaptic vesicle cycle in the basal ganglia.

Introduction

Neurotransmission, the process by which adjacent neurons typically communicate, is a tightly regulated process. Dozens of proteins converge to effectively initiate and terminate appropriate neuronal communication. Though many of the details of neurotransmission have been elegantly demonstrated in the last twenty years, much of the process remains enigmatic. The synaptic vesicle glycoprotein 2 (SV2) family participates in neurotransmission, though its exact role is not currently known.

The synaptic vesicle glycoprotein 2 (SV2) is integrally important to vesicular function, though its exact role remains elusive. SV2 is sorted with high precision; every neurosecretory vesicle expresses five copies of the protein with very little deviation between vesicles (Mutch, 2011). Such precision and universal vesicular expression bespeaks the importance of SV2 to neurotransmission. Three isoforms exist in mammals, SV2A, B, and C, which share approximately 60% sequence homology (Janz et al., 1999). SV2A and B are ubiquitously expressed throughout the brain. Loss of SV2A reduces cortical GABA and hippocampal glutamate release. Animals that lack SV2A develop a severe seizure phenotype and die within three weeks of birth. Alternatively, SV2B animals are viable, but show neurotransmission deficits in the retina, where SV2B is the primary expressed isoform. Less is known about the role of SV2C, which is enriched in evolutionarily old brain regions, including the basal ganglia (Bajjalieh, 1994; Bajjalieh et al., 1992; Dardou, 2011; Janz, 1999). Given the importance of SV2 to neurotransmission and the localization of SV2C to vesicles in dopaminergic regions, we hypothesized that SV2C modulates dopamine neurotransmission and contributes to dopaminergic behavior, including reward. To address these questions, we generated SV2C-KO mice and utilizing

fast-scan cyclic voltammetry, radioactive uptake, and behavioral metrics demonstrate a role for the protein in regulating both dopamine release and dopaminergic vesicular function.

Methods

Fast-scan cyclic voltammetry (FSCV). Brains from WT and SV2C-KO male and female adult mice (2-6 months) were sliced on a vibratome in ice-cold oxygenated artificial cerebral spinal fluid ((aCSF [in mM]: NaCl [126], KCl [2.5], NaH_2PO_4 [1.2], $CaCl_2$ [2.4], $MgCl_2$ [1.2], and glucose [11], pH 7.4) with 194 mM sucrose at 300 μm. Slices were transferred to oxygenated plain aCSF and incubated at room temperature for 30 minutes. Slices were then transferred to the recording chamber, where they were perfused with 30˚ C oxygenated aCSF. Following a 30-minute incubation, the pronged stimulating electrode was inserted 70 μm into the site of interest (nucleus accumbens or ventral pallidum). A carbon fiber microelectrode was inserted 250 μm from the stimulating electrode to a similar depth, forming an equilateral triangle. Stimulation (*NAc*: 1 pulse, 700 μA, 4 ms and 5 pulse, 100 Hz, 700 μA, 4 ms; *ventral pallidum:* 60 hertz, 60 pulse, 600 μA, 2 ms; *stim trains:* 0.5 hertz, 15 pulse, 700 μA, 4 ms) was applied via the stimulating electrode. Dopamine release was measured as a background subtracted current generated via dopamine oxidation and reduction at the carbon fiber microelectrode, which had a -0.4 to 1.3V cyclic voltage ramp applied. Dopamine release from NAc and ventral pallidum was averaged from 3-4 independent sites with 4 recordings at each site. Stimulation trains were conducted once at each site from 4-6 independent sites in the nucleus accumbens. Following the experiment, electrode sensitivity was calibrated to known standards.

Radioactive uptake.

Mice: ^{3}H-dopamine uptake was performed as previously described (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 dopamine (0.03, 0.1, 0.3, 1, 3, or 10 µM dopamine with 2% ^{3}H-dopamine tracer) for five minutes at 30˚C then harvested with a vacuum manifold (Brandel) on GF/F filter paper (Whatman). Specific dopamine uptake was determined by subtracting counts from VMAT2-inhibited (10 µM tetrabenazine) reactions at each data point. Reactions were normalized to total protein, determined by bovine serum albumin assay (Pierce).

Cell culture: Human embryonic kidney cells stably transfected with VMAT2 were transiently transfected with SV2C or pcDNA3.1 (empty vector). Postnuclear fractions were collected by homogenization and centrifugation (8000 x g, 8 minutes, 4˚ C). Samples were aliquoted into glass sample tubes and incubated with 1 µM dopamine with a 2% ^{3}H-dopamine tracer for 5 minutes at 30˚ C. Samples were harvested on filter paper (Brandel) and read via liquid scintillation counting (Beckman Coulter).

Vesicular leak. Vesicles were isolated from WT and SV2C-KO mice, as described above. Samples were kept on ice and sequentially added to a 30ºC water bath. Samples were incubated for five minutes and then [3H] dopamine spiked in to a final concentration of 30 nM. Uptake proceeded for 10 minutes, at which time VMAT2 inhibitor tetrabenazine was added to the samples to yield a final concentration of 10 uM. This addition effectively froze the transporter, preventing additional uptake of radiolabeled dopamine. Vesicular leak proceeded for 0, 1, 2.5, 5, 7.5, and 10 minutes. The experiment was arranged such that all samples concluded at the same time. Each

time point was assessed in three specific and one nonspecific samples per animal. Nonspecific uptake, measured from samples that lacked ATP, was subtracted from total counts to generate specific uptake counts. Counts are shown as percentages of the zero leak time point.

Animal behavior.

Locomotor activity. Male and female WT and SV2C-KO animals (2-6 months) were habituated to locomotor activity chambers the day prior to the experiment. The following day, animals were injected with saline and locomotor behavior measured (beam breaks, Photobeam Activity System, San Diego Instruments) for 1 hour. For the next two weeks, animals received increasing injections of free base-corrected methamphetamine hydrochloride (METH, Sigma) intraperitoneally (IP) at 0.1, 0.3, 1, 3, and 10 mg/kg and beam breaks were recorded for 1 hour. Data are shown as consecutive beam breaks, indicating horizontal movement in the 60-minute time bin, and fine beam breaks, defined as repetitive breaks of a single beam, which represent stereotypied behavior such as grooming.

Conditioned place preference. Male and female WT and SV2C-KO animals (2-6 months) were conditioned with METH as previously described (Lohr, 2015). A three chambered arena was used, with one side containing a rough floor, striped walls, and a lemon scent and the other side containing a smooth floor, dark walls, and a peppermint scent, with the two chambers separated by metal rod flooring. On day one animals were tested for side preference and injection side chosen to counterbalance side bias. On days two through four, animals received IP injections of saline in the morning and were placed in the non-paired side of the chamber for 30 minutes. In the afternoon, animals received

IP METH (1 or 2.5 mg/kg) and were confined to the paired-side for 30 minutes. On day five, partitions were removed and side preference measured. Data are represented as time spent in the paired side and analyzed by two-way repeated measure analysis of variance with Newman-Keuls posthoc analysis (GraphPad prism).

Locomotor sensitization. Sensitization was measured as previously published (Stout et al., 2016). Mice (2-6 months, male and female) were habituated to the chamber the day before the dosing regimen began. On the first day, animals were injected with 1 mg/kg METH and locomotor behavior (PAS, beam breaks) recorded for 45 minutes. On days 2-7 animals received 2.5 mg/kg METH. After a 7-day washout, on day 14, animals received 1 mg/kg METH and locomotor activity was recorded. Data shown is ambulatory (consecutive beam breaks) and fine motor (repetitive beam breaks). Data were analyzed as two-way repeated measure analysis of variance with Newman-Keuls posthoc analysis (GraphPad prism).

Marble burying. Group housed mice were taken from their home cage to a temporary cage. A grid of marbles (4 by 5) was placed in the home cage and mice returned individually to the cage for 30 minutes. The animal was then removed and marbles more than 2/3 buried were counted. Marbles were cleaned and dried between animals to eliminate confounding odor cues.

Forced swim test. Group housed animals were placed in glass cylinders (24 x 16 cm) filled with 15 cm of 25˚C water for 6 minutes. Swimming was recorded (white iPhone 5S) and minutes 2-6 scored for immobility time, which was defined as passive, nondirectional floating. Scoring was blinded and conducted by two individuals to eliminate bias.

Sucrose preference. Individually housed animals were given two pre-weighed identical water bottles, one containing 2% sucrose for five days. Bottles were weighed daily. Data are shown as sucrose preference (percent of total liquid intake that contained sucrose) and as mg of sucrose consumed per day, normalized to body weight.

Immunoblotting.

Western blotting: Samples (10-50 µg of protein depending on sample concentration) underwent gel electrophorysis on 10% bis-tris gels (NuPAGE). Gels were transferred overnight at 4˚ C, blocked with milk, then incubated overnight on a nutator with appropriate primary antibody (Table 1). The following day, blots were rinsed and incubated at room temperature for 1 hour with the appropriate secondary antibody. Blots were imaged with chemiluminescence (Thermo). Densitometry was conducted using Image Lab software (Bio Rad).

Immunohistochemistry: WT and SV2C-KO animals were transcardially perfused with 4% paraformaldehyde (PFA). Brains were incubated in 4% PFA for 24 hours at 4˚ C, then transferred to 30% sucrose for 2 days. Brains were sectioned to 40 µm on a freezing microtome and stored in cryoprotectant (30% ethylene glycol, 30% glycerol, 40% tris buffered saline, pH 7.4). Slices were rinsed in phosphate buffered saline with 0.2% Triton X-100 (PBST). All slices underwent antigen retrieval in citra buffer (Biogenex) for 1 hour at 70˚ C and hydrogen peroxide quenching (3%, 10 min, room temp) for slices undergoing 3,3'-diaminobenzidine (DAB) peroxidase staining. All slices were blocked in 3% normal horse serum (NHS) for 1 hour, then incubated free floating in the appropriate primary antibody diluted in 3% NHS at 4˚ C overnight with gentle

agitation. The following day, slices were rinsed in PBST and incubated in appropriate secondary antibody at room temperature for 1 hour, followed by rinsing. Immunofluorescent slices were then mounted and coverslipped on slides with hardset mounting media with DAPI (VECTASHIELD). DAB slices were incubated in ABC solution (VECTASTAIN) for 1 hour to amplify staining. Slices were rinsed in PBST and then processed in DAB solution until tissue just began to show staining. Slices were again rinsed, mounted on glass slides, and dried overnight. The following day, slides were dehydrated by progressive incubation in increasing ethanol concentrations followed by xylene incubation, then coverslipped. DAB staining was imaged on a NeuroLucida brightfield microscope (MBF). Immunofluorescent slices were imaged on an Fluoview 1000 confocal microscope (Olympus).

Results

SV2C is expressed in regions associated with reward. DAB immunohistochemistry revealed that SV2C is expressed in the core and shell of the NA and in the midbrain (Figure 1B, C). Immunofluorescence of brain slices identified co-expression of SV2C and dopaminergic neuronal markers, TH and DAT, in the NA (Figures 1D, E), ventral pallidum (Figures 1D, E), and VTA (Figures 1D, E). SV2C is co-expressed in nearly 90% of TH- and DAT- positive dopamine neuron terminals and 84% of dopamine cell bodies in the VTA (JACOP analysis, ImageJ, Figure 1D, E). By both DAB and fluorescent staining, SV2C expression is strongest in the ventral pallidum. Though dopamine neuron markers in the region are sparsely expressed, they do colocalize with SV2C expression. In addition to labeling dopaminergic neurons, SV2C is

present in non-dopaminergic terminals in the ventral pallidum and NA (Figures 1D, E). Further, SV2C labels cell bodies within the nucleus accumbens (white arrows, Figure 1E).

*Generation of SV2C-KO mice***.** To assess the importance of SV2C to dopamine release and related behavior, we generated knockout mice from embryonic stem cells (ESCs). ESCs with the "knockout-first" insertion (Skarnes, 2011) targeting SV2C were purchased from the European Conditional Mouse Mutagenesis Program (EUCOMM). The cells, which are derived from C57BL/6N-A/a mice, were implanted into female C57B/6 mice. Heterozygous knockout-first (KOF) mice (Figure 2) were bred with FLP homozygous mice (Charles River) to excise the LacZ/neo cassette and restore a functional gene, with exon 2 flanked by loxP sites. Flanked SV2C produces a slightly larger PCR product, allowing for delineation from wildtype SV2C, which lacks a loxP site. SV2C Heterozygous SV2C-floxed mice positive for FLP were crossed with nestin-CRE mice (Charles River), then bred to homozygosity to generate global neuronal knockout of SV2C (SV2C-KO).

Protein expression in SV2C-KO mice. Loss of SV2C does not reduce dopaminergic arborization (Figure 3). In fact, SV2C-KO animals have increased expression of the dopamine transporter (DAT) compared to WT mice ($p=0.028$, two-way ANOVA, Figure 3). No difference was observed in expression of tyrosine hydroxylase (TH) or the vesicular monoamine transporter 2 (VMAT2). Additionally, no compensatory upregulation of SV2A or SV2B was observed (Figure 3B, C). Finally, no changes were observed in SNAP25 expression, suggesting no presynaptic structural deficits in SV2C-KO animals.

SV2C-KO mice have reduced dopamine content and metabolites. Dopamine content was assessed by high performance liquid chromatography in striatal dissections from WT and SV2C-KO animals. SV2C-KO animals have a 33% decrease in total dopamine content ($p=0.007$, $n=7$, Figure 4B). Within the brain, dopamine is metabolized to 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-methoxytryptamine (3-MT). DOPAC content content was decreased in SV2C-KO animals ($p=0.026$, Figure 4C), with a trend toward decreased 3-MT content ($p=0.071$, Figure 4D). The ratio between dopamine and metabolites is indicative of dopamine turnover. SV2C-KO animals have a trend towards increased dopamine synthesis and metabolism ($p=0.097$, Figure 4E). No deficits were observed in norepinephrine (NE) content between WT and SV2C-KO animals ($p=0.725$, Figure 4F).

Dopamine release is reduced in animals lacking SV2C. Brain slices containing the ventral striatum, caudle ventral pallidum (CVP), and rostal ventral pallidum (RVP) were isolated from SV2C-KO mice and littermate controls. FSCV experiments revealed a 40% decrease in the NA (Figure 5A,D: $p=0.04$, $n=10$, two-tailed t-test) and a 66.9% decrease in the RVP (Figure 5B,E, $p=0.02$, $n=6$-7, two-tailed t-test). Interestingly, no difference in release was observed in the CVP (Figure 4C,F: $p=0.85$, $n=6$, two-tailed t-test). Further analysis of this effect revealed that loss of SV2C completely ablated subregional differences within the ventral pallidum compared to WT mice, which have a 278.6% increase in RVP signaling compared to CVP (data not show, $p<0.05$, $n=7$-8, two-way ANOVA with Bonferroni posthoc analysis).

SV2C-KO mice have increased dopamine clearance and dopamine transporter expression. The time constant tau is significantly lower in the NA of SV2C-KO mice, indicating enhanced synaptic clearance compared to WT mice (Figure 4H: p=0.04, n=6, two-tailed t-test). No difference between genotypes was observed in clearance in RVP or CVP (data not shown). Increased clearance is coupled with increased striatal DAT expression, as measured by radioactive DAT ligand WIN 35,428 binding (p=0.035, n=4).

SV2C-KO animals have reduced responsiveness to stimulation trains. As the importance of SV2 to maintaining the readily releasable pool of vesicles has been demonstrated in SV2A knockout animals (Xu, 2001), we assessed vesicle pool depletion using stimulus train application in brain slices containing the NA from WT and SV2C-KO animals. 15 stimulations were applied at 2 second intervals and resultant dopamine release measured (Figure 7A-B). As expected, SV2C-KO animals recapitulated the reduced dopamine release observed in single pulse stimulation experiments (Figure 7C). Stimulations were normalized to the first stim release to assess differences in the capacity of the neuron to respond to repeated stimulation (Figure 7D-E). SV2C-KO brain sites showed reduced ability to respond to stimulation trains.

Phasic release can be modelled using FSCV by administering a 5 pulse, 100 Hz stimulation (Zhang, 2009). In the nucleus accumbens, phasic stimulation parameters result in augmented dopamine release compared with single pulse stimulation. This effect was observed in littermate controls but not SV2C-KO animals (Figure 7F), again demonstrating a reduced capacity to respond to large stimulation.

SV2C contributes to vesicular storage of dopamine. It has been repeatedly postulated in the literature that SV2s may contribute to vesicular storage capacity. The

luminal loop of SV2 is heavily glycosylated and thought to be the largest contributor to the proteoglycan matrix within the vesicle lumen (Reigada, 2003). Luminal proteoglycans bind neurotransmitter and ATP, which may reduce the concentration gradient within the lumen, alleviating the neurotransmitter gradient between the lumen and the cytosol which vesicular transporters must oppose. The contribution of SV2 to this phenomenon has not been directly assessed or reported in the literature. To address this question we performed radioactive dopamine uptake experiments. HEK cells, though non-neuronal, have neuronal characteristics and form vesicle-like compartments capable of storing neurotransmitter when transfected with vesicular transporters. HEK cells stably expressing human VMAT2 transfected with human SV2C had augmented dopamine storage capacity compared to cells transfected with empty vector (Figure 8A, $p=0.007$, $n=3$). Interestingly, this effect was not observed in knockout animals: no observable difference in vesicular storage capacity between WT and SV2C-KO mice was observed in isolated synaptic vesicles (Figure 8B, C).

Vesicles are inherently leaky (Floor, 1995). Instead of directly affecting vesicular storage capacity, we next hypothesized that SV2C may control how tightly a vesicle retains its stored neurotransmitter, i.e. that vesicles lacking SV2C may be leakier. To test this hypothesis, we pre-loaded vesicles with radiolabeled dopamine and then titrated in a saturating concentration of VMAT2-inhibitor, tetrabenazine. This effectively froze the transporter, allowing us to compare a time course of vesicular leak to uninhibited vesicles. SV2C-KO animals had significantly reduced dopamine retention: half-life of vesicular dopamine was 1.98 s in KO animals compared with 3.32 s in WT controls ($p=0.029$, one-phase exponential decay, Figure 8D).

SV2C-KO animals have altered locomotor and METH-stimulated behavior. Circadian rhythm: As locomotor behavior cycles with the dark period, activity was analyzed over a 24-hour period (Figure 9A). SV2C-KO animals exhibited a 26.0% reduction in total locomotor behavior (Figure 9B: p=0.02, n=7-8, two-tailed t-test). In general, mice exposed to novel environments have increased locomotor behavior. Novelty-induced activity was assessed by quantifying locomotor activity during the first two hours of the 24-hour period. Locomotor activity was reduced by 23.5% in SV2C-KO animals compared to WT controls (Figure 9C: p=0.03, n=7-8, two-tailed t-test). SV2C-KO animals have a trend towards decreased locomotor activity (15.5% decrease) during the active period, but this measure failed to reach significance (Figure 9D: p=0.11, n=7-8, two-tailed t-test). It seems that the reduction during the active period is driven by reduced activity immediately prior to onset of the light cycle. Looking at just the last two hours of the dark cycle revealed that SV2C-KO animals have a 46.0% reduction in circadian induced locomotor ambulatory behavior (Figure 9E: p=0.01, n=7-8, two-tailed t-test). No difference was observed in the light cycle (p=0.61, data not shown, n=7-8, two-tailed t-test).

Altered meth efficacy: Locomotor activity and conditioned place preference experiments were used to assess behavioral response to METH administration in WT and SV2C-KO animals. SV2C-KO animals are more sensitive to the stimulatory effects of METH administration, as evidenced by a leftward shift in the dose response curve (Figure 10A-B). No difference was observed in methamphetamine-induced locomotor sensitization (Figure 10B). We performed conditioned place preference experiments to assess reinforcement in SV2C-KO animals. At 2.5 mg/kg, both WT and SV2C-KO

animals form a preference for the drug-paired side, but SV2C-KO animals spend markedly less time in the paired-side than WT mice (Figure 10D, drug-induced variance: $p=0.0001$, genotype-induced variance, $p=0.15$, interaction: $p=0.05$, Bonferonni post-hoc test shows SV2C-KO and WT animals differ in place preference, $p<0.05$). To assess whether this effect was due to potential adverse effects at the higher dose, mice were conditioned at 1 mg/kg (Figure 10C, drug-induced variance: $p=0.053$, genotype-induced variance: $p=0.61$, interaction: $p=0.68$, $n=6$, two-way ANOVA). Though a trend towards place preference was identified, the experiment failed to meet statistical significance. SV2C-KO animals show no difference in normal affective behavior, with no significant difference from WT animals in marble burying (Figure 10E), forced swim test (Figure 10F), or sucrose preference (Figure 10G) or consumption (Figure 10H) experiments.

Discussion

SV2C is primly positioned to contribute to dopamine neurotransmission. This work adds to the SV2 literature in several key ways, including: characterizing the viability of SV2C-KO animals, revealing reductions in dopamine content and release in KO animals, investigating the mechanistic contributions of SV2C to synaptic transmission, and detailing altered response to stimulant administration.

SV2C-KO animals are viable. SV2C-KO animals are viable, in contrast to animals lacking SV2A, which die within weeks of birth. Due to this lethality, all experimentation in SV2A null animals must be done in early postnatal time frames. This is a major caveat, given that SV2 expression is substantially altered during development (Cohen, 2011; Crèvecoeur, 2013). Thus, the lesser phenotype is actually an advantage, allowing assessment of the functional relevance of SV2 in adulthood, at the peak of

protein expression. The reasons for developmental differences in SV2 expression have yet to be defined. Temporal control of ablation of each SV2 isoform using inducible knockout animals is paramount to fully understanding the importance of this protein to neuronal transmission and resultant disease states.

Additionally, SV2C-KO animals do not exhibit compensatory changes in the expression of SV2A or SV2B. Compensatory upregulation of SV2C has been reported in SV2A-null chromaffin cells (Xu, 2001). The lack of upregulation of SV2A or B is interesting, given that each vesicle expresses 5 copies of SV2 (Mutch, 2011). Thus, SV2C-KO mice either have reduced vesicle SV2 copy number or decreased vesicle number. Electron microscopy studies are currently underway to address this question.

Reduced dopamine level in SV2C-KO mice. SV2C-KO animals have a 33% reduction in striatal dopamine level, with a strong trend towards increased dopamine turnover in KO animals. This reduction is likely due to enhanced dopamine clearance via increased DAT expression coupled with reduced vesicular retention of dopamine in SV2C-KO animals, which results in enhanced cytosolic dopamine and subsequently enhanced dopamine metabolism.

Reduced dopamine release in SV2C-KO mice: mechanistic considerations. We assessed dopamine release in SV2C-KO animals using FSCV, which allows for direct assessment of presynaptic release. Capacitance analysis of vesicular fusion in SV2A and B knockout animals has yielded a large amount of interesting data, but capacitance neither represents actual transmitter content/release nor identifies which type of vesicle fuses (Oberhauser et al., 1996). Thus FSCV analysis represents a significant advantage in assessing the contribution of SV2 to synaptic release. Loss of SV2C

substantially reduced stimulated dopamine release in the NA and RVP. This loss could be solely due to reduced dopamine content in SV2C-KO animals. However, the data suggest a more complicated scenario. Repetitive stimulations were used to deplete reserve vesicle pools. Normalizing data to the first stimulation revealed that loss of SV2C resulted in reduced capability to repeatedly respond to stimulation. As substantial metabolism is not likely occurring within the stimulation train, this suggests impaired vesicular cycling, due either to aberrant vesicular fusion deficits, as previously demonstrated in animals lacking SV2A and SV2B (Chang, 2009; Janz et al., 1999; Morgans et al., 2009), altered endocytosis, which has not been investigated, or a combination of the two.

SV2C-KO mice have altered behavioral response. SV2C-KO animals have reduced behavioral response to METH administration. This is of particular interest, given the mechanism of METH action. METH is a stimulus-independent drug, meaning that aberration in vesicular trafficking or fusion should not alter its effectiveness. Thus, altered METH response is likely due to decreased dopamine content. The behavioral response in SV2C-KO mice is reminiscent of VMAT2 heterozygous mice, which have augmented locomotor response and decreased preference for stimulants (Fukushima et al., 2007; Takahashi, 1997).

In summary, loss of SV2C results in reduced stimulated dopamine release in the ventral striatum and pallidum and limited ability to appropriately respond to large or repetitive stimuli. Though SV2C presence does augment vesicular dopamine storage in isolated cell systems, no deficit in vesicle storage was observed in animals lacking SV2C, likely due to increased expression of SV2A. These neurochemical changes are correlated

with decreased behavioral response to METH, in the absence of other behavioral deficits, identifying SV2C as a potential target for therapeutic intervention in addiction.

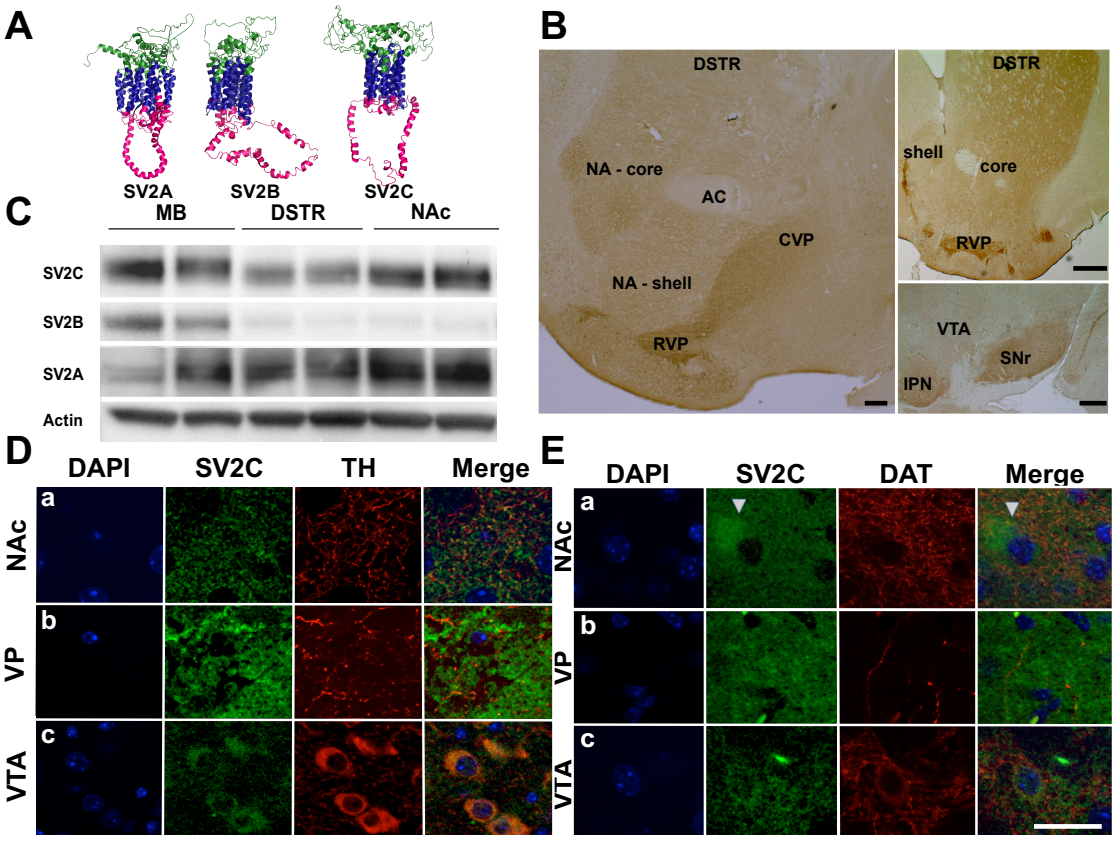
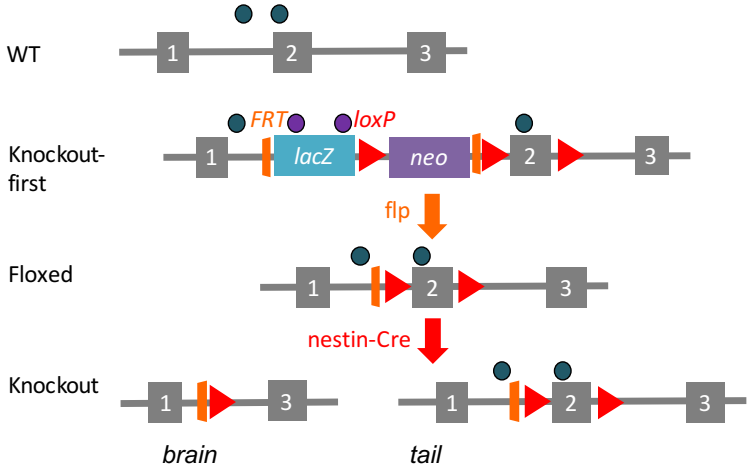


Figure 4- 1. SV2C localizes to dopamine neurons in reward regions.

SV2C is a 12-transmembrane domain protein that is homologous to family members SV2A and SV2B. A: Predictive models were created using Swiss PDB Viewer. Cytosolic domains are shown in green, transmembrane domains in blue, and intraluminal/extracellular domains in pink. B,C: SV2C is enriched in the basal ganglia, including the dorsal striatum (DSTR), nucleus accumbens (NA), midbrain (MB), and ventral pallidum (VP). D,E: SV2C co-localizes with tyrosine hydroxylase (TH) and dopamine transporter (DAT) positive dopamine neurons within the NA, VP, and ventral tegmental area (VTA). SV2C is also expressed in non-dopaminergic cell bodies in the NA (white triangle).

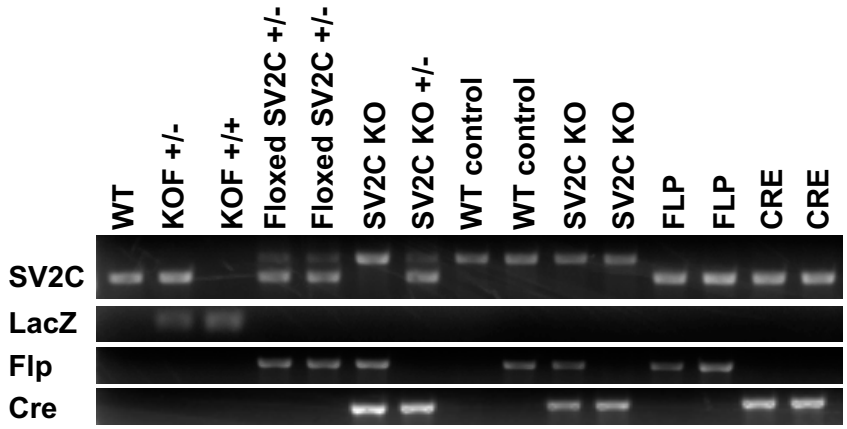
A



B

	Housekeeping gene	LacZ	Exon 2 (FRT2 to LOXP3)	FLP	CRE
WT	+	-	Lower band	-	-
KOF	+	+	-	-	-
Floxed	+	-	Upper band	+	-
KO	+	-	Upper band	-/+	+
WT controls	+	-	Upper band	-/+	-

C



D

Primer	Sequence
SV2C (exon 2)	F: 5' - TCA TCT AGA AGG GTT AAG GTC TGG - 3' R: 5'- ACC ATC ATC CCG AGG TAC AC – 3'
LacZ	F: 5'- GTC GTT TGC CGT CTG AAT TT - 3' R: 5'- CAT TAA AGC GAG TGG CAA CA – 3'
Flp	F: 5'-CAC TGA TAT TGT AAG TAG TTT GA -3' R: 5'- CTA GTG CGA AGT AGT GAT CAG G – 3'
Cre	F: 5'- CCT GGA AAA TGC TTC TGT CCG TTT GCC -3' R: 5'- GAG TTG ATA GCT GGC TGG TGG CAG ATG – 3'

Figure 4- 2. Generation of SV2C-KO animals.

Embryonic stem cells containing the 'knockout-first' cassette targeting SV2C exon 2 (A) were purchased from EUCOMM and implanted into female C57 mice. Mice were bred and genotyped according to the diagrammed breeding scheme (B), using primers for exon 2 of SV2C, LacZ, Flp, and Cre (D). SV2C-KO mice are homozygous for the upper SV2C band (indicating two copies of floxed exon 2) and express Cre under the control of the nestin promotor (C). As nestin is not expressed in the tail, exon 2 is not excised from tail DNA. Wildtype animals are also homozygous for floxed SV2C (upper band) but do not express Cre (C).

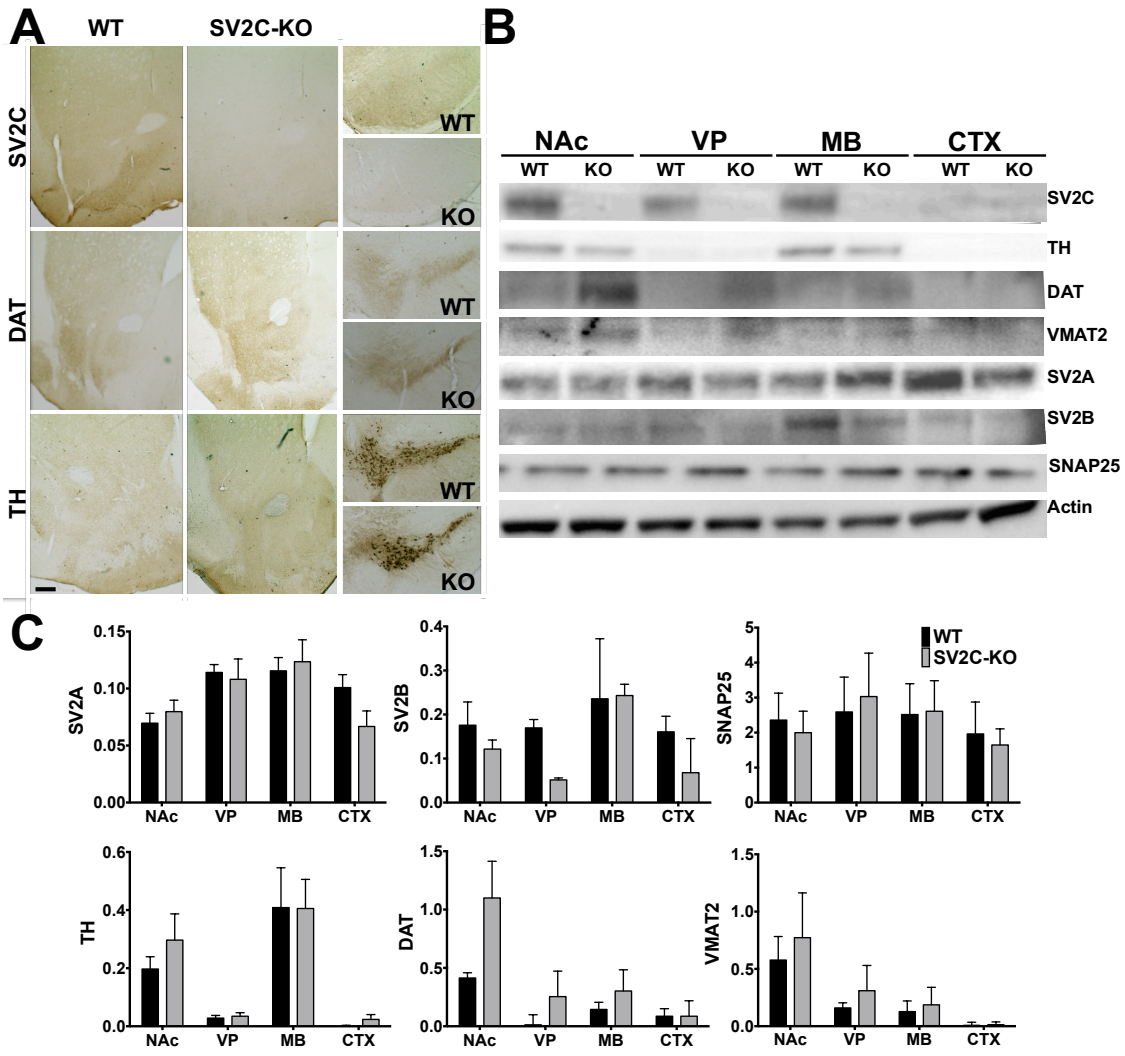

A
WT
SV2C-KO
SV2C
DAT
TH
WT
KO
B
NAc
VP
MB
CTX
WT
KO
SV2C
TH
DAT
VMAT2
SV2A
SV2B
SNAP25
Actin
C
WT
SV2C-KO
SV2A
SV2B
SNAP25
TH
DAT
VMAT2
NAc
VP
MB
CTX

Figure 4- 3. Protein expression in SV2C-KO and WT mice.

Compensatory changes in protein expression following genetic ablation of SV2C were investigated by immunochemistry. A: DAB immunohistochemistry of brain slices containing striatum and midbrain revealed that dopaminergic terminal markers, dopamine transporter (DAT) and tyrosine hydroxylase (TH), are slightly increased in SV2C-KO mice compared to WT controls. Scale bar = 100 μm. B/C: Western blot analysis of tissue punches revealed DAT is upregulated in SV2C-KO animals compared with WT mice (p:0.028, two-way ANOVA). No difference was observed in TH, VMAT2, SV2A, SV2B, or SNAP25 expression.

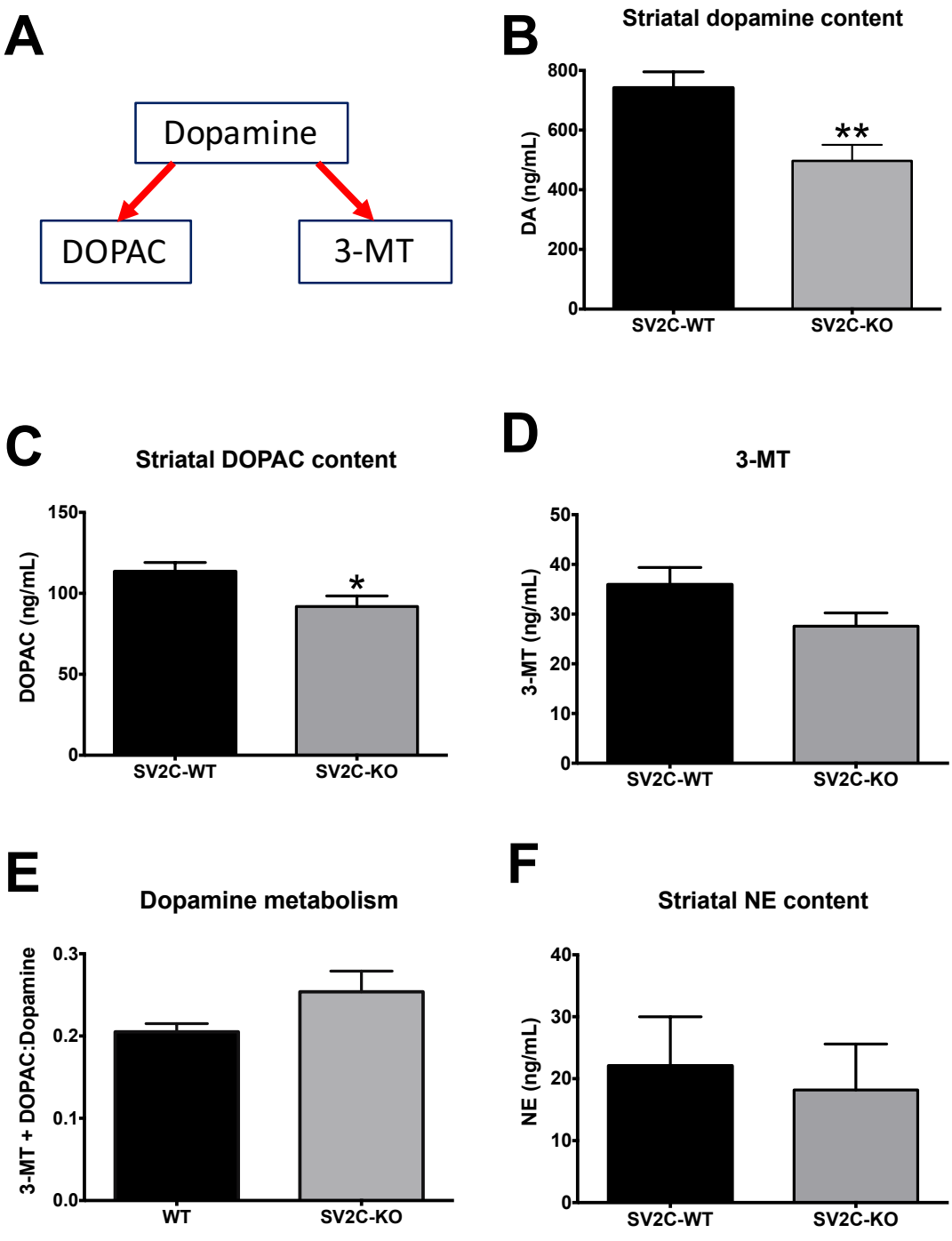

A
Dopamine
DOPAC
3-MT
B
Striatal dopamine content
DA (ng/mL)
800
600
400
200
0
**
SV2C-WT
SV2C-KO
C
Striatal DOPAC content
DOPAC (ng/mL)
150
100
50
0
*
SV2C-WT
SV2C-KO
D
3-MT
3-MT (ng/mL)
50
40
30
20
10
0
SV2C-WT
SV2C-KO
E
Dopamine metabolism
3-MT + DOPAC:Dopamine
0.3
0.2
0.1
0.0
WT
SV2C-KO
F
Striatal NE content
NE (ng/mL)
40
30
20
10
0
SV2C-WT
SV2C-KO

Figure 4- 4. SV2C-KO animals have reduced dopamine levels.

Dopamine is metabolized by monoamine oxidase and catechol-O-methyltransferase to 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-methoxytryptamine (3-MT) (A). HPLC from striatal dissections of WT and SV2C-KO animals revealed a 33% decrease in dopamine level in SV2C-KO animals ($p=0.007$, $n=7$, B). This was coupled with decreased DOPAC ($p=0.026$, C) and a trend towards both decreased 3-MT ($p=0.071$, D) and increased DA metabolism ($p=0.097$, E). No decrease was observed in striatal NE content ($p=0.725$, F).

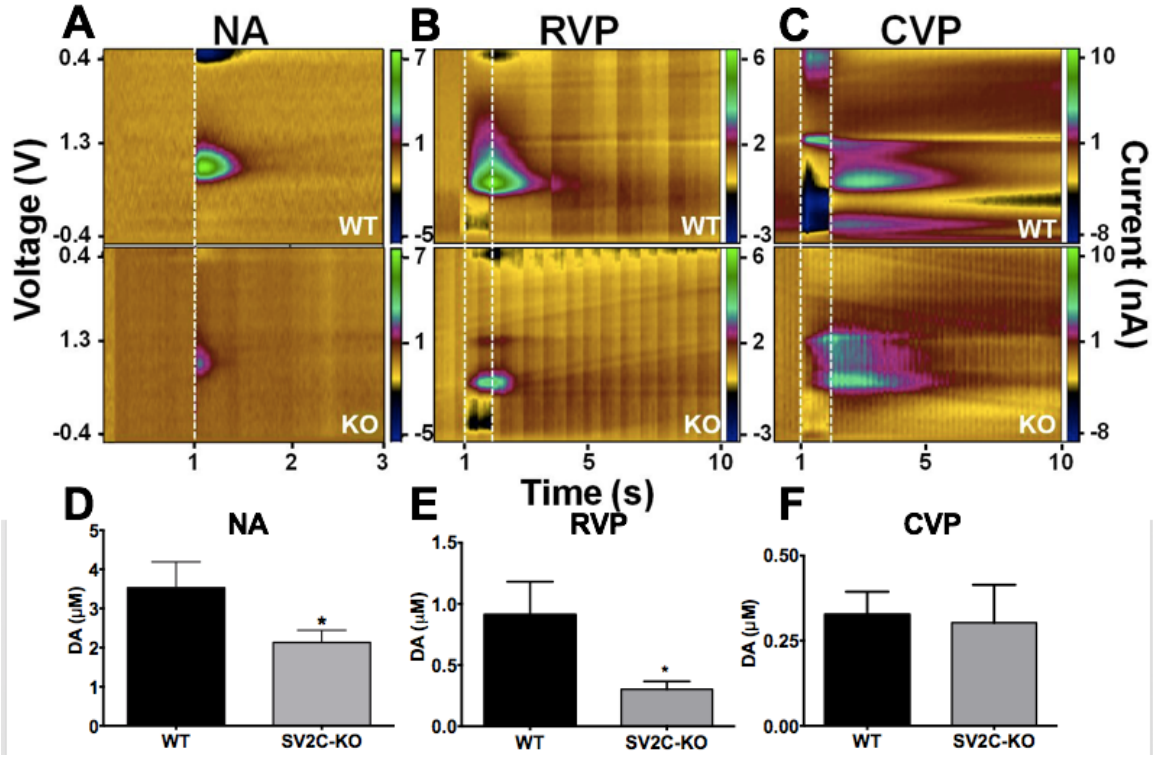

A
NA
B
RVP
C
CVP
Voltage (V)
Current (nA)
Time (s)
WT
KO
D
NA
E
RVP
F
CVP
DA (μM)
WT
SV2C-KO

Figure 4- 5. SV2C-KO animals have reduced dopamine release and content in ventral striatum and pallidum.

Dopamine release was detected by fast-scan cyclic voltammetry in brain slices of SV2C-KO and wildtype mice. Dopamine release was reduced in the NA (A,D: p=0.04, n=10, two-tailed t-test). In the ventral pallidum, dopamine release is substantially decreased in the RVP of SV2C-KO animals compared to WT (B,E: p=0.01, n=6,7, one-tailed t-test) but not in the CVP (C,E: p=0.85, n=6, one-tailed t-test).

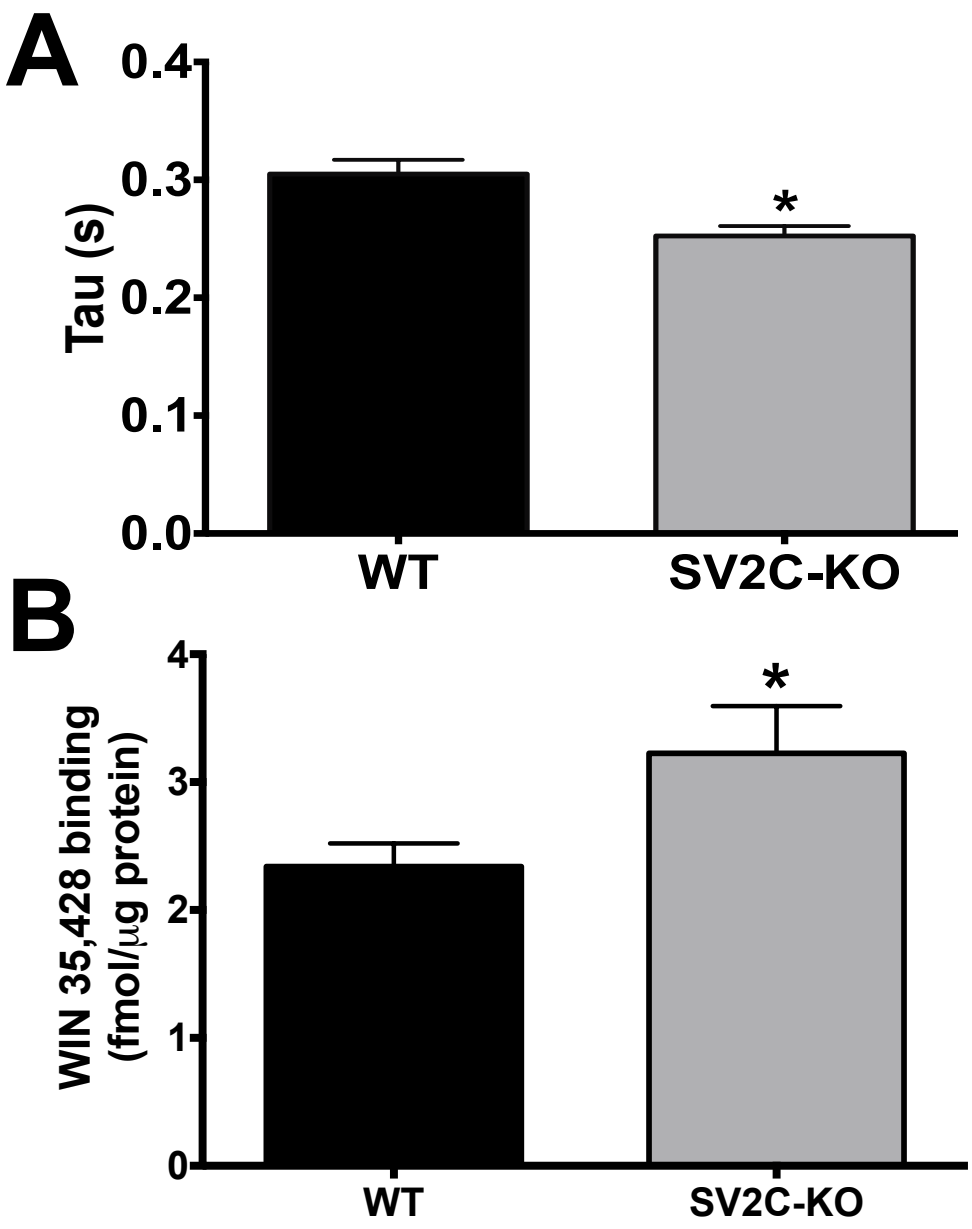

A
0.4
0.3
0.2
0.1
0.0
Tau (s)
*
WT
SV2C-KO
B
4
3
2
1
0
WIN 35,428 binding
(fmol/μg protein)
*
WT
SV2C-KO

Figure 4- 6. SV2C-KO animals have augmented dopamine clearance and enhanced expression of the dopamine transporter.

Dopamine clearance was accelerated, as measured by decreased time constant, tau (A: p=0.04, n=10, two-tailed t-test)in the nucleus accumbens of SV2C-KO mice compared to WT. Augmented clearance is likely due to increased expression of DAT in KO animals, as measured by WIN 35,428 binding (B: p=0.035, n=4, one-tailed t-test).

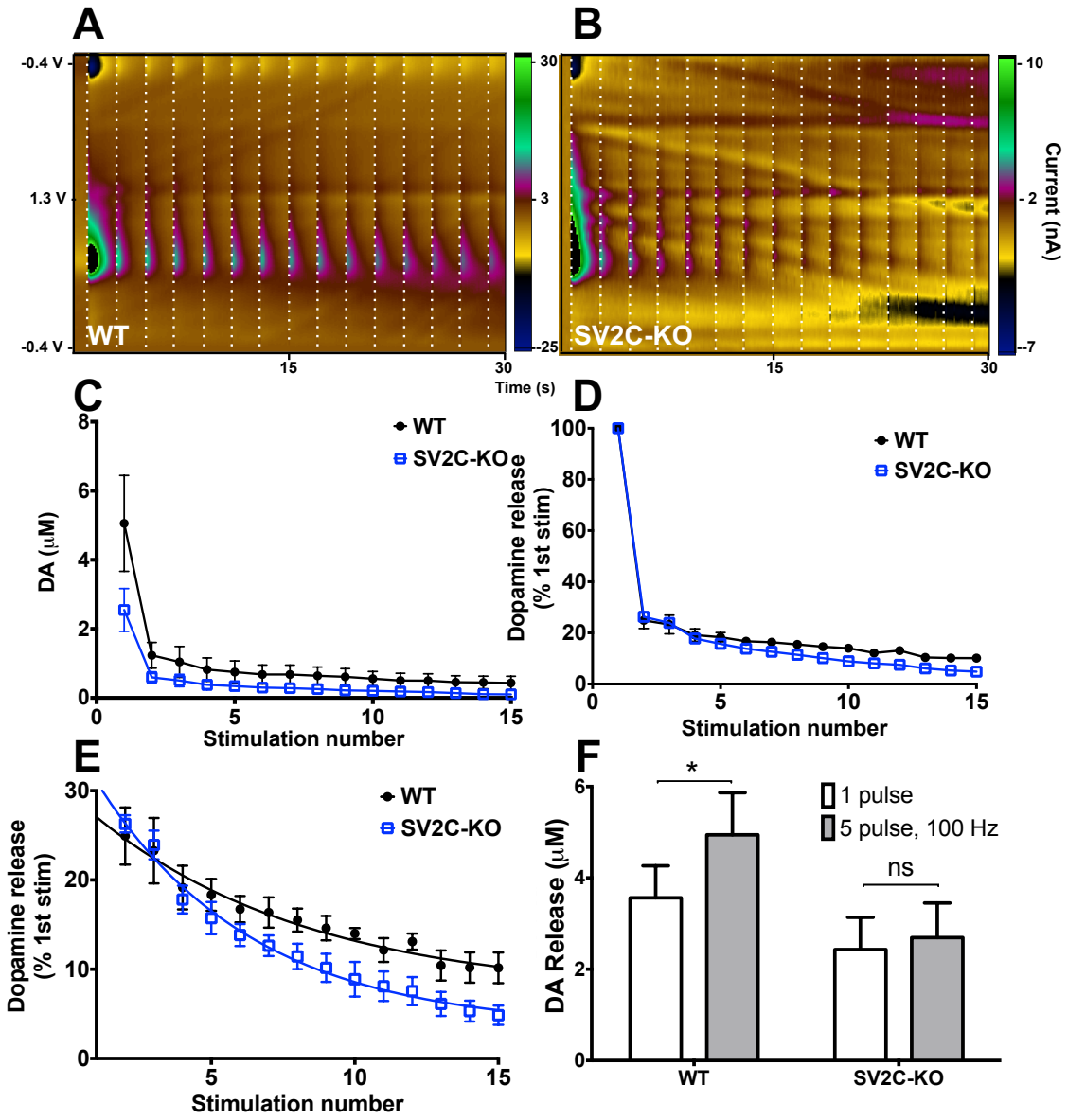

A
B
-0.4 V
1.3 V
-0.4 V
WT
SV2C-KO
30
3
-25
10
2
-7
Current (nA)
15
30
Time (s)
C
D
DA (μM)
Dopamine release (% 1st stim)
WT
SV2C-KO
Stimulation number
E
F
Dopamine release (% 1st stim)
DA Release (μM)
1 pulse
5 pulse, 100 Hz
*
ns
WT
SV2C-KO

Figure 4- 7. SV2C modulates neuronal response to repetitive stimulation in the ventral striatum.

Brain slices from SV2C-KO animals were deficient in responding to stimulus trains (15 pulse, 0.5 Hz) compared to WT slices. As observed in previous figures, total release was reduced by 65.1% (C). Normalizing to peak release shows an acceleration of vesicular depletion in SV2C-KO animals (D,E). Decay curves plotted from the second stimulus following normalization reveal significantly different depletion (E: one phase decay, $p<0.001$, n=4,5). Additionally, modeling of tonic (1 pulse) versus phasic (5 pulse, 100 Hz) dopamine release in the nucleus accumbens differentially effects dopamine release in WT and SV2C-KO animals, with increased phasic release in WT animals (F: $p<0.01$, n=6, Bonferonni posthoc two-way ANOVA) but no change between differing stimulation parameters in SV2C-KO animals (F: $p>0.05$, n=6, Bonferonni posthoc two-way ANOVA).

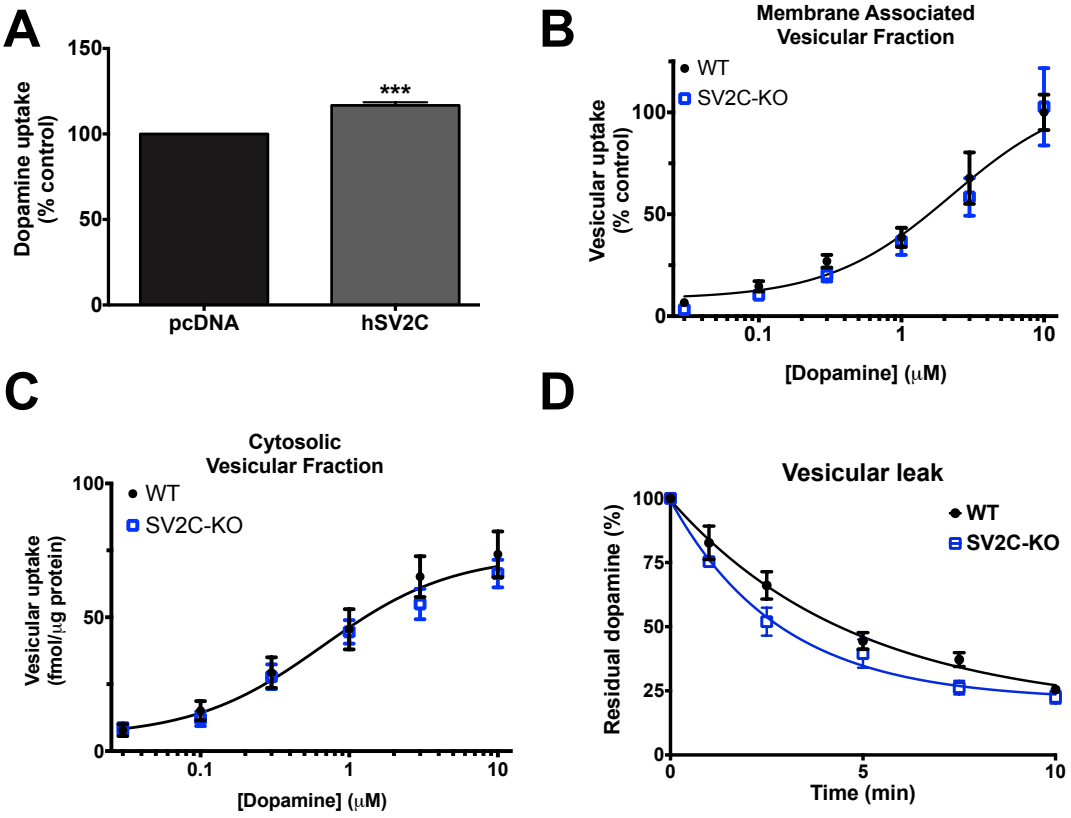

A
Dopamine uptake (% control)
150
100
50
0
***
pcDNA
hSV2C
B
Membrane Associated Vesicular Fraction
WT
SV2C-KO
Vesicular uptake (% control)
100
50
0
0.1
1
10
[Dopamine] (μM)
C
Cytosolic Vesicular Fraction
WT
SV2C-KO
Vesicular uptake (fmol/μg protein)
100
50
0
0.1
1
10
[Dopamine] (μM)
D
Vesicular leak
WT
SV2C-KO
Residual dopamine (%)
100
75
50
25
0
0
5
10
Time (min)

Figure 4- 8. SV2C expression dictates vesicular filling and retention.

HEK cells co-expressing VMAT2 and SV2C have a 20% increase in storage capacity compared to cells only expressing VMAT2 (A: p=0.007, n=3). This increased capacity is not observed in membrane-associated or cytosolic vesicles isolated from WT and SV2C-KO animals (B: p=0.65, n=8. C: p=0.45, n=8). Synaptic vesicles are inherently leaky and, as SV2 is thought to bind free intravesicular neurotransmitter, we investigated the contribution of SV2C to vesicular leak. SV2C-KO animals had significantly increased vesicular leak compared to WT animals (D: WT half-life: 3.3 min, KO half-life: 1.98 min, p=0.003, n=4, one-phase exponential decay).

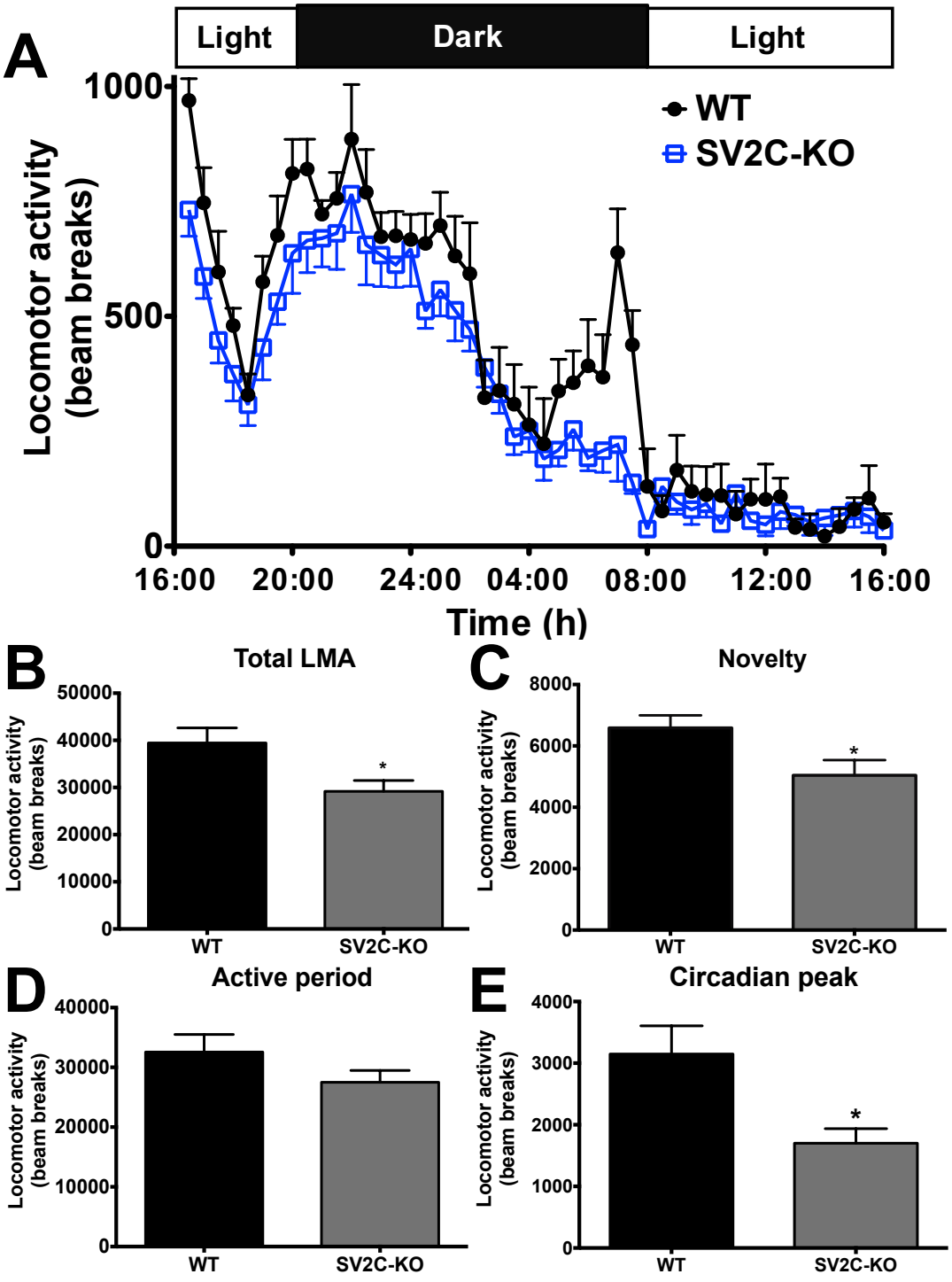

A
Light
Dark
Light
1000
500
0
Locomotor activity (beam breaks)
WT
SV2C-KO
16:00
20:00
24:00
04:00
08:00
12:00
16:00
Time (h)
B
Total LMA
50000
40000
30000
20000
10000
0
Locomotor activity (beam breaks)
*
WT
SV2C-KO
C
Novelty
8000
6000
4000
2000
0
Locomotor activity (beam breaks)
*
WT
SV2C-KO
D
Active period
40000
30000
20000
10000
0
Locomotor activity (beam breaks)
WT
SV2C-KO
E
Circadian peak
4000
3000
2000
1000
0
Locomotor activity (beam breaks)
*
WT
SV2C-KO

Figure 4- 9. SV2C-KO animals have altered locomotor behavior.

24-hour circadian locomotor behavior assessment (A) revealed that SV2C-KO animals have a 26.0% reduction in total locomotor activity (B: p=0.02, n=7-8, two-tailed t-test). SV2C-KO animals display 23.5% less novelty-induced locomotor activity, analyzed from summation of the first two hours of the test, prior to the dark cycle (C: p=0.03, n=7-8, two-tailed t-test). SV2C-KO animals have a trend towards decreased locomotor activity (D: 15.5% decrease) during the active period, but this measure failed to reach significance (D: p=0.11, n=7-8, two-tailed t-test). Quantification of the two hours prior to onset of the light cycle revealed that SV2C-KO animals have a 46.0% reduction in circadian induced locomotor ambulatory behavior (E: p=0.01, n=7-8, two-tailed t-test).

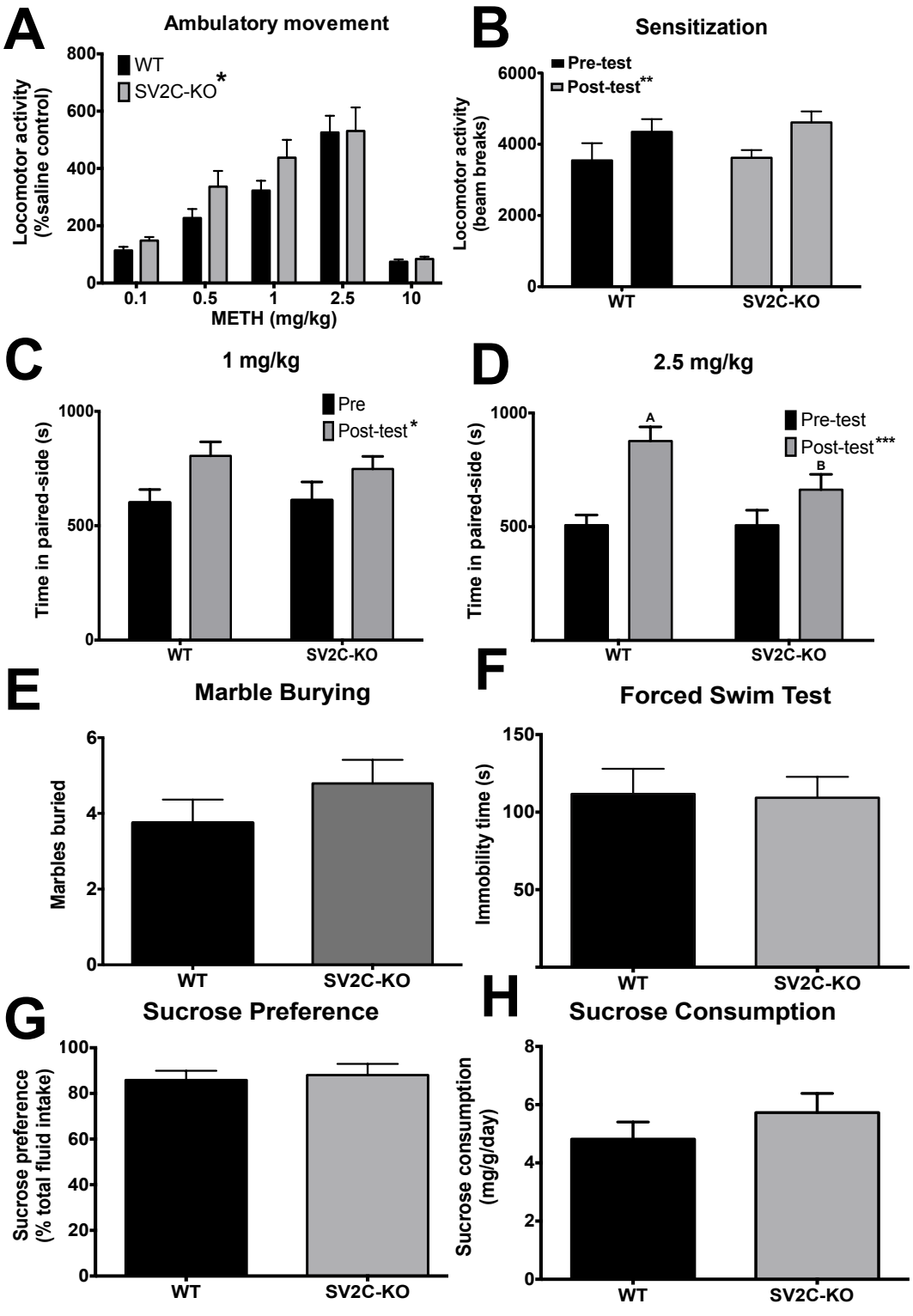

A
Ambulatory movement
Locomotor activity (%saline control)
WT
SV2C-KO*
METH (mg/kg)
B
Sensitization
Locomotor activity (beam breaks)
Pre-test
Post-test**
WT
SV2C-KO
C
1 mg/kg
Time in paired-side (s)
Pre
Post-test*
WT
SV2C-KO
D
2.5 mg/kg
Time in paired-side (s)
Pre-test
Post-test***
A
B
WT
SV2C-KO
E
Marble Burying
Marbles buried
WT
SV2C-KO
F
Forced Swim Test
Immobility time (s)
WT
SV2C-KO
G
Sucrose Preference
Sucrose preference (% total fluid intake)
WT
SV2C-KO
H
Sucrose Consumption
Sucrose consumption (mg/g/day)
WT
SV2C-KO

Figure 4-10. SV2C-KO animals have altered reward behavior.

SV2C-KO animals have slightly augmented response to the locomotor stimulant effects of METH (A: $p=0.0497$, variance due to genotype; $p<0.0001$, variance due to drug dose. Repeated measure two-way ANOVA, normalized to baseline locomotor activity following saline administration, n=16). Animals did not differ in locomotor sensitization behavior (B: variance due to sensitization, $p=0.009$, variation due to genotype, $p=0.734$, two-way ANOVA, n=8). At 1 mg/kg, SV2C-KO and WT animals have similar meth induced place preference (C: $p=0.559$, variance due to genotype; $p=0.0001$, variance due to side preference, repeated measure two-way ANOVA, n=15). However, at 2.5 mg/kg WT animals formed a strong side preference for the METH paired side, which was not observed in SV2C-KO animals (D: $p=0.08$, variance due to genotype; $p=0.0001$, variance due to side preference, Sidak's multiple comparison: variance between WT pre and post test, $p<0.0001$, SV2C-KO no significant variance). No significant difference was observed in marble burying (E: $p=0.23$, n=30, two-tailed t-test), forced swim test (F: $p=0.91$, n=14, two-tailed t-test), sucrose preference (G: $p=0.75$, n=5, two-tailed t-test), or sucrose consumption (H: $p=0.34$, n=8, two-tailed t-test).

Chapter 5: Future directions -- Sex differences dictate behavioral reduction of METH effect in SV2C-KO animals

Introduction

Methamphetamine (METH) is a psychostimulant with high abuse liability that carries devastating personal and societal costs. METH abuse is a significant public health concern, with over 100,000 new METH users and an estimated societal cost of more than $24 billion in the United States annually (den Hollander et al., 2013; Nicosia et al., 2009) . Males over the age of 18 are almost twice as likely as women to be substance dependent (Robinson et al., 2014). However, women ages 12-25 account for 49% of stimulant users (Becker and Hu, 2008), due to a drastic preference for METH over other drugs of abuse in women (Compton et al., 2000); SAMHSA, 2014). Women appear to be at greater risk for addiction to stimulants, given that they: begin using stimulants at a younger age (Griffin et al., 1989), progress to dependence more rapidly (Brady and Randall, 1999), have increased craving to drug related cues during abstinence (Robbins et al., 1999), and have longer periods of drug use following relapse (Gallop et al., 2007). Interestingly, subjective response to acute administration of cocaine in females is highly variable with estrous cycle, with response inversely related to progesterone level (Evans and Foltin, 2006; Evans et al., 2002; Sofuoglu et al., 1999). Further, administration of progesterone during the luteul phase of estrous, characterized by high estrogen and low progesterone, diminishes reported subjective response to cocaine (Evans and Foltin, 2006; Sofuoglu et al., 2002). Whereas administration of estradiol during the luteul phase augments subjective response to d-amphetamine (Justice and de Wit, 2000). Data collected from animal models support the observed sex differences in response to stimulant administration. Female mice show increased sensitivity to stimulants, as measured by: increased amphetamine-induced locomotor stimulation (Van Swearingen et

al., 2013), enhanced sensitivity to cocaine-induced conditioned place preference (Russo et al., 2003; Zakharova et al., 2009), decreased time and dose required for acquisition of cocaine self-administration (Lynch and Carroll, 1999), and augmented cocaine reinstatement (Bobzean et al., 2010). While it is clear that these effects are influenced by the estrous cycle, the exact molecular mechanisms of augmented stimulant effect in females remains unclear.

Our laboratory recently discovered that female mice express significantly more of the synaptic vesicle glycoprotein 2C (SV2C, Figure 1) than their male littermates. SV2C is preferentially expressed in synaptic vesicles of the basal ganglia (Bajjalieh, 1994; Janz, 1999). SV2C is associated with both movement and reward related dopamine release: SV2C-KO animals have reduced dopamine release in the dorsal and ventral striatum (Dunn, 2016; Stout, 2016). Further, animals lacking SV2C have reduced locomotor stimulation and conditioned place preference in response to METH administration (Stout, 2016). The importance of differential SV2C expression in females to METH behavioral response has not been reported. Here we provide preliminary evidence to suggest that SV2C may contribute to augmented METH response in female mice.

Methods

Immunoblotting

Western blotting: Striatal dissections were homogenized in buffer and underwent differential centrifugation to prepare crude synaptic fractions, as previously described (Caudle, 2007). Samples (10-50 μg of protein depending on sample concentration)

underwent gel electrophorysis on 10% bis-tris gels (NuPAGE). Gels were transferred overnight at 4˚ C, blocked with milk, then incubated overnight on a nutator with appropriate primary antibody [polyclonal rabbit anti-mouse SV2C 1:2500, mouse anti-actin(Millipore), 1:5000). The following day, blots were rinsed and incubated at room temperature for 1 hour with the appropriate secondary antibody (1:5000). Blots were imaged with chemiluminescence (Thermo). Densitometry was conducted using Image Lab software (Bio Rad).

Immunohistochemistry: WT and SV2C-KO animals were transcardially perfused with 4% paraformaldehyde (PFA). Brains were incubated in 4% PFA for 24 hours at 4˚ C, then transferred to 30% sucrose for 2 days. Brains were sectioned to 40 μm on a freezing microtome and stored in cryoprotectant (30% ethylene glycol, 30% glycerol, 40% tris buffered saline, pH 7.4). Slices were rinsed in phosphate buffered saline with 0.2% Triton X-100 (PBST). All slices underwent antigen retrieval in citra buffer (Biogenex) for 1 hour at 70˚ C and hydrogen peroxide quenching (3%, 10 min, room temp) for slices undergoing 3,3'-diaminobenzidine (DAB) peroxidase staining. All slices were blocked in 3% normal horse serum (NHS) for 1 hour, then incubated free floating in the appropriate primary antibody diluted in 3% NHS at 4˚ C overnight with gentle agitation. The following day, slices were rinsed in PBST and incubated in appropriate secondary antibody at room temperature for 1 hour, followed by rinsing. Immunofluorescent slices were then mounted and coverslipped on slides with hardset mounting media with DAPI (VECTASHIELD). DAB slices were incubated in ABC solution (VECTASTAIN) for 1 hour to amplify staining. Slices were rinsed in PBST and then processed in DAB solution until tissue just began to show staining. Slices were

again rinsed, mounted on glass slides, and dried overnight. The following day, slides were dehydrated by progressive incubation in increasing ethanol concentrations followed by xylene incubation, then coverslipped. DAB staining was imaged on a NeuroLucida brightfield microscope (MBF). Immunofluorescent slices were imaged on an Fluoview 1000 confocal microscope (Olympus).

Animal behavior

Locomotor activity. Male and female WT and SV2C-KO animals (2-6 months) were habituated to locomotor activity chambers the day prior to the experiment. The following day, animals were injected with saline and locomotor behavior measured (beam breaks, Photobeam Activity System, San Diego Instruments) for 1 hour. For the next two weeks, animals received increasing injections of free base-corrected methamphetamine hydrochloride (METH, Sigma) intraperitoneally (IP) at 0.1, 0.3, 1, 3, and 10 mg/kg and beam breaks were recorded for 1 hour. Data are shown as consecutive beam breaks, indicating horizontal movement in the 60-minute time bin.

Conditioned place preference. Male and female WT and SV2C-KO animals (2-6 months) were conditioned with METH as previously described (Lohr., 2015). A three chambered arena was used, with one side containing a rough floor, striped walls, and a lemon scent and the other side containing a smooth floor, dark walls, and a peppermint scent, with the two chambers separated by metal rod flooring. On day one animals were tested for side preference and injection side chosen to counterbalance side bias. On days two through four, animals received IP injections of saline in the morning and were placed

in the non-paired side of the chamber for 30 minutes. In the afternoon, animals received IP METH (1 or 2.5 mg/kg) and were confined to the paired-side for 30 minutes. On day five, partitions were removed and side preference measured. Data are represented as time spent in the paired side and analyzed by two-way repeated measure analysis of variance with Newman-Keuls posthoc analysis (GraphPad prism).

Locomotor sensitization. Sensitization was measured as previously published (my citation hopefully). Mice (2-6 months, male and female) were habituated to the chamber the day before the dosing regimen began. On the first day, animals were injected with 1 mg/kg METH and locomotor behavior (PAS, beam breaks) recorded for 45 minutes. On days 2-7 animals received 2.5 mg/kg METH. After a 7-day washout, on day 14, animals received 1 mg/kg METH and locomotor activity was recorded. Data were analyzed as two-way repeated measure analysis of variance with Newman-Keuls posthoc analysis (GraphPad prism).

Marble burying. Group housed mice were taken from their home cage to a temporary cage. A grid of marbles (4 by 5) was placed in the home cage and mice returned individually to the cage for 30 minutes. The animal was then removed and marbles more than 2/3 buried were counted. Marbles were cleaned and dried between animals to eliminate confounding odor cues.

Forced swim test. Group housed animals were placed in glass cylinders (24 x 16 cm) filled with 15 cm of 25˚C water for 6 minutes. Swimming was recorded (white iPhone 5S) and minutes 2-6 scored for immobility time, which was defined as passive, nondirectional floating. Scoring was blinded and conducted by two individuals to eliminate bias.

Sucrose preference. Individually housed male and female animals (2-3 months) were given two pre-weighed identical water bottles, one containing 2% sucrose for five days. Bottles were weighed daily. Data are shown as sucrose consumption per day, normalized to body weight.

Results

SV2C is more robustly expressed in female mice. Immunohistochemistry and western blotting revealed increased expression of SV2C in wildtype female versus male C57Bl/6 mice (Figure 1). Quantification of western blots from striatal tissue of male and female mice revealed that males express 35.3% less SV2C protein than their female littermates (Figure 1, $p=0.002$, female $n=5$, male $n=3$).

Genetic ablation of SV2C differentially affects locomotor stimulation in female and male mice. SV2C-KO mice have reduced responsiveness to the stimulatory effects of METH administration (Stout, 2016). Preliminary data suggest that female mice drive the behavioral reduction in locomotor activity, though it should be noted that this data represents small cohorts ($n=4$-6 per group) and bears repeating for conclusivity. It should also be noted that this data represents untransformed results due to baseline differences in locomotor behavior. Female SV2C-KO mice have significantly reduced locomotor stimulation to METH administration compared to WT female mice (Figure 2B, $p=0.004$, $n=4$, two-way ANOVA). No difference was observed between male SV2C-KO and WT mice (Figure 2A, $p=0.43$, $n=4$, two-way ANOVA). At 2.5 mg/kg, the dose with the greatest differential in response between WT and SV2C-KO animals, female SV2C-KO

animals have no augmented METH response comparative to male animals, as seen in WT animals, where females have substantially increased locomotor response (Figure 2D, $p<0.01$, n=4 per group, Bonferonni post-test, two-way ANOVA). Juxtaposition of motor response revealed that, rather than having decreased sensitivity to METH administration, SV2C-KO animals lack sex differences in METH stimulation (Figure 2C). Further, female animals trend towards enhanced expression of locomotor sensitization compared to male mice, irrespective of genotype (Figure 4B, $p=0.15$, n=2 males, 4 females per group).

SV2C-KO animals trend towards less robust place preference in female mice. Conditioned place preference experiments indicated that females may have enhanced sensitivity to METh reward at low dose METH administration (Figure 3A). At higher doses, WT females may have greater expression of conditioned place preference compared to male counterparts, while this effect seems normalized in SV2C-KO animals (Figure 3B). However, these experiments did not have sufficient animal numbers to reach significance. Further experiments are needed to fully assess place preference in male versus female WT and SV2C-KO animals.

Affective behavior is differentially affected in WT and SV2C-KO animals. Female mice had increased sucrose consumption compared to male mice, irrespective of genotype ($p=0.02$, n=3, two-way ANOVA). Interestingly, WT males show dramatically decreased compulsive behavior compared to SV2C-KO males and all females ($p<0.05$, n=10, two-

way ANOVA with Bonferonni posthoc test). No observable difference was measured in by forced swim test between genotype or sex (n=9 male, 6 female).

*Sex differences in circadian locomotor behavior***.** SV2C-KO animals have decreased ambulatory behavior during 24-hour locomotor locomotor recording (p=0.02, n=4 females, 3 males per group, two-way ANOVA). SV2C-KO animals have significantly ablated motor response during the circadian peak, defined as the elevated motor response seen in WT animals in the three hours prior to the start of the light cycle (p=0.0004, n=4 females, 3 males per group). Additionally, SV2C-KO animals show decreased novelty induced locomotor behavior, defined as locomotor response to a new environment quantified from the first two hours of the experiment (p=0.003, n= 4 females, 3 males per group, two-way ANOVA).

Discussion

Our initial hypothesis, upon realization of the sex disparity in SV2C expression, was that female animals may have increased behavioral and neurochemical effects of SV2C loss, as they have a greater change from baseline. Upon stratification of the data, however, it seems that our initial hypothesis was flawed. Rather, it seems loss of SV2C normalizes augmented METH-effects in female animals, which drives the observed behavioral reduction in METH response. Though this interesting is inherently interesting, these experiments must be repeated to ensure that the effects are reproducible. Further, these experiments should be conducted in overectomized females.

Additionally, SV2C may dictate female neuroprotection to methamphetamine toxicity. These experiments are currently ongoing in the laboratory.

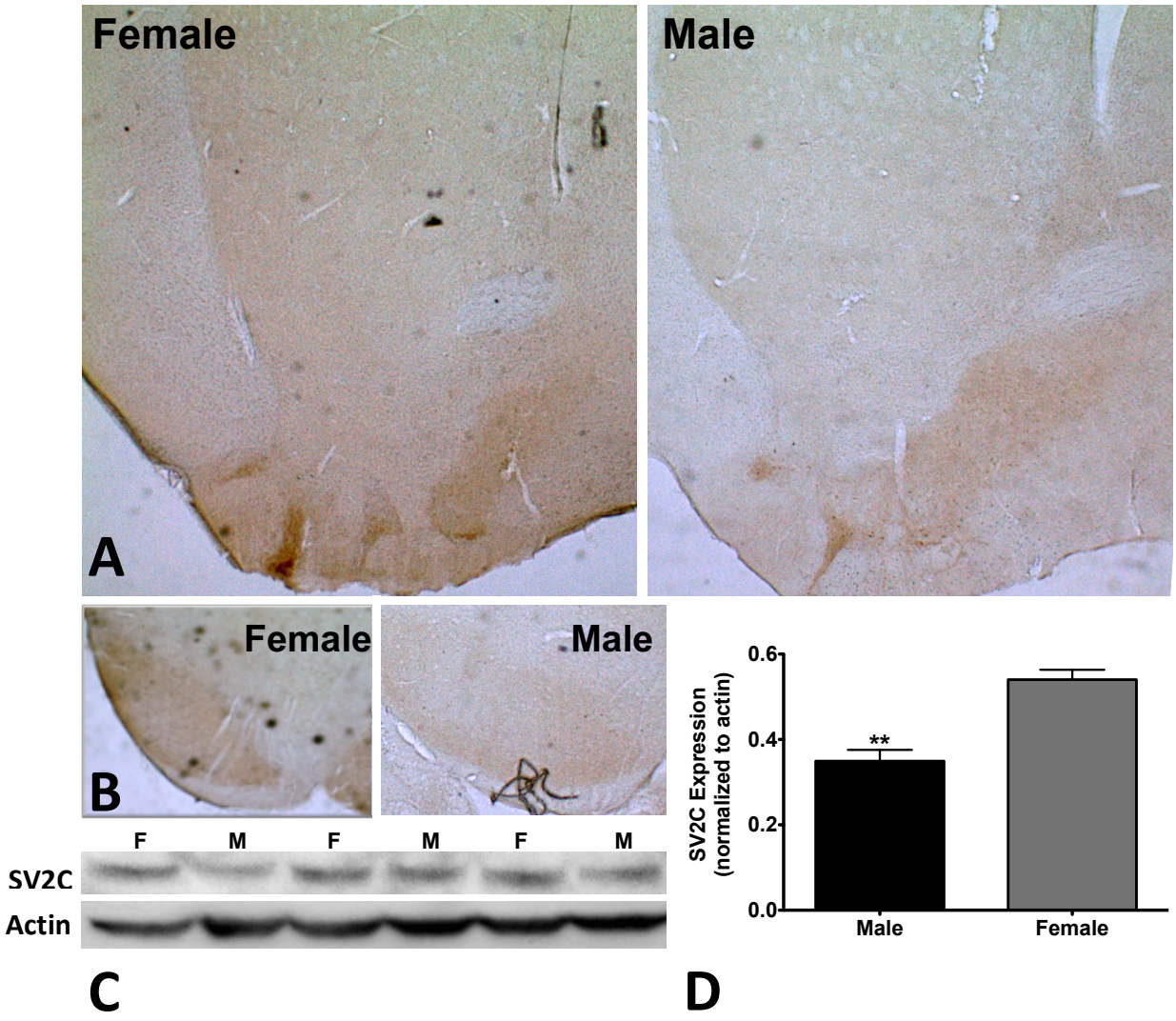

Female
Male
A
Female
Male
B
F
M
F
M
F
M
SV2C
Actin
C
SV2C Expression (normalized to actin)
0.6
0.4
0.2
0.0
**
Male
Female
D

Figure 5- 1. SV2C expression is enhanced in female mice.

Female mice have augmented SV2C expression compared to male mice by immunohistochemistry in striatum (A) and midbrain (B) and western blot of midbrain dissections (C). Quantification of western blot revealed significant difference in protein expression (D: $p=0.002$, $n= 5$ female, 3 male, two-tailed t-test).

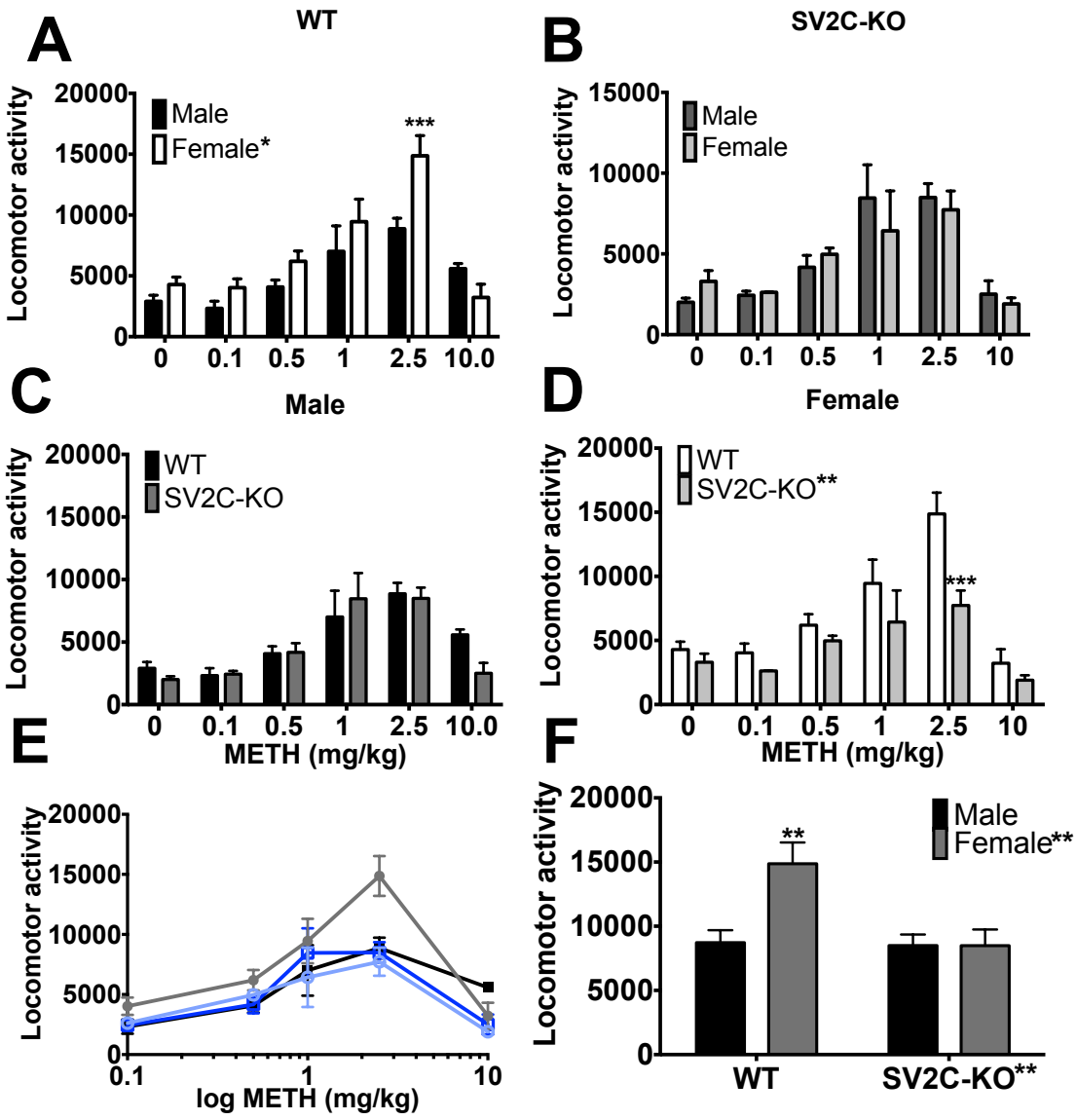

A
WT
Locomotor activity
20000
15000
10000
5000
0
Male
Female*
***
0
0.1
0.5
1
2.5
10.0
B
SV2C-KO
Locomotor activity
15000
10000
5000
0
Male
Female
0
0.1
0.5
1
2.5
10
C
Male
Locomotor activity
20000
15000
10000
5000
0
WT
SV2C-KO
0
0.1
0.5
1
2.5
10.0
METH (mg/kg)
D
Female
Locomotor activity
20000
15000
10000
5000
0
WT
SV2C-KO**
***
0
0.1
0.5
1
2.5
10
METH (mg/kg)
E
Locomotor activity
20000
15000
10000
5000
0
0.1
1
10
log METH (mg/kg)
F
Locomotor activity
20000
15000
10000
5000
0
**
Male
Female**
WT
SV2C-KO**

Figure 5-2. SV2C-KO mice have differential responsiveness to METH administration by sex.

WT female mice have augmented response to METH administration (A, $p=0.01$, $n=4$, two-way ANOVA). No sex difference is observed in SV2C-KO animals (B). No difference in locomotor behavior was seen between male WT and SV2C-KO animals (C). However, female SV2C-KO animals had substantially less locomotor stimulation than WT animals (D, $p=0.004$, $n=4$, two-way ANOVA). Juxtaposition of data reveal that female SV2C-KO animals were substantially similar to male animals in motor response to METH (E, Black: WT male, Grey: WT female, Dark blue: SV2C-KO male, Light blue: SV2C-KO female). At 2.5 mg/kg, WT female had substantially augmented motor response, which was normalized in SV2C-KO animals (F, $p<0.01$, $n=7$, two-way ANOVA with Bonferonni posthoc analysis).

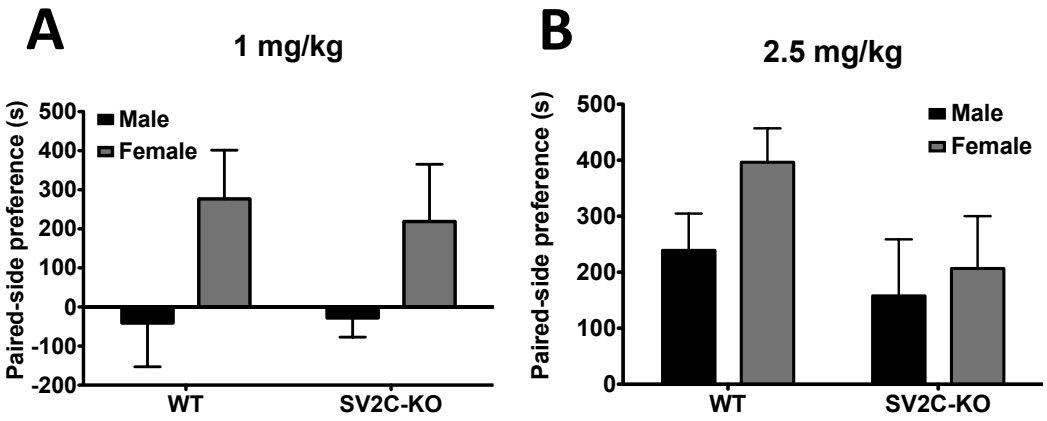

A
1 mg/kg
Paired-side preference (s)
500
400
300
200
100
0
-100
-200
Male
Female
WT
SV2C-KO
B
2.5 mg/kg
Paired-side preference (s)
500
400
300
200
100
0
Male
Female
WT
SV2C-KO

Figure 5- 3. Conditioned place preference by sex in WT and SV2C-KO animals.

Conditioned place preference experiments show a trend towards altered behavior by sex in WT and SV2C-KO animals. At low dose METH administration, no difference is readily apparent (A). However, at 2.5 mg/kg female SV2C-KO animals appear to have reduced augmented preference compared to WT females (B). Additional experiments required to reach significance.

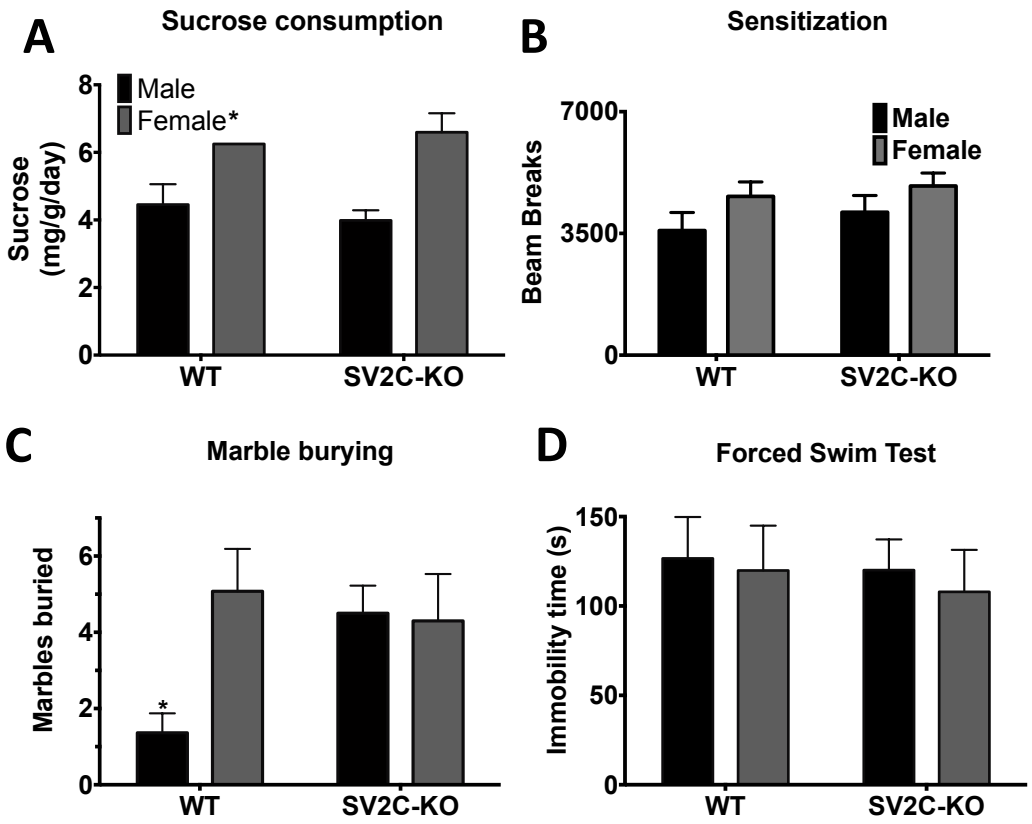

A
Sucrose consumption
Male
Female*
Sucrose (mg/g/day)
8
6
4
2
0
WT
SV2C-KO
B
Sensitization
Male
Female
Beam Breaks
7000
3500
0
WT
SV2C-KO
C
Marble burying
Marbles buried
6
4
2
0
*
WT
SV2C-KO
D
Forced Swim Test
Immobility time (s)
150
100
50
0
WT
SV2C-KO

Figure 5- 4. Sex differences in affective behavior in WT and SV2C-KO animals.

Female mice have increased sucrose consumption compared to male mice (A: $p=0.02$, $n=3$, two-way ANOVA). No difference was seen in sensitization or forced swim test (B,D). However, male WT mice buried significantly less marbles than any other group (C: $p<0.05$, $n=10$, two-way ANOVA with Bonferroni posthoc analysis).

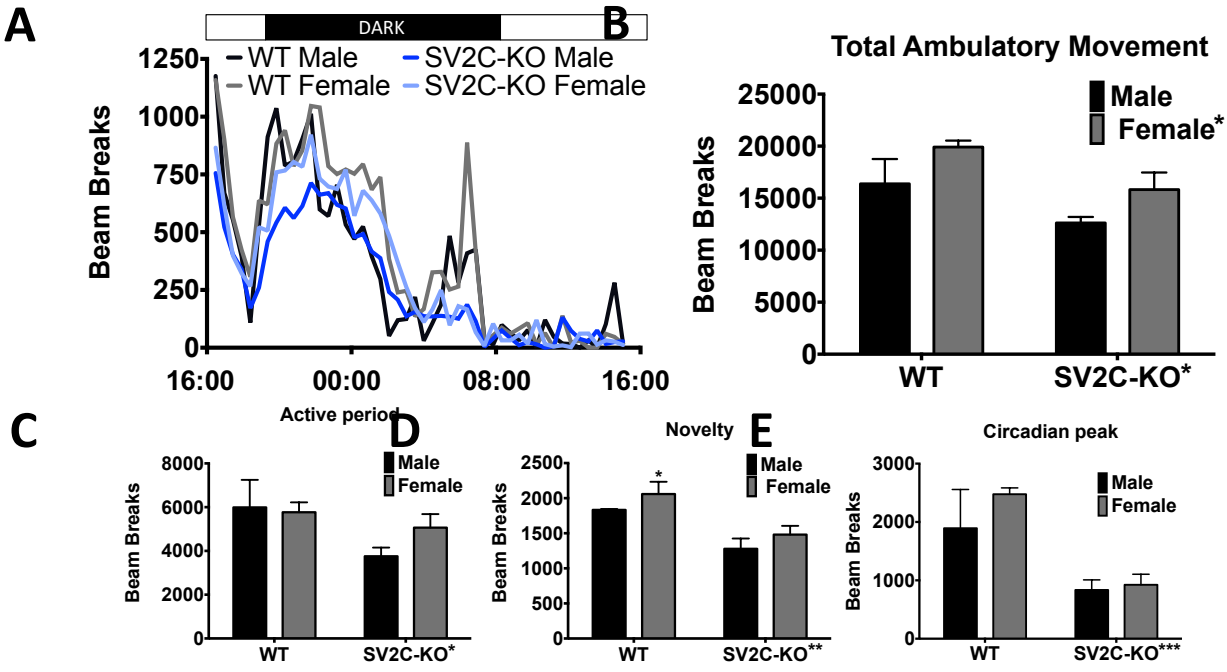

A
DARK
WT Male
WT Female
SV2C-KO Male
SV2C-KO Female
Beam Breaks
1250
1000
750
500
250
0
16:00
00:00
08:00
16:00
B
Total Ambulatory Movement
Male
Female*
Beam Breaks
25000
20000
15000
10000
5000
0
WT
SV2C-KO*
C
Active period
Male
Female
Beam Breaks
8000
6000
4000
2000
0
WT
SV2C-KO*
D
Novelty
Male
Female
Beam Breaks
2500
2000
1500
1000
500
0
*
WT
SV2C-KO**
E
Circadian peak
Male
Female
Beam Breaks
3000
2000
1000
0
WT
SV2C-KO***

Figure 5- 5. Sex differences in circadian rhythm in SV2C-KO animals.

Locomotor behavior was recorded for 24-hours to assess changes in circadian rhythm in male and female WT and SV2C-KO mice (A). SV2C-KO animals had decreased locomotor behavior compared to WT animals (B: $p=0.02$), additionally female mice had augmented locomotor behavior compared to males, irrespective of genotype (B: $p=0.04$). SV2C-KO mice had significantly decreased locomotor activity during the active period (C: $p=0.048$). This difference was largely driven by decreased circadian locomotion prior to light cycle initiation (E: $p=0.0004$). A trend towards increased circadian locomotor activity was observed in WT mice, with no apparent trend in SV2C-KO mice (E). SV2C-KO animals also exhibited decreased novelty-induced locomotion compared to WT mice (D: $p=0.003$), with WT female mice exhibiting augmented motor behavior and no sex difference in SV2C-KO animals (D: $p<0.05$). Data analyzed by two-way ANOVA with Bonferroni posthoc analysis, n=4 females, 3 males, two-way ANOVA.

Chapter 6: Concluding remarks

At the outset of this project, very little was known about SV2C. To quote Richard Janz and Thomas Sudhof, “SV2C, finally, can basically be ignored because it is restricted to a very small subset of neurons (Janz, 1999)." I’m pleased that this assertion proved false; SV2C is not a protein that should be ignored.

This work clearly demonstrates that SV2C positively effects dopamine neurotransmission and helps regulate reward response. Brain slices from mice lacking SV2C have dramatically reduced stimulated dopamine release and limited capability to respond to high impulse or repetitive stimulation. Though SV2 has been purported to contribute to vesicular storage capacity, no difference was seen in mice lacking SV2C. This result may be confounded by SV2A and SV2B expression in the striatum of SV2C-KO animals. Work is ongoing to determine the effect of SV2C knockdown to vesicular storage in N2a cells, which only express SV2C. Also, the contribution of SV2C to vesicular retention of stored dopamine is currently being addressed. Additional experiments are underway to better understand the contribution of SV2C to the synaptic vesicle cycle, including: calcium sensitivity of dopamine release by FSCV, vesicular internalization in WT versus SV2C-KO mice using total internal resonance fluorescence microscopy of endocytotic TM dyes, and ATP sensitivity of vesicular uptake in WT and SV2C-KO animals. especially Though the exact mechanism of reduced release is not clear, it is readily apparent that SV2C-KO mice have reduced behavioral response to METH administration, as demonstrated by reduced locomotor stimulation and conditioned place preference to drug treatment. Interestingly, this effect seems to be driven by sex differences.

Stimulants seem to elicit augmented responses in females, both in human and animal models (reviewed in chapter 5). In our hands, WT female mice show augmented METH-stimulated locomotor response compared to males and a trend towards augmented place preference. Female SV2C-KO animals have no such increases in METH effect. This is particularly exciting, given that female mice express about 40% more SV2C than male mice. Though more work is needed to fully elucidate sex effects in METH responsiveness of WT and SV2C-KO mice, it is fascinating to think that enhanced SV2C expression may drive augmented behavioral response to stimulant administration in females.

Also potentially interesting is the role SV2C may play in neuroprotection. Our lab has repeatedly and consistently published the importance of vesicle function to dopamine neuron viability (Alter, 2013; Caudle, 2007; Dunn, 2016; Guillot, 2008; Guillot et al., 2008; Lohr, 2014, 2015; Taylor, 2014) Given its localization to the synaptic vesicle and its clear interaction with vesicular function, it is likely that reduced SV2C expression may augment cellular loss following administration of neurotoxins. Neurotoxicity experiments using MPTP and methamphetamine are currently underway. Augmented SV2C expression in females may also affect neurotoxicity. Compared to males, female mice are protected from neurotoxic regimens of METH and MPTP, and this effect is estrogen dependent (Dluzen and McDermott, 2006; Dluzen et al., 1996a; Dluzen et al., 1996b). Estrogen-induced neuroprotection is thought to converge upon mitochondrial function, increasing resilience to toxins (Klinge, 2008; Wen et al., 2011). Recently, mitochondrial expression of SV2A was identified and proposed to contribute to mitochondrial dysfunction in Alzheimer's disease (Stockburger et al., 2015). The

functional relevance of mitochondrial SV2 expression is not known, nor has mitochondrial expression of SV2C been established. However, mitochondrial function is thought to be the linchpin of neurotoxicity. Thus, one potential mechanism for estrogen neuroprotection could be enhanced expression of mitochondrial SV2. The inner mitochondrial space is defined as a matrix, due to the observation that the environment is more viscous than the aqueous environment of surrounding spaces. The composition of this matrix has not been well defined but it stands to reason that, similar to their hypothesized role in the vesicle lumen, proteoglycans such as SV2s may contribute to matrix formation and act as an ionic and ATP buffer, stabilizing gradients generated by electron transport. In support of this hypothesis, it was recently reported that SV2 binds ATP (Yao and Bajjalieh, 2008). Though it has not been investigated, if augmented SV2C expression in females drives resilience to neurotoxic regimens, then SV2C-KO female and male mice should have equivalent losses of dopamine neuron and terminal markers due to toxin administration.

This work provides the foundation of the work that will reveal the importance of SV2C to dopamine neuron function. A plethora of interesting and timely questions remain. It was incredibly difficult to put these experiments down in order to produce this document. And I am excited to return to the bench for a few more months to play with these ideas more. It is an exciting time to be a Miller lab member.

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