

Rapid purification and metabolomic profiling of synaptic vesicles from mammalian brain

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Abstract

1 Neurons communicate by the activity-dependent release of small-molecule neurotransmitters
2 packaged into synaptic vesicles (SVs). Although many molecules have been identified as
3 neurotransmitters, technical limitations have precluded a full metabolomic analysis of synaptic vesicle
4 content. Here, we present a workflow to rapidly isolate SVs and to interrogate their metabolic contents at
5 a high-resolution using mass spectrometry. We validated the enrichment of glutamate in SVs of primary
6 cortical neurons using targeted polar metabolomics. Unbiased and extensive global profiling of SVs
7 isolated from these neurons revealed that the only detectable polar metabolites they contain are the
8 established neurotransmitters glutamate and GABA. Finally, we adapted the approach to enable quick
9 capture of SVs directly from brain tissue and determined the neurotransmitter profiles of diverse brain
10 regions in a cell-type specific manner. The speed, robustness, and precision of this method to interrogate
11 SV contents will facilitate novel insights into the chemical basis of neurotransmission.

12 **Introduction**

13

14 Critical to the function of the brain are neurotransmitters, a diverse class of small molecules that
15 act as chemical messengers between neurons. Neurotransmitters are stored in synaptic vesicles (SVs),
16 membrane-bound organelles located within presynaptic axon terminals and whose activity-dependent
17 release is essential for proper transmission of information within the brain (Jahn and Südhof, 1994). Upon
18 electrical excitation of a neuron via action potentials, SVs rapidly fuse with the membrane to release their
19 neurotransmitters, which are detected by transmembrane receptors on a postsynaptic neuron (Südhof,
20 2013; Traynelis et al., 2014) . This binding event can trigger diverse consequences to the postsynaptic
21 neuron, depending on the identity of the neurotransmitter and the receptors to which it binds. Whereas
22 some neurotransmitters cause acute electrical activation or inhibition of a neuron by opening ion
23 channels, others result in longer-term modulation of its signaling network by activating G-protein coupled
24 receptors (Nicoll et al., 1990). Thus, the functional contribution of a neuron to a circuit is defined by the
25 neurotransmitters it releases and the postsynaptic cells that it contacts.

26

27 Although neurotransmitters were discovered more than a century ago, our understanding of what
28 molecules are used as neurotransmitters and how they change during life is likely incomplete. Neurons
29 were classically believed to release only one fast-acting neurotransmitter, whose identity was fixed
30 throughout the lifetime of the neuron (Strata and Harvey, 1999). However, neurons in many brain regions
31 have recently been discovered to release multiple neurotransmitters (Hnasko and Edwards, 2012; Jonas
32 et al., 1998; Root et al., 2014; Shabel et al., 2014; Tritsch et al., 2012). Further increasing the complexity,
33 these neurotransmitters may be packaged within the same or different SV pools or released from distinct
34 axon terminals (Hnasko and Edwards, 2012; Saunders et al., 2015). Each possibility has unique effects
35 on functionality and plasticity within circuits. In addition, neurons in the developing and mature brain have
36 been found to lose, add, or switch the neurotransmitters they release in an activity-dependent manner
37 (Dulcis et al., 2013; Spitzer, 2012). Finally, there exist synapses in which the neurotransmitters released
38 remain unknown. Although many neurons have been found to contain the machinery to synthesize and
39 potentially release neurotransmitters, it remains to be established if this release occurs and whether it has
40 functional consequences (Mickelsen et al., 2017; Trapp and Cork, 2020). Altogether, these discoveries
41 greatly expand how neurotransmitters control brain circuitry and reveal the complexities that remain to be
42 deduced.

43

44 Current techniques to infer the neurotransmitter identity of neurons rely on detecting the
45 molecular machinery involved in synthesizing, packaging, or binding to neurotransmitters; however, these
46 methods have several caveats which limit their applicability. Low mRNA and protein expression levels
47 coupled with poor reagents for detection lead to false negatives and inaccurate conclusions (Hnasko and
48 Edwards, 2012). Moreover, these approaches do not account for neurons that use unknown

49 neurotransmitters or non-canonical pathways for neurotransmitter synthesis (Kim et al., 2015; Tritsch et
50 al., 2012). In addition, the substrate specificities for many neurotransmitter receptors and vesicular
51 transporters remain unclear (Yelin and Schuldiner, 1995). Alternatively, neurotransmitter identity is often
52 inferred by pharmacological analysis of the receptors that mediate postsynaptic effects; for instance, a
53 synaptic current is assumed to be induced by GABA release if it is blocked by an antagonist of ionotropic
54 GABA receptors. However, many neurotransmitter receptors can be activated or allosterically modulated
55 by diverse sets of small molecules that are found within cells, making this pharmacological approach
56 difficult to interpret (Macdonald, 1994; Patneau and Mayer, 1990).

57

58 Many of these concerns can be addressed by direct profiling of SV contents using mass
59 spectrometry (MS), a powerful tool that identifies diverse metabolites in a systematic, sensitive and robust
60 manner (Patti et al., 2012). Indeed, MS has greatly expanded our understanding of organellar biology by
61 providing insight into their rich and dynamic metabolomes (Abu-Remaileh et al., 2017; Chen et al., 2016).
62 To accurately profile the metabolic contents of SVs, quick and specific purification methods for SVs are
63 required. However, current protocols are optimized for proteomic characterizations of SVs and require
64 several hours to days to complete, during which the activity of transporters and biosynthetic enzymes
65 may alter the contents of purified SVs (Ahmed et al., 2013; Chen et al., 2016). More importantly, these
66 protocols are difficult to apply to specific neuronal populations in complex tissue.

67

68 To overcome these caveats, we developed a method to rapidly immunopurify SVs from both
69 cultured mouse neurons and intact mouse brains within half an hour. In combination, we employed MS to
70 directly and comprehensively interrogate the metabolic contents of SVs. With this workflow, we
71 characterized the neurotransmitter profiles for diverse brain regions in a cell-type specific manner. This
72 method will serve as a foundation to discover and understand the diverse ways that neurons
73 communicate with one another to control brain function.

74

75 **Results**

76

77 **A method for rapid and specific capture of SVs from cultured neurons**

78

79 In developing a method to dramatically reduce SV purification times while maintaining purity, we
80 were inspired by immunoprecipitation (IP)-based workflows for organellar isolation which are highly
81 specific and do not require time-consuming differential centrifugation techniques classically used to
82 isolate organelles (Abu-Remaileh et al., 2017; Chen et al., 2016; Ray et al., 2020). We developed SV-
83 Tag, a construct in which a hemagglutinin (HA) tag is fused to the C-terminus of synaptophysin, an SV-
84 specific, integral membrane protein and an ideal candidate to tag due to its ubiquitous presence and high
85 abundance on SVs (Figure 1A) (Takamori et al., 2006). In addition, we appended a tdTomato sequence

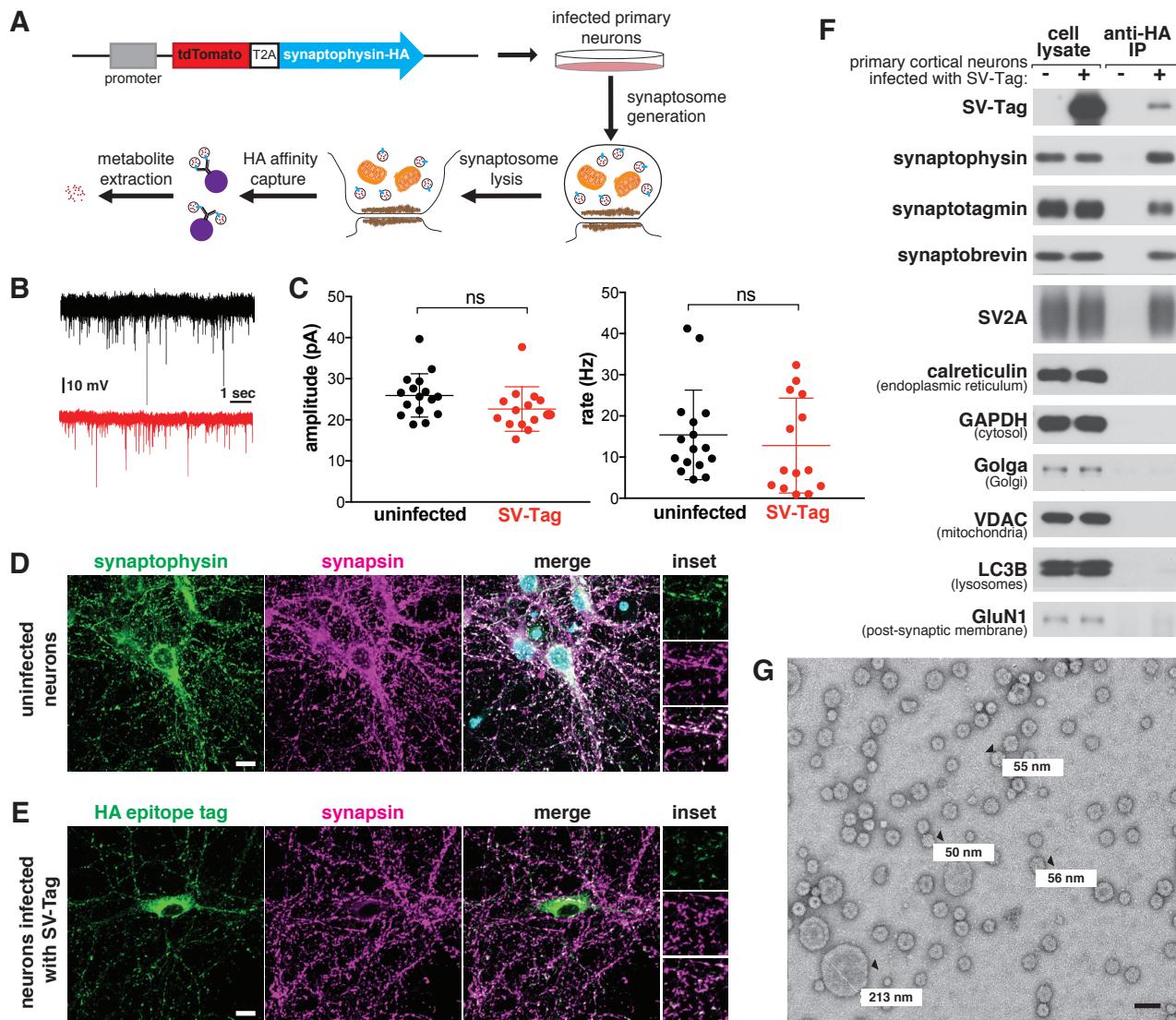


Figure 1: A method for rapid and specific isolation of synaptic vesicles from mouse primary cortical cultures **(A)** Construct design for tagging synaptic vesicles and schematic of the workflow used to isolate synaptic vesicles. **(B)** Representative trace of mEPSC responses from uninfected neurons (black) and neurons infected with SV-Tag (red). **(C)** Summary of the average amplitude (\pm standard deviation (std)) and rate of mEPSC responses from uninfected neurons and neurons infected with SV-Tag ($V_{hold} = -70$ mV, 1 μ M TTX, 10 μ M gabazine). Non-significant p value = n.s. **(D)** Immunostaining of uninfected primary neurons for endogenous synaptophysin (green) and synapsin (magenta). Insets represent selected fields that were magnified 1.6X. Scale bars: 10 μ m. **(E)** Immunostaining of infected primary neurons expressing SV-Tag (green) and synapsin (magenta). Insets represent selected fields that were magnified 1.6X. Scale bars: 10 μ m. **(F)** Immunoblot analysis of protein markers for synaptic vesicles and indicated subcellular compartments in whole cell lysates, purified synaptic vesicles, and control immunoprecipitates. Lysates were prepared from neurons infected with lentivirus encoding SV-Tag. **(G)** Electron microscope image of vesicles isolated with the workflow. Values denote diameter of indicated particles, specified by black arrows. Scale bar: 100 nm

86 followed by a self-cleaving T2A sequence to allow quick identification of infected cells via fluorescence.
87 This strategy has several advantages compared to using an antibody against endogenous synaptophysin
88 to isolate SVs. First, the SV-Tag construct can be easily modified to express in genetically defined
89 subpopulations, which is crucial given the heterogeneity of neurons and brain tissue. Furthermore, the HA
90 antibody is highly specific, sensitive, and well-characterized. Finally, the SV-Tag strategy enables this
91 method to be generalizable as the HA tag is easily appended to other proteins and is compatible with
92 many other applications, including metabolic and proteomic studies (Chen et al., 2017; Huttlin et al.,
93 2015).

94 Because our workflow relies on immuno-affinity purification of subcellular compartments labeled
95 with an ectopically expressed construct, it is necessary to ensure that this fusion protein does not perturb
96 neurotransmitter release and is properly localized to SVs. In cultured mouse cortical neurons, the
97 expression of SV-Tag did not alter the amplitude nor the rate of intrinsic glutamate release from these
98 cells, as determined by recording spontaneous miniature excitatory post synaptic currents (mEPSCs) at a
99 holding potential of -70 mV in the presence of TTX and gabazine (Figure 1B-C). To assess the
100 localization of SV-Tag, we compared its distribution to that of synapsin-1, a synaptic vesicle protein that
101 prominently marks presynaptic boutons (Camilli et al., 1983). Although SV-Tag colocalizes with synapsin,
102 a fraction of it is also detected in the soma in apparently synapsin-free areas (Figure 1E). In contrast,
103 endogenous synaptophysin completely colocalizes with synapsin, with a small fraction of the signal in the
104 perinuclear area (Figure 1D). We therefore pursued multiple optimization routes to improve the targeting
105 of SV-Tag. We moved the HA tag to the N-terminus, lowered expression levels, tagged endogenous
106 synaptophysin using CRISPR, tested other epitope tags (FLAG, GFP), and tagged other SV resident
107 proteins (SV2A, VAMP2, synaptotagmin) (Figure S1A). Surprisingly, for all of these approaches the
108 epitope tagged protein exhibited somatic localization comparable to that of SV-Tag (Figure S1A). This
109 suggests that for recombinant SV proteins, it is difficult to achieve the correct level of expression to
110 ensure that they are trafficked from the soma to the boutons. Alternatively, it could indicate that a
111 population of endogenous synaptophysin is somatically localized but not accessible by the synaptophysin
112 antibody. This latter hypothesis is supported by the appearance of a somatic pool of synaptophysin when
113 an HA tag was introduced into the endogenous gene (Figure S1A, top row). Therefore, we decided to use
114 our original synaptophysin-based SV-Tag construct, due to the advantages of synaptophysin being a
115 protein that is easy to express, abundant, and ubiquitously present on SVs (Takamori et al., 2006).
116

117 Based on a series of classical SV purification methods (Camilli et al., 1983; Craige et al., 2004;
118 Huttner et al., 1983; Nagy et al., 1976), we developed a workflow to immunoisolate SVs from cultured
119 cortical neurons within 30 minutes, a substantial reduction in time compared to the multiple hours to days
120 needed for classical methods (Figure 1A). First, we formed synaptosomes, which are isolated synaptic
121 terminals (Figure S1C). Unlike direct cell lysis to release SVs, this process affords us a key advantage of
122 separating and discarding the soma, along with its mislocalized SV-Tag. We isolated synaptosomes

123 within just seven minutes by optimizing the homogenization steps needed to generate synaptosomes and
124 the speed and duration of spins that are necessary to separate unlysed cells from synaptosomes. ATP
125 was added throughout the purification to maintain vATPase function, which establishes the proton
126 gradient across the SV membrane that is necessary for the import of neurotransmitters and the
127 maintenance of their levels within SVs (Burger et al., 1989). Following hypotonic lysis of the
128 synaptosomes to release SVs, we immunoprecipitated SVs using HA antibodies conjugated to solid
129 magnetic beads. The reduced porosity and magnetic properties of these beads enable cleaner, quicker
130 capture compared to standard agarose beads, which can trap metabolite contaminants within their porous
131 bead matrix (Chen et al., 2016). A series of high salt washes post-immunoprecipitation disrupted non-
132 specific protein and metabolite interactions and further reduced contaminants. During our initial attempts
133 to IP SVs, we used a triple HA tag. Although this was sufficient to capture SVs as assessed by
134 immunoblotting, the yield was low (Figure S1B). We reasoned that additional repeats of the HA epitope
135 would increase capture efficiency by enhancing the probability that the tag will encounter an antibody
136 during the IP period. Indeed, an extended tag of nine tandem HA sequences increased the yield of SVs
137 despite expressing at a lower level than the triple HA tag (Figure S1B).

138

139 To assess the quality and integrity of SVs isolated by this rapid procedure, we characterized the
140 isolate remaining at the end of the purification for multiple key features of SVs. Using immunoblotting, we
141 confirmed the enrichment of SV protein markers such as synaptotagmin and SV2A and the concomitant
142 depletion of markers for other subcellular organelles (Figure 1F). We then used mass spectrometry to
143 profile the proteome of isolated SVs in depth and found that the most significantly enriched proteins were
144 SV resident proteins, including glutamate transporters, synaptobrevin, and vATPase subunits
145 (Supplementary Table 1) (Figure S1D) (Gronborg et al., 2010; Takamori et al., 2006). Furthermore,
146 transmission electron microscopy revealed that the majority of particles isolated from our workflow are
147 homogeneous spheres of ~30-50 nm in diameter (Eshkind and Leube, 1995), as expected for SVs
148 (Figure 1G). A minority of larger particles (>100 nm) are also present, which may be large dense core
149 vesicles (Gondré-lewis et al., 2012) or contaminating organelles and cellular debris. Finally, SVs isolated
150 with SV-Tag are comparable to those purified via lengthier, traditional differential centrifugation protocols,
151 as assessed by immunoblot (Figure S1E) and electron microscopy (Figure S1F), albeit with a reduction in
152 yield that comes as a tradeoff for the speed of isolation.

153

154 Although these analyses indicate we were able to enrich for SVs, they do not provide evidence
155 that the SVs are intact, which is crucial for subsequent metabolite analysis. If the integrity of isolated SVs
156 is not compromised, they should contain glutamate, the principal neurotransmitter of cultured cortical
157 neurons (Beaudoin III et al., 2012). Using a luminescence-based assay to detect glutamate, we observed
158 an enrichment of glutamate in isolated SVs compared to the material obtained when the same protocol
159 was applied to uninfected neurons (Figure S1G). Importantly, glutamate was depleted upon treatment of

160 neurons with BaflomycinA (BafA), a vATPase inhibitor that dissipates the proton gradient of SVs that is
161 essential for the import of glutamate into SVs (Bowman et al., 1988). Taken together, multiple lines of
162 evidence demonstrate that our SV-Tag workflow enables rapid and specific high-affinity capture of intact
163 SVs.

164

165 **Targeted and global metabolite profile of SVs from cultured neurons**

166

167 To interrogate the metabolite contents of SVs in a precise and robust manner, we initially used
168 targeted gas chromatography-mass spectrometry (GC/MS), a method with high sensitivity for many
169 analytes, low cost and ease of operation, all of which are important considerations for optimization studies
170 (Beale et al., 2018). We selected a panel of amino acids to profile, which included bona fide
171 neurotransmitters (glutamate and glycine) (Gundersen et al., 2005), a putative neurotransmitter
172 (aspartate) (Fleck and Palmerv, 1993), and non-neurotransmitter amino acids to assess the cleanliness of
173 our preps. To identify metabolites that are enriched in SVs, we compared the signal of metabolites
174 present in HA immunoprecipitates from SV-Tag infected neurons versus the signal from
175 immunoprecipitates of uninfected neurons, which served as a control for metabolites that non-specifically
176 adhere to HA beads (Chen et al., 2017) (Supplementary Table 3). In primary cortical cultures, glutamate
177 was the sole metabolite that was significantly enriched in SVs when compared to control (Figure 2A) and
178 it was the only metabolite depleted by BafA treatment (Figure 2B), consistent with the excitatory and
179 glutamatergic identity of these neurons. Importantly, non-neurotransmitter amino acids were not enriched,
180 demonstrating that our SV preparations are of high purity as they lack contaminating metabolites. Of note,
181 aspartate was not detected in these vesicles, suggesting that it does not function as a neurotransmitter in
182 these cells. The same conclusion was reached via electrophysiological studies for hippocampal excitatory
183 synapses (Herring et al., 2015).

184

185 To demonstrate the applicability of this method to profile other neuron types, we isolated SVs
186 from inhibitory neuron cultures (Figure S2A), prepared from the medial ganglionic eminence (MGE)
187 (Franchi et al., 2018). Unlike cortical cultures, these neurons are GABAergic, as evidenced by their
188 expression of VGAT, a GABA transporter (Wojcik et al., 2006), and their lack of VGLUT1 protein, a
189 glutamate transporter (Pines et al., 1992) (Figure S2B). Reflecting the differences in the neurotransmitter
190 identities of cortical and MGE-derived neurons, GC/MS analysis revealed that SVs isolated from MGE
191 cells are enriched for GABA but not glutamate, whereas the converse is observed for cortical cultures
192 (Figure S2C). Highlighting the specificity of our method, MGE SVs were not significantly enriched for any
193 other amino acids profiled. Thus, by combining the SV-Tag isolation workflow with GC/MS, we can
194 successfully obtain neurotransmitter profiles of diverse neuron subtypes.

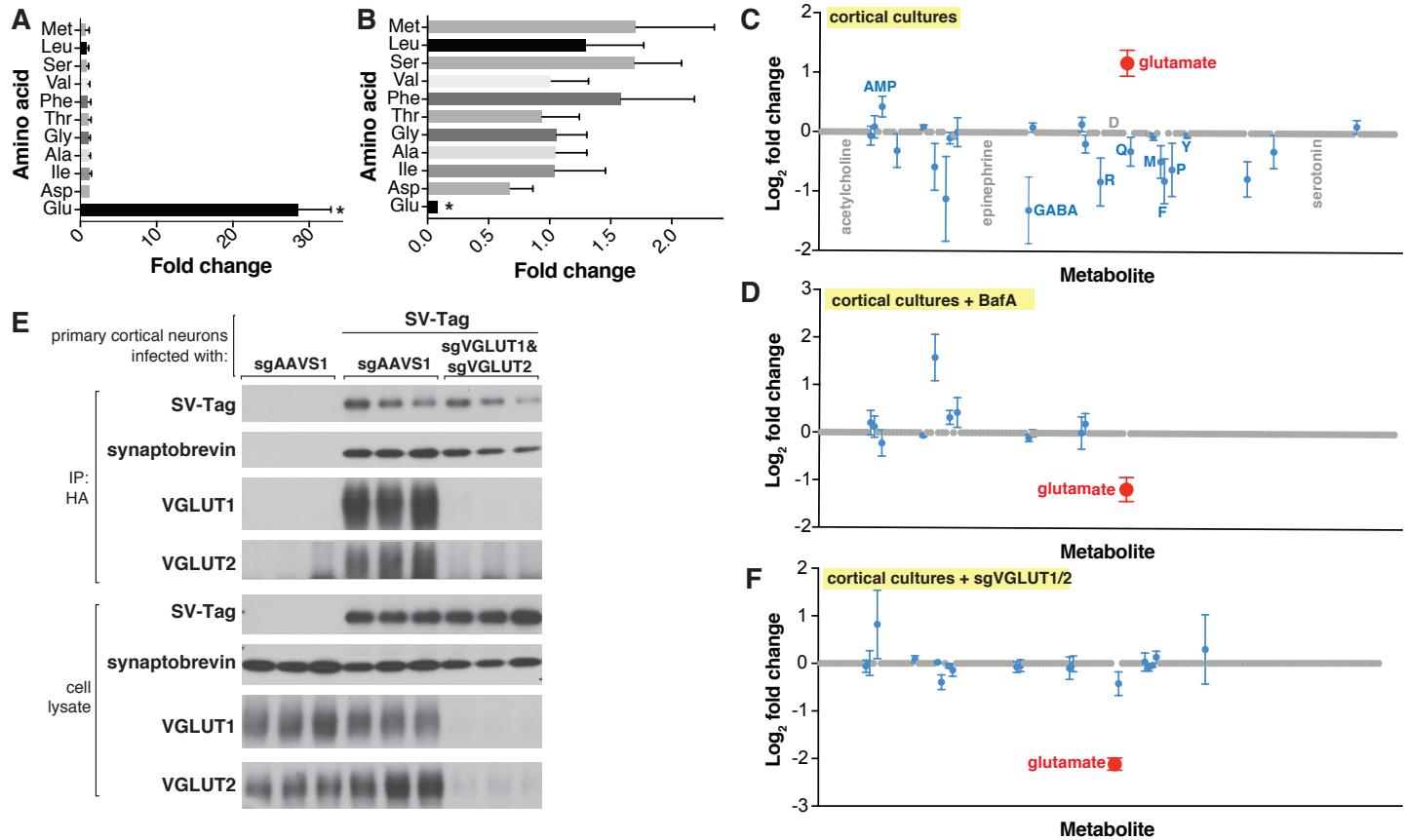


Figure 2: Targeted metabolite profile of purified synaptic vesicles from cultured neurons

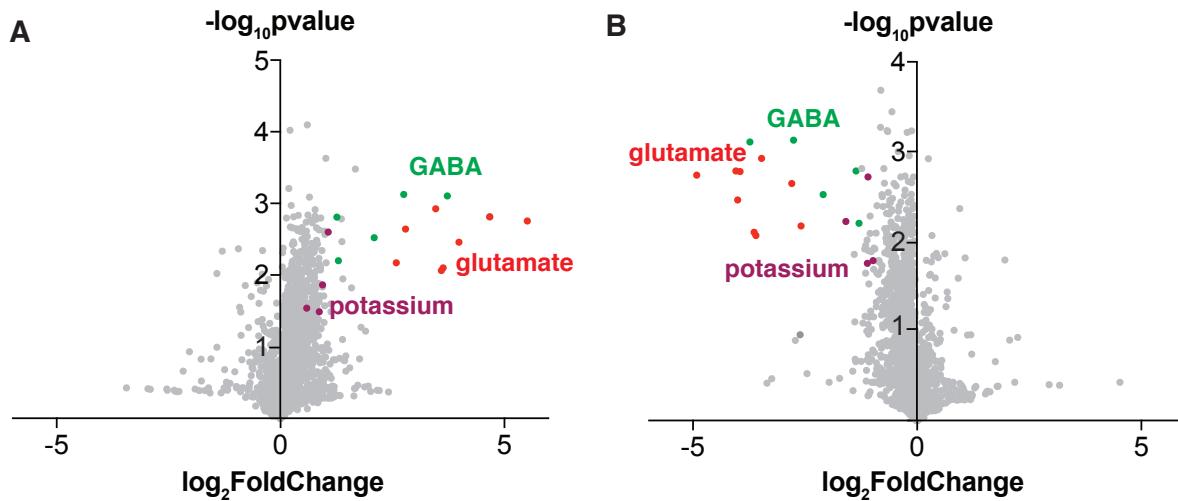
(A) Fold change (mean \pm standard error of the mean (SEM), n=3) of selected amino acids detected by GC/MS in purified SVs versus control IPs. Asterisk denotes a statistically significant difference (p value < 0.05) of the abundance of the indicated amino acid in SVs profiled from cells infected with SV-Tag compared to uninfected cells. (B) Effect of pretreatment of neurons with BafilomycinA on the abundance of selected amino acids in purified synaptic vesicles, as detected with GC/MS (mean \pm SEM, n=3) (C) Relative abundance via LC/MS of 153 polar metabolites present in purified SVs derived from SV-Tag infected cells, compared to preps from uninfected cells (mean \pm SEM, n = 3-4). Red indicates p value <0.05, blue indicates p value > 0.05, and gray indicates that the metabolite was undetected in all samples. Single letter codes annotate selected amino acids. Metabolites are listed in alphabetical order, and their corresponding identities can be found in Supplemental Table 2. (D) Effect of BafilomycinA on the presence of a panel of polar metabolites in purified synaptic vesicles profiled with LC/MS (mean \pm SEM, n = 3-4). Fold changes are color coded using the same specifications as in (C). (E) Immunoblot analysis of neurons expressing control guides (sgAAVS1) or guides targeting glutamate transporters (sgVGLUT1 and sgVGLUT2). Lysates were prepared from neurons infected with lentivirus encoding the indicated constructs. Fold changes are color coded using the same specifications as in (C). (F) LC/MS metabolite profile of synaptic vesicles isolated from cells with glutamate transporter knockdown compared with control cells expressing the control guide.

195 Although GC/MS provides a strong starting point for analysis, it is limited in its capacity to detect
196 a wide range of polar metabolites, due to the need for polar metabolite volatilization and separation on a
197 GC column (Lei et al., 2011; Sobolevsky et al., 2003; Stenerson, 2011). Therefore, we transitioned to
198 metabolomic analysis by liquid chromatography – high resolution mass spectrometry (LC/MS), which can
199 detect a much broader range of metabolites with nanomolar sensitivity (Lei et al., 2011). We quantified
200 the relative abundance of 153 polar compounds, which included neurotransmitters such as acetylcholine,
201 serotonin, and epinephrine, as well as key molecules in metabolic pathways and subcellular
202 compartments (Supplementary Table 2). Corroborating our earlier findings with GC/MS, glutamate was
203 the sole detected metabolite that is significantly enriched within SV-Tag isolated SVs (Figure 2C) and
204 depleted upon BafA treatment (Figure 2D) (Supplemental Table 5). All other metabolites were either
205 undetected or detected at statistically insignificant levels. Reflecting the specificity of our workflow for
206 isolating SVs, markers for other subcellular compartments were not enriched, including cystine, which is
207 characteristic of lysosomes (Pisoni and Thoene, 1991), and aspartate and phosphocholine, which are the
208 most enriched metabolites in mitochondria (Chen et al., 2016).

209
210 Because vATPase resides in other organelles (Kanazawa and Wada, 2000), the observation that
211 its inhibition depletes glutamate is not sufficient to provide certainty that the glutamate detected is within
212 SVs. To address this concern, we took advantage of the fact that glutamate is imported into SVs via
213 glutamate transporters VGLUT1 and VGLUT2 (Takamori et al., 2001), which, unlike vATPase, are SV-
214 specific proteins (Fremeau Jr et al., 2001). Reassuringly, CRISPR-mediated depletion of these
215 transporters from cortical neurons by targeting the genes *Slc17a6* (VGLUT1) and *Slc17a7* (VGLUT2)
216 significantly reduced glutamate levels from isolated SVs (Figure 2E, 2F). Taken together, these results
217 demonstrate that our purification workflow isolates SVs of high purity and integrity which are compatible
218 for robust and sensitive profiling with multiple MS methods.

219
220 Previous work has proposed that other polar molecules may function as neurotransmitters,
221 including taurine, gamma-hydroxybutyrate, β -alanine, and agmatine (Cash, 1994; Clarke and Haig, 1971;
222 Kilb and Fukuda, 2017; Reis and Regunathan, 2000; Tiedje et al., 2010). To gain a more comprehensive
223 and unbiased view of the SV metabolome, we performed a global metabolomics screen for polar
224 metabolites (Figure 3A, 3B). The LC/MS runs were optimized for detection of a broad range of polar
225 retentivities using a HILIC stationary phase and fast polarity switching MS method to capture analytes
226 which ionize exclusively in positive or negative mode. Overall, we detected 2,724 representative features
227 which were defined by an observed m/z (25 ppm) at a specific retention time (± 0.25 min) with a minimum
228 intensity and signal to noise threshold (Supplemental Table 4).

229



metabolite	polarity	m/z	retention time	SV-Tag IP/control IP		SV-Tag IP BafA/DMSO	
				\log_2 fold change	pvalue	\log_2 fold change	pvalue
glutamate	-	146.0447	11.53	5.52	0.0018	-4.92	0.0018
glutamate	+	148.0602	11.54	4.69	0.0015	-4.04	0.0016
glutamate (isotope)	+	149.0635	11.52	3.99	0.0035	-4.00	0.0035
glutamate	+	149.0601	11.52	4.68	0.0015	-3.95	0.0017
glutamate (sodium adduct)	+	170.0421	11.54	3.63	0.0079	-3.63	0.0079
glutamate (in-source fragment)	+	130.0497	11.54	3.60	0.0086	-3.60	0.0086
glutamate (CO ₂ adduct)	+	190.0240	11.58	3.47	0.0012	-3.47	0.0012
glutamate (sodium adduct)	-	168.0269	11.53	2.79	0.0023	-2.79	0.0023
glutamate (in-source fragment)	+	84.0448	11.57	2.59	0.0067	-2.59	0.0067
GABA (in-source fragment)	+	87.0445	11.36	3.73	0.0008	-3.73	0.0008
GABA (in-source fragment)	+	86.0604	11.32	2.76	0.0007	-2.76	0.0007
GABA (M+1 isotope)	+	105.0741	11.34	2.09	0.0030	-2.09	0.0030
GABA	+	104.0708	11.35	1.37	0.0016	-1.37	0.0016
GABA (peak tail)	+	104.0708	11.37	1.30	0.0063	-1.30	0.0063
potassium	+	751.7706	22.88	0.87	0.0326	-1.59	0.0060
potassium	-	705.7961	22.86	0.59	0.0288	-1.11	0.0176
potassium	-	461.8357	23.21	0.94	0.0136	-1.10	0.0019
potassium	-	724.7686	22.86	0.45	0.0025	-0.98	0.0164

Figure 3: Unbiased polar metabolomics profile of purified synaptic vesicles from cultured cortical neurons

(A) Global polar metabolomics analysis via LC/MS of purified synaptic vesicles, compared to an IP from uninfected cells. Green indicates glutamate and its associated derivatives generated during the LC/MS run. Red indicates GABA and its derivatives. Purple indicates potassium. Each dot represents the average of three replicate samples. **(B)** Global polar metabolomics analysis on purified synaptic vesicles from Bafilomycin-treated versus DMSO treated neurons. Legend is same as in Figure 3A. **(C)** Summary of metabolites from global analysis which are significantly enriched in SV-Tagged SVs and significantly depleted by BafilomycinA treatment.

230 To identify SV-specific metabolites, we filtered for peaks that were significantly enriched by at
 231 least two-fold in SVs and concomitantly depleted by BafA by at least two-fold. To assign the peak identity
 232 of this small subset of features, we used a variety of manual approaches including spectral library search,
 233 accurate mass formula search and isotope fine structure. To our surprise, only three metabolites satisfied
 234 these criteria – glutamate, GABA, and potassium (Figure 3C, Supplemental Table 4). In these samples,

235 GABA likely originates from a minority population of interneurons in cultured cortical neurons, consistent
236 with the low expression level of VGAT, the GABA transporter, observed in these cultures (Figure S2B).
237 The variability of the number of GABAergic interneurons across different cortical culture preps likely
238 contributes to the variation in the GABA levels detected across MS runs. In addition to GABA, we
239 identified a peak that is associated with potassium, which arises from the putative pairing of this ion with
240 carbonate ions in the LC buffers used in the IP/MS workflow. However, potassium is a ubiquitous
241 component of multiple reagents and sample-to-sample fluctuations in their levels can contribute to altered
242 potassium content. Follow-up studies using atomic absorption spectroscopy or inductively coupled
243 plasma mass spectrometry (ICP-MS) will be necessary to establish how much potassium these SVs
244 quantitatively contain, whether these weakly enriched potassium peaks are indeed internalized within
245 SVs, and if they contribute to SV function.

246

247 **Adaptation of the method for SV isolation and profiling from brain tissue**

248

249 Given their relative homogeneity of neuron types and ease of preparation, cultured cortical
250 neurons provide an ideal setting to optimize and test purification protocols. However, in the brain,
251 molecularly and functionally distinct neurons are intermingled in an intricate and heterogeneous
252 environment, and they rely on the uptake of extracellular metabolites found in this environment for
253 neurotransmitter synthesis (Elsworth and Roth, 1997; Mathews and Diamond, 2003; Schousboe et al.,
254 2013). To gain a more complete understanding of neurotransmission and potentially identify unknown
255 endogenous neurotransmitters, it is necessary to profile SVs isolated from their native environment. We
256 therefore adapted the method for use in brain tissue (Figure 4A). First, we expressed SV-Tag in the brain
257 by transducing desired brain regions of mice via stereotaxic injections of adeno-associated viruses (AAV)
258 encoding Cre-independent SV-Tag. To ensure neuron specific expression, the expression of SV-Tag was
259 driven by the synapsin promoter (Kugler et al., 2003). SV-Tag readily expresses in diverse areas, as
260 evidenced by the abundance of tdTomato positive neurons in targeted regions (Figure 4B). To ensure
261 that SV-Tag does not impair neurotransmission *in vivo*, we examined synaptic transmission at well-
262 characterized Schaffer collateral synapses between hippocampal CA3 and CA1 pyramidal cells (Figure
263 S4A) (Jackman et al., 2014). Paired-pulse facilitation, which indicates changes in probability of release
264 from presynaptic terminals, was assessed using pairs of closely spaced electrical stimuli (Figure S4B).
265 Paired pulse ratios, measured at an inter-stimulus interval of 50 ms, were unaffected by SV-Tag
266 expression in presynaptic neurons, which indicated that SV-Tag did not significantly alter neurotransmitter
267 release probability at this synapse (Figure S4C).

268

269 Our initial attempts to isolate SVs from brain tissue using the same strategy developed for
270 cultured neurons resulted in preps with poor yield and high background. This is not surprising given the
271 complex structure and composition of brain tissue compared to cultured neurons, which grow as a

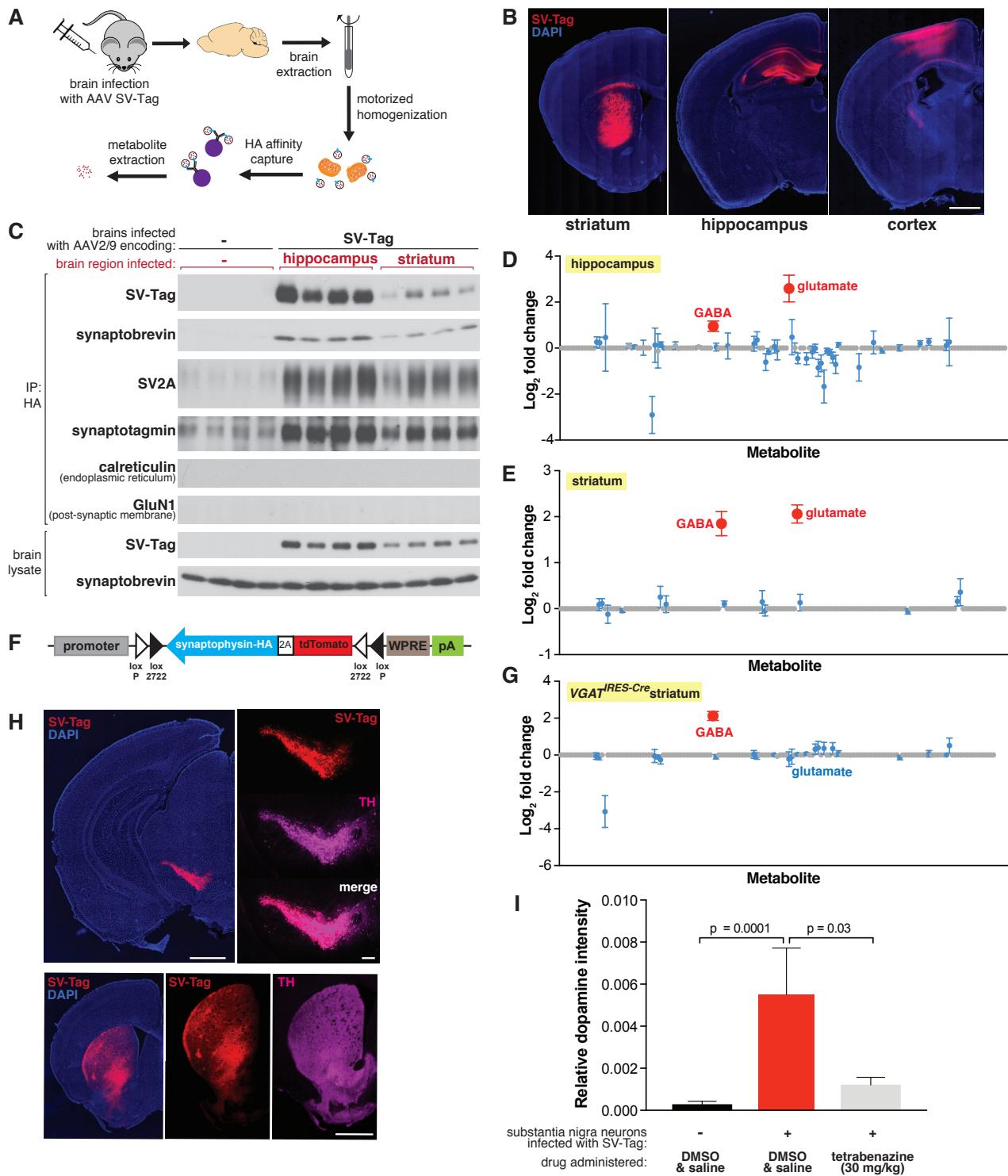


Figure 4: Adaptation of the workflow for rapid and specific isolation and metabolite profiling of synaptic vesicles directly from mouse brain tissue

(A) Schematic of the workflow used to isolate synaptic vesicles from mouse brain tissue. **(B)** Immunofluorescence images of coronal sections from wild type mouse brains transduced with SV-Tag in the indicated brain regions. Neurons are labeled with DAPI nuclear stain (blue) and SV-Tag (red). Scale bar = 1 mm. **(C)** Immunoblot analysis of indicated protein markers present in brain lysates, control

immunoprecipitates from uninfected brains, and HA immunoprecipitates from hippocampi and striatum that were infected with SV-Tag. **(D)** LC/MS profile of synaptic vesicles isolated from wild type mice brains infected with SV-Tag in hippocampus compared to a control IP from uninfected brains. (mean \pm SEM, n=4). Color code and legend is the same as in Figure 2C. **(E)** LC/MS profile of synaptic vesicles isolated wild type mice brains infected with SV-Tag in striatum compared to uninfected brains. (mean \pm SEM, n=4) **(F)** Construct design for expression of SV-Tag in neurons in a Cre-dependent manner. **(G)** LC/MS profile of synaptic vesicles isolated from *Slc32a1*^{IRES-Cre/wt} (*VGAT*^{IRES-Cre}) mice brains infected with SV-Tag in striatum compared to uninfected brains. (mean \pm SEM, n=4) **(H)** Coronal sections from a *Slc6a3*^{IRES-Cre/wt} mouse transduced with Cre-dependent SV-Tag in dopaminergic neurons of the midbrain. Dopamine neurons are immunolabelled for tyrosine hydroxylase (TH, magenta), DAPI nuclear stain (blue) and SV-Tag (red). **(I)** Targeted LC/MS profiling of dopamine in synaptic vesicles isolated from *Slc6a3*^{IRES-Cre/wt} mice transduced with Cre-dependent SV-Tag in dopaminergic neurons of the midbrain. Indicated mice were subjected to with saline injection or tetrabenazine injection intraperitoneally 2 hours prior to harvesting of synaptic vesicles.

272 homogeneous monolayer. To resolve these issues, we compared several lysis protocols for their
273 efficiency in releasing SVs from neurons. Compared to forming and lysing synaptosomes, homogenizing
274 the whole brain to immediately free SVs greatly improved yields (Figure S4D). Importantly, a rotarized
275 homogenizer was necessary for effective and rapid lysis. The addition of a final five-minute high-salt
276 incubation after the IP further reduced background contamination. In combination, these changes enabled
277 SV isolation in under 30 minutes from diverse brain regions, including hippocampus, cortex, and striatum
278 (Figure 4C, S4E). Moreover, SVs had minimal contamination of other subcellular organelles as
279 demonstrated by immunoblot and electron microscope analyses (Figure 4C, S4E, S4F).

280

281 Utilizing the LC/MS workflow, we determined if our method is capable of distinguishing the
282 content of SVs isolated from brain regions with different neurotransmitter profiles. We profiled SVs from
283 the hippocampus, which is composed of roughly 90% glutamatergic pyramidal neurons and 10%
284 GABAergic interneurons (Olbrich and Braak, 1985), and the striatum, which is comprised primarily of
285 GABAergic cells (Yoshida and Precht, 1971). The metabolite profiles of hippocampal SVs are expected,
286 with glutamate and GABA detected and glutamate being the more abundant neurotransmitter (Figure 4D).
287 Similar to cortical culture SVs, no other metabolites other than these two known neurotransmitters were
288 significantly present.

289

290 In contrast, striatal SVs were contaminated by a significant amount of glutamate. Because SV-
291 Tag was expressed in a Cre independent manner in wild type mice, this could result from isolation of SVs
292 from glutamatergic cortical neurons that lie above striatum and became infected with SV-Tag due to
293 pipette withdrawal and viral leak or via retrograde transduction due to axonal-uptake of AAV.
294 Alternatively, it could be due to contamination by cortical and thalamic glutamatergic terminals that
295 heavily innervate the striatum. To distinguish between these possibilities, we generated a Cre-dependent
296 construct of SV-Tag to enable cell type specific expression of this IP handle (Figure 4F) (Atasoy et al.,

297 2008). Striatal SVs from *Slc32a1*^{IRRES-Cre} mice, where SV-Tag expression is restricted to GABAergic
298 neurons (Vong et al., 2011) and SVs from *Adora2a*^{Cre} mice, which restrict expression further to a
299 subpopulation of GABAergic striatal neurons (Durieux et al., 2009)(Figure S4G), reveal the enrichment of
300 GABA and the concomitant depletion of glutamate (Figure 4G and S4H). Altogether, these results
301 indicate that cell-type specific expression of SV-Tag permits analysis of SV content on material isolated
302 directly from complex brain tissue.

303

304 Thus far, we focused on the detection of neurotransmitters whose polar and stable nature is
305 compatible for sensitive detection by LC/MS. However, some neurotransmitters are less stable, such as
306 the rapidly oxidizable dopamine, a crucial neurotransmitter whose loss underlies debilitating motor
307 ailments such as Parkinson's disease (Damier et al., 1999). To test if, despite its labile nature, our rapid
308 SV isolation workflow enables detection of dopamine, we expressed SV-Tag in midbrain substantia nigra
309 neurons of *Slc6a3*^{IRRES-Cre} mice where Cre recombinase is restricted to cells expressing DAT, the plasma
310 membrane dopamine transporter. As has been previously demonstrated in the injection target, Cre-
311 expressing cells in this mouse line match well with the population of neurons that express VMAT2, the
312 vesicular dopamine transporter (Edwards, 1992; Lammel et al., 2015). We validated specificity of
313 expression by immunohistochemical analysis, which indicated selective expression of SV-Tag in
314 dopaminergic cells as seen by colocalization with tyrosine hydroxylase, a dopamine synthetic enzyme
315 (Daubner et al., 2011) both in the soma and in axonal projections to striatum (Figure 4H). In a cohort of
316 mice expressing SV-Tag, we administered tetrabenazine, a reversible inhibitor of VMAT2 (Erickson et al.,
317 1996; Scherman et al., 1983), at a concentration validated to deplete dopamine from SVs in acute striatal
318 slices (Figure S4I, S4J). Using a modified LC/MS method for the detection of dopamine, we find that
319 substantia nigra SVs are enriched for dopamine and these stores are depleted by VMAT2 inhibition
320 (Figure 4I). Taken together, we developed a workflow to enable rapid and specific purification of SVs
321 directly from the brain, and to obtain the neurotransmitter profiles of a wide range of neurons in a cell-type
322 specific manner.

323

324 **Discussion**

325

326 Neurons use a broad array of small molecules for fast chemical neurotransmission. The ability
327 for different neuron classes to release different transmitters, along with the diversity of neurotransmitter
328 specific postsynaptic receptors, enables complex and intermingled excitatory, inhibitory, and
329 neuromodulatory trans-synaptic signaling in dense brain tissue. Furthermore, individual neurons are able
330 to release multiple transmitters, sometimes releasing different neurotransmitters to different postsynaptic
331 targets, as well as switch neurotransmitter identity in a developmental and activity dependent manner.
332 This richness of neurotransmitter usage in the brain highlights the need for new methods to deduce
333 neurotransmitter identity in an accurate and cell type specific manner. To address these needs, we

334 developed a workflow that combines high-affinity capture of epitope tagged SVs with MS-based
335 metabolomics to comprehensively profile SV contents. We adapted our method to isolate SVs with speed
336 and ease from both primary cultured neurons and genetically-defined neurons in brain tissue, which will
337 allow complementary *in vitro* and *in vivo* studies in the future. Our study confirms the utility of combining
338 IP and MS based methods to characterize organelles such as SVs, and underscores the importance of
339 performing global analyses to draw unbiased conclusions of the metabolic contents of organelles.

340

341 Our analyses of polar metabolites in SVs profiled in this study reveal that these organelles have a
342 minimal metabolic content and contain only previously recognized *bona fide* neurotransmitters, which is
343 consistent with their primary role in neurotransmitter storage and release. We find that glutamatergic
344 vesicles, isolated from either cultured neurons or directly from the brain, contain only glutamate and lack
345 detectable amounts of other proposed neurotransmitters (such as aspartate). Indeed, untargeted
346 analysis of polar metabolites failed to detect any additional molecule that was enriched within these
347 vesicles. It is possible that non-polar molecules and ions could also be imported into SVs. Indeed, zinc
348 has been proposed to function as a neurotransmitter at excitatory synapses (Vergnano et al., 2014). In
349 future studies, it will be beneficial to modify our workflow to be compatible with MS methods for atomic ion
350 and non-polar metabolite detection. In addition, further optimization of the immunocapture procedure to
351 increase SV yields is necessary to utilize MS methods for atomic ion detection, which require high
352 amounts of starting material.

353

354 Although our method is rapid and specific, improvements to increase its sensitivity for
355 neurotransmitter detection, particularly from SVs isolated from the brain, would be beneficial, even for the
356 detection of polar metabolites. We were able to identify dopamine in SVs from substantia nigra neurons,
357 but not GABA and glutamate (Tritsch et al., 2016; Zhang et al., 2015), which are known to be released by
358 these cells. In addition, we observed a robust GABA signal in SVs from striatal cells but did not detect
359 acetylcholine, which is released by a small population of interneurons that reside in this region (Cox and
360 Witten, 2019). As cholinergic neurons represent about 2% of striatal neurons (Zhou et al., 2002), this
361 suggests a lower limit for the ability of our current protocol to detect minor neurotransmitters. The yield of
362 SVs captured by our method and thus the detection limit would likely be increased by the use of a mouse
363 line in which synaptophysin is endogenously tagged with HA in a conditional manner. This would permit
364 more uniform and complete expression of SV-Tag in desired cells without ectopic overexpression, and
365 thus increase SV yields and the consistency between preps. Furthermore, it would eliminate the need for
366 tedious injections and facilitate greater turnaround time of experiments.

367

368 With a method to identify molecules that are enriched in SVs, we can pursue many intriguing
369 questions and avenues. To determine whether specific molecules function as neurotransmitters,
370 candidates can be easily profiled for their presence inside SVs using our workflow. For instance, in the

371 context of cultured neurons and the brain regions profiled in this study, neither aspartate nor taurine -
372 molecules long debated to be neurotransmitters - were enriched within SVs, thus bringing into question
373 their potential synaptic functions. Analysis in the hippocampus also failed to find evidence in support of
374 aspartate being a neurotransmitter (Herring et al., 2015). Another important avenue of research is
375 elucidating the function of transporter-like SV proteins such as SV2A and defining the substrate
376 specificities of promiscuous vesicular transporters, including VMAT2 (Lynch et al., 2004; Yelin and
377 Schuldiner, 1995). Loss of function experiments combined with global MS profiling will provide a powerful
378 strategy to interrogate how these proteins affect SV function. Finally, this method can be easily adapted
379 to other applications to resolve long-standing questions about SVs. For instance, the release properties of
380 SVs have long been known to be heterogeneous, and only a fraction of SVs known as the rapidly
381 releasable pool fuse upon electrical excitation of a neuron (Rizzoli and Betz, 2005). Proteomic profiling of
382 these different SV populations following immunoisolation could reveal the molecular basis for these
383 differences, which has long eluded researchers. In combination, these possibilities highlight the potential
384 of SV immunoisolation and metabolomics to address a wide range of biological questions.

385

386 In conclusion, the robustness and ease of our SV isolation and profiling methodology will serve as
387 a platform upon which we can gain a deeper understanding of the diverse ways that these organelles
388 control neurotransmission.

389 **Materials and Methods**

390

391 **Preparation of neuronal cultures and drug treatments** – Primary dissociated cortical cultures were
392 prepared from cortices of E16-E18 embryos of CD-1 mice (Charles River stock # 022) as described
393 (Sciarretta and Minichiello, 2010). Cultured inhibitory neurons were prepared from the MGE of E13-E14
394 mice. The following modifications were made to enhance culture health: tissue pieces were digested with
395 papain (Worthington) instead of trypsin-EDTA, DNase (Sigma) was added to break down released
396 genomic DNA and aid in more efficient trituration, and 1 ml pipette tips (PipetOne) were used to titurate
397 the tissue instead of fire polished pipettes for more consistency.

398 For immunohistochemistry experiments, ~1 million cells were plated onto 24-well plates pre-
399 coated with Poly-D-Lysine (PDL) (Sigma) and laminin (Invitrogen). For biochemical isolations, 4-5 million
400 cells were plated onto 10 cm plates pre-coated with Poly-D-Lysine (PDL) and laminin. Cells were
401 maintained at 37°C and 5% CO₂. A third of the media was replaced every 3 days with fresh Neurobasal
402 media, and 1 µM Arabinoside C (Sigma) was added on days in vitro (DIV) 5 to prevent overgrowth of
403 astrocytes and microglia.

404 In experiments where SVs were isolated from BafilomycinA (BafA) treated neurons, prior to the
405 isolation cells were treated for 2 hrs with 500 nM BafA (EMD Millipore) or the corresponding DMSO
406 volume.

407

408 **Lentiviral constructs and production** – The following sense (S) and antisense (AS) oligonucleotides
409 encoding the guide RNAs were cloned into a pLentiCRISPR vector (Addgene 52961):

410

411 sgAAVS1 (S): caccgTCCCCTCCACCCCACAGTG

412 sgAAVS1 (AS): aaacCACTGTGGGTGGAGGGGAc

413

414 sgVGLUT1 (S): caccgGGAGGAGTTTCGGAAGCTGG

415 sgVGLUT1 (AS): aaacCCAGCTTCCGAAACTCCTCCc

416

417 sgVGLUT2 (S): caccgAGAGGACGGTAAGCCCCTGG

418 sgVGLUT2 (AS): aaacCCAGGGGCTTACCGTCCTCTc

419

420 Lentiviruses were produced by transfection of viral HEK-293T cells with SV-Tag in combination
421 with the VSV-G envelope and CMV ΔVPR packaging plasmids. Twenty-four hours after transfection, the
422 media was changed to fresh DMEM (Invitrogen) with 20% heat inactivated fetal bovine serum (Gemini
423 BioProducts). Forty-eight hours after transfection, the virus containing supernatant was collected from the
424 cells and centrifuged at 1000g for 5 minutes to remove cells and debris. Supernatants were stored for up
425 to 1 week at 4°C and added to plated cortical neurons at DIV 3.

426

427 **SV isolation from cortical cultures** – Five million neurons plated on a 10 cm tissue culture dish at
428 DIV12-14 were used for each immunoisolation. No more than two plates were processed at a time to
429 increase the speed of isolation. All buffers and tubes used were prechilled on ice, with the exception of
430 the metabolite extraction mix, which was kept on dry ice, and all steps were performed swiftly. Neurons
431 were placed on ice to chill, rapidly rinsed with ice-cold phosphate buffered saline (PBS) to remove
432 residual media, and gently scraped into 1 ml PBS. Cells were pelleted by a brief centrifugation step at
433 2400g for 40 seconds. The PBS was aspirated and 1 ml of homogenization buffer (320 mM sucrose, 4
434 mM HEPES NaOH, pH 7.4) supplemented with cComplete EDTA-free protease inhibitor (Roche) and 1
435 mM ATP NaOH, pH 7.4. The cell pellet was uniformly resuspended with a 1 ml large bore tip (Fisher
436 Scientific) and transferred to a 2 mL homogenizer (VWR International). To generate synaptosomes, the
437 cells were homogenized with 25 steady strokes, with care taken to minimize formation of air bubbles. The
438 homogenate was centrifuged at 2400g for 40 seconds to pellet unbroken cells. The supernatant was then
439 transferred to a new tube and centrifuged at 14000g for 3 minutes to pellet synaptosomes. The
440 supernatant was carefully removed and the pellet resuspended in 100 μ l of homogenization buffer using a
441 200 μ l large bore tip (Fisher Scientific). 900 μ l of ice cold ddH₂O (MS grade) was added, and the liquid
442 was immediately transferred to a 2 mL homogenizer. To lyse synaptosomes, the cells were homogenized
443 with 12 steady strokes. Osmolarity was restored following homogenization by the addition of ATP,
444 HEPES NaOH and cComplete EDTA-free protease inhibitor to the same concentrations as in the
445 homogenization buffer. Finally, the homogenate was centrifuged at 17000g for 3 minutes to remove any
446 unbroken synaptosomes and debris. This process takes a total of ~12 minutes.

447

448 To immunoisolate SVs, 150 μ l of suspended, prewashed magnetic HA beads (Thermo Fisher
449 Scientific) were added to the supernatant and incubated at 4°C with end-over-end rotation for 15 minutes.
450 For washes, beads were captured with a DynaMag Spin Magnet (Thermo Fisher Scientific) for 40
451 seconds. Four washes were performed in succession by the addition of 1 ml of KPBS (136 mM KCl, 10
452 mM KH₂PO₄, pH 7.25 in MS grade water) (Chen et al., 2016). Following the final wash, 25% of the
453 KPBS-bead suspension was aliquoted for immunoblot analysis and the remaining 75% was subjected to
454 metabolite extraction with 100 μ l of 80% methanol/20% water supplemented with 500 μ M internal amino
455 acid standards. To ensure complete extraction, the beads were incubated with extraction mix for at least
456 10 minutes on dry ice prior to being separated from the mix. Extracted metabolites were subjected to a
457 final 17000g spin for 3 minutes to remove any particulates and stored at -80°C until the MS run.

458

459 **Immunoblotting** – Protein from lysates were denatured by the addition of 50 μ l of sample buffer. For
460 whole cell lysates, 0.5 μ l of Benzonase (EMD Millipore) was added and incubated with the lysates for at
461 least 5 minutes to break down genomic DNA. Samples were resolved by 8%–16% SDS-PAGE,
462 transferred for 2 hrs at room temperature at 45 V to 0.45 mm PVDF membranes, and analyzed by

463 immunoblotting as described previously (Chantranupong et al., 2016). Briefly, membranes were blocked
464 with 5% milk prepared in TBST (Tris-buffered Saline with Tween 20) for at least 5 min at room
465 temperature, then incubated with primary antibodies in 5% BSA TBST overnight at 4°C with end-over-end
466 rotation. Primary antibodies targeting the following proteins were used at the indicated dilutions and
467 obtained from the denoted companies: synaptophysin 1:2000 (SySy Cat # 101002), SV2A 1:2000 (SySy
468 # 119003), HA 1:1000 (CST #C29F4), NMDAR 1:1000 (SySy #114011) (1:1000), synaptobrevin 1:5000
469 (SySy #104211), calreticulin 1:300 (CST #12238), VDAC 1:200 (CST#4661), LC3B 1:300 (CST #2775),
470 GAPDH 1:2000 (CST #2118), synaptotagmin 1:1000 (SySy #105011), VGLUT1 1:2000 (SySy #135303),
471 and VGLUT2 1:500 (SySy #135421) . Following overnight incubation, membranes were washed three
472 times, 5 min each, with TBST and incubated with the corresponding secondary antibodies in 5% milk
473 (1:5000) for 1 hour at room temperature. Membranes were then washed three more times, 5 min each,
474 with TBST before being visualized using enhanced chemiluminescence (Thermo Fisher Scientific).
475

476 **Endogenous tagging of synaptophysin** – An endogenous triple HA tag was appended onto the C-
477 terminus of synaptophysin using the vSLENDR method (Nishiyama et al., 2017). An AAV construct
478 (below) containing a guide targeting the C-terminus of synaptophysin (underlined text), gRNA scaffold
479 (bold italic text), 5' and 3' homology arms flanking this region (normal text), and the triple HA tag (gray
480 highlighted text) replaced the mEGFP-cmak2a HDR cassette in the backbone of the pAAV-HDR-mEGFP-
481 camk2a (Addgene #104589). This construct, which we term pAAV-HDR-sphys-3XHA, along with pAAV-
482 EFS-SpCas9 were packaged at Boston Children's Viral Core. Cortical neurons cultured on a cover slip
483 were coinjected on DIV3 with 3.45e⁷ genome copies (GC) of AAV-EFS-SpCas9 and 1.42e¹⁰ GC of AAV-
484 HDR-sphys-3XHA. Neurons were processed for immunostaining at DIV12-14.
485

486 5' TTCTCCAATCAGATGTAATC**GTTTAGAGCTAGAAATAGCAAGTTAAATAAGGCTAGTCGTTATCAACTTG**
487 **AAAAAGTGGCACCGAGTCGGCTTTTGAAATTCTTTGGTTGAGACAGGATCTACTTATGTGACCT**
488 GGCTGTCCCTGGAACTCACTACTCAGACCAGACTGGCCTCAAACACTCACAGACCTCTGCTTGCCTCTGCCTCCTGA
489 GTACTAAGATGAAGACTGCACCACCACCCCAGCCCCAAATGAGTTGTTGAGGCTGACTTCATGTTGCACAG
490 GCTAGCCTCAAACATGAAATTAAAGGTAGACTTGAATTCTGGTAGTGGAGGCAGAGACAGGCGACTTCTATG
491 AGTTCCAGGCCAGCCTGGTCTACAGAGTGAATTCCAGGACAGGCCAGGGCTGCAGAGAGACCCCTGTCCTCAAAAAA
492 AAAAAAGCTAGCCTGAAGTGATCGCCCTGCCTCCAGCTTCTTAAGATTACAAGATGTGGGCCTTCAGACTTGT
493 CCATGTAAACACTGATAGAAGTTGAACATCATGGGAATCTAACACACACACACTCCAAAGTTTCTGTACA
494 CTGATAGTCATAGAGGCCACGAATTATGCCCTAAAGGCCATTCTGTTCACTCAGCCTCAAAGACCCCTGG
495 GGCTGCCGAGGCAATGGTAAGAGACAACAGCTTGGTCATGTCTCCCTGCAGGTGTTGGCTTCTGAACCTG
496 GTGCTCTGGTTGGCAACCTATGGTCGTGTTCAAGGAGACAGGCTGGCCGCCCCATTCTATGCGCGCACCTC
497 CAGGCCAGAAAGCAACCAGCTCCTGGCGATGCCTACGGCGATGCGGGCTATGGCAGGGCCCCGGAG
498 GCTATGGCCCCAGGACTCCTACGGCCTCAGGGTGGTTCAACCCGATTACGGCAGCCAGGCCAGCGCG
499 GTGGCGTGGCTACGGCCTCAGGGCGACTATGGCAGCAAGGCTACGGCAACAGGGTGGCCACAGGATCCTACCCCT
500 TCTCCAACCAAATGGGAGGGAGCGGCTATCCCTATGACGTGCCTGATTACGCCGGCACAGGATCCTACCCCTAT
501 **GATGTGCCTGACTACGCTGGCAGGCCGGATACCCCTATGATGTGCCTGATTATGCTTAATCTGGTGAGTGACA**

502 ACTGGCGGATGCGGTAGGCAGGGAGCATAAGGAGTGAAGTTGAAGGAACCAATAGATAGGCAGAACCAA
503 AAAAAAAAAAAGGTGAACTTGGTAAACTAGCCAATGAGAGGAACCGTGAGAAGGAAGGGACGGAGCAGTGC
504 TCAGAGTAACCAATGAAAGGAGTGTAGGGCACTTGCAGTGGAGAATCACCAAAGTGGTAGGTTCCAGG
505 AAGGGAAAGGGAGGGAGGTCTTGAATCATTGGTAAACCAATAGGCGTAACGCCAGTAGGTGGAAGAAGGT
506 AAACACGTTGGGTTTGAAGGGCGTAGCGCTAACAGCAGGATGTAGGTAGCTGCTACCTCTCCTAACCTTT
507 AATGAAAGAGAGAGTTGAAATTCAAATGAGGAAAAGGGAGGGCTGGAGGCCTAGAAACACGAGTATGCCT
508 TTTTGTGGGCCTTAAAAAATGAATGCCGCCGACGGTGGAGGCGCACGCCCTTAATCCCAGCACTGGGAGG
509 CAGAAGCAGGGCGAGTTTGAGTTGAGGCTGAGGCCAGCCTGGTCTACAAAGTGAGTCCAGGACAGCCAGGGCTAT
510 ACAGAGAAACCTGTCGCAAAAAAAAAACCTGCCTGGTGTGATGGAGCACATATAATCCC
511 AGCACTTGAGAGGTAGAGGCATGGGATTGCAAGTCTCGAGTCCTGCAGTGGCAGTATAGCCCAATCCTGTCT
512 TAAACAGAGACGGTAACAGCATCTAGGTGGAGCAGATGTGGCCTGGTGAGCCTCTACAGCAACCCACATT
513 TAATTGTTTAAACTCCTGGACAGGCTCTGAGACACACCTTAAGCACAGCTCTGGGGATTAGAGACAGGC
514 CTAGGTCTCTGTTGCAAAGCAATTCCAGGCTGC 3'

515

516 **Immunohistochemistry** – Cells were fixed in 4% PFA 4% sucrose in PBS for 10 minutes, and washed in
517 PBS twice for 10 minutes each with shaking at room temperature. Cells were then blocked in 1 ml of
518 blocking solution (10% BSA, 10% normal goat serum, PBS) for 30 minutes at room temperature. Cells
519 were washed with 1 ml of TBST (0.2% TritonX-100 in PBS) for 10 minutes. Primary antibodies were
520 suspended in BTBST (1% BSA, 1% normal goat serum PBS) and 300 ul was added to each slip. The
521 following concentrations were used: synapsin 1:500 (CST #5297), synaptophysin 1:1000 (SySy 101004),
522 synaptotagmin 1:1000, and HA 1:500 (CST#2367). Cells were incubated overnight at 4°C or at room
523 temperature for 1.5 hours. Following this, cells were washed three times, 10 minutes each, in 1 ml of PBS
524 with rocking at room temperature. The following secondary antibodies (Thermo Fisher Scientific) were
525 diluted in BTBST and added at a 1:500 dilution: goat anti-rabbit Alexa Fluor 488, goat anti-guinea pig
526 Alexa Fluor 488, goat anti-rabbit Alexa Fluor 647. Cells were covered from light and incubated at room
527 temperature for 2 hours, washed 3 more times in PBS for 10 minutes each and mounted on cover slides
528 with Floromount G (Thermo Fisher Scientific). Slips were imaged an Olympus VS120 slide scanning
529 microscope.

530

531 **Transmission Electron Microscopy** – To free SVs from beads, we relied on a protease strategy as the
532 HA binding strength to its cognate antibody is too strong to be dissociated by peptide competition. We
533 used ficin (Sigma), a cysteine protease that can rapidly digest murine monoclonal IgGs (Mariani et al.,
534 1991). Following the IP, SVs were equilibrated with three washes of ficin buffer (50 mM Tris pH 7.0, 2 mM
535 EDTA, 1 mM cysteine). The supernatant was removed and replaced with 100 μ l of ficin buffer
536 supplemented with 5 mg/ml ficin (Sigma). SVs were incubated at 37°C for 20 minutes to enable ficin to be
537 active. Supernatant was removed and immediately chilled on ice prior to EM analysis. Images were
538 acquired at the Electron Microscopy Core at Harvard Medical School. 5 μ l of the sample was adsorbed for
539 1 minute to a carbon coated grid that had been made hydrophilic by a 30 second exposure to a glow
540 discharge. Excess liquid was removed with a filter paper (Whatman #1) and the samples were stained

541 with 0.75% uranyl formate for 30 seconds. After removing the excess uranyl formate with a filter paper the
542 grids were examined in a JEOL 1200EX Transmission electron microscope or a TecnaiG² Spirit BioTWIN
543 and images were recorded with an AMT 2k CCD camera.

544

545 **Luminescence Assay for Glutamate** – Following immunoisolation, SVs were permeabilized with 70 µl of
546 Triton elution buffer (1% TritonX-100, 500 mM NaCl, 5 mM HEPES pH 7.4) and incubated at 37°C for 20
547 minutes to ensure complete permeabilization. 15 l of this eluent was saved for immunoblot analyses. To
548 detect glutamate, the Glutamate Glo Assay Kit (Promega) was used. 50 µl of eluent was combined into a
549 96 well plate with a 50 µl mix of Luciferin detection solution, which contains reductase, reductase
550 substrate, glutamate hydrogenase, and NAD as specified. The mixture was incubated at room
551 temperature for 1 hr and luminescence was detected with a fluorescent plate reader (BioTek).

552

553 **Proteomics run and analysis** – To ensure sufficient yields for proteomic analysis, each sample
554 combined cells from three plates, for a total of 15 million neurons. Following immunoisolation, SVs were
555 permeabilized with 70 µl of Triton elution buffer at 32°C for 20 minutes. Eluents from the three plates were
556 pooled into a common tube. 20 µl of this mix was saved for immunoblot analysis. The remaining eluent
557 was transferred to a new tube and subjected to TCA precipitation. Briefly, the volume of eluent was raised
558 to 400 µl with MS grade, ice cold water. 100 µl of 100% trichloroacetic acid (TCA) was added to this
559 mixture.

560 Precipitated proteins were submitted to the Taplin Mass Spectrometry Core for proteomic
561 analysis. There, samples were digested with 50 µl of 50 mM ammonium bicarbonate solution containing 5
562 ng/µl modified sequencing-grade trypsin (Promega, Madison, WI) at 4°C. After 45 min., the excess
563 trypsin solution was removed and replaced with 50 mM ammonium bicarbonate solution to just cover the
564 gel pieces. Samples were then placed in a 37°C room overnight. Peptides were later extracted by
565 removing the ammonium bicarbonate solution, followed by one wash with a solution containing 50%
566 acetonitrile and 1% formic acid. The extracts were then dried in a speed-vac (~1 hr). The samples were
567 then stored at 4°C until analysis.

568 On the day of analysis the samples were reconstituted in 5 - 10 µl of HPLC solvent A (2.5%
569 acetonitrile, 0.1% formic acid). A nano-scale reverse-phase HPLC capillary column was created by
570 packing 2.6 µm C18 spherical silica beads into a fused silica capillary (100 µm inner diameter x ~30 cm
571 length) with a flame-drawn tip (Peng and Gygi, 2001). After equilibrating the column each sample was
572 loaded via a Famos auto sampler (LC Packings, San Francisco CA) onto the column. A gradient was
573 formed and peptides were eluted with increasing concentrations of solvent B (97.5% acetonitrile, 0.1%
574 formic acid).

575 As peptides eluted they were subjected to electrospray ionization and then entered into an LTQ
576 Orbitrap Velos Pro ion-trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Peptides were
577 detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for

578 each peptide. Peptide sequences (and hence protein identity) were determined by matching protein
579 databases with the acquired fragmentation pattern by the software program, Sequest (Thermo Fisher
580 Scientific, Waltham, MA) (Eng et al., 1994). All databases include a reversed version of all the
581 sequences and the data was filtered to between a one and two percent peptide false discovery rate.
582

583 **Mice** – The following mouse strains/lines were used in this study: CD-1® IGS (Charles River
584 Laboratories, Stock # 022); C57BL/6J (The Jackson Laboratory, Stock # 000664); DAT-IRES-Cre (The
585 Jackson Laboratory, Stock #006660)(referred to as *Slc6a3*^{lRES-Cre} mice); VGAT-IRES-Cre (The Jackson
586 Laboratory, Stock #016962) (referred to as *Slc32a1*^{lRES-Cre} mice); genetically targeted *Adora2a*-Cre BAC
587 transgenic mice (GENSAT, founder line KG139), which express Cre under transcriptional control of the
588 adenosine A2A receptor genomic promoter (Durieux et al., 2009). All animals were kept on a regular
589 12:12 light/dark cycle under standard housing conditions. All experimental manipulations were performed
590 in accordance with protocols approved by the Harvard Standing Committee on Animal Care following
591 guidelines described in the US National Institutes of Health Guide for the Care and Use of Laboratory
592 Animals.

593
594 **SV isolation from whole brain** – Mice were rapidly anesthetized with isoflurane and brains were quickly
595 extracted from mice on ice. To ensure more efficient homogenization, each brain was divided in half
596 along the midline and transferred immediately to a homogenizer containing 1.5 mls of ice-cold lysis buffer
597 (KPBS supplemented with 1 mM ATP and cOmplete EDTA-free protease inhibitor). Brains were rapidly
598 lysed with 30 strokes using a motorized homogenizer, taking care not to introduce bubbles. Lysates were
599 transferred to prechilled 2 ml tubes and centrifuged at 17000 g for 3 minutes to pellet unbroken cells,
600 contaminating organelles and debris. Supernatants were transferred to new 1.5 ml tubes and subjected to
601 IP with 150 ul HA beads for 15 minutes with end-over-end rotation. Following the IP, the beads were
602 washed 4 times in KPBS supplemented with 500 mM NaCl to enhance cleanliness. In the final wash, the
603 IP was incubated with end-over-end rotation at 4°C for 5 minutes to further remove contaminants.
604 Following this final wash, immunoprecipitates were processed for immunoblot and metabolite analysis as
605 described for cortical culture SVs described above.

606
607 **Stereotaxic injections** – Injections were performed as previously described (Huang et al., 2019). AAVs
608 for SV-Tag was obtained from Boston Children's Virus Core and infused at a concentration of ~10¹²
609 GC/ml. AAVs were infused into target regions at approximately 50 nl/min using a syringe pump (Harvard
610 Apparatus, #883015), and pipettes were slowly withdrawn (<10 µm/s) at least 8 min after the end of the
611 infusion. All coordinates are relative to Bregma along the anterior-posterior (AP) axis and medial-lateral
612 (ML) axis, and relative to the pial surface along the dorsoventral axis (DV). Coordinates for injections are
613 as follows: cortex site 1: AP = -2.5 mm, ML = -2.0 mm, DV = -0.4 mm, cortex site 2: AP = -1.0 mm,
614 ML = -1.5 mm, DV = -0.4 mm , striatum: AP = +0.6 mm, ML = 1.7 mm, DV = -3.33 mm, hippocampus AP

615 = -2.5 mm, ML = -1.5 mm, DV = -1.5 mm; hippocampus transverse (for paired pulse ratio
616 measurements): AP = -2.9 mm, ML = -3.15 mm, DV = -3.3 mm, substantia nigra: AP = -3.3 mm, ML = -
617 1.5 mm, DV = -4.3 mm. Bilateral injections were performed for all mice used, with the exception of cortex,
618 in which 4 injections were performed to maximally cover the cortical area. Following wound closure, mice
619 were placed in a cage with a heating pad until their activity was recovered before returning to their home
620 cage. Mice were given pre- and post-operative oral carprofen (MediGel CPF, 5 mg/kg/day) as an
621 analgesic, and monitored daily for at least 4 days post-surgery.

622

623 **Immunohistochemistry** – Mice were anaesthetized by isoflurane inhalation and perfused cardially with
624 PBS followed by 4% PFA in PBS. Brains were extracted and stored in 4% PFA PBS for at least 8 hours.
625 Brains were sliced into 70 μ m thick free-floating sections with a Leica VT1000 s vibratome. Selected
626 slices were transferred to a clean 6 well plate and rinsed three times, 5 minutes each in PBS. They were
627 then blocked with rotation at room temperature for an hour in blocking buffer (5% normal goat serum
628 (Abcam), 0.2% TritonX-100 PBS). Blocking buffer was removed and replaced with 500 μ l of a solution
629 containing a 1:500 dilution of anti- tyrosine hydroxylase antibody (Millipore Sigma). Slices were incubated
630 overnight with side to side rotation at 4°C. The next day, slices were transferred to a clean well and
631 washed 5 times, 5 minutes each in PBST (PBS with 0.2% TritonX-100). Following the final wash, slices
632 were incubated for 1.5 hours in 500 μ l of secondary antibody (goat anti mouse Alexa Fluor 647) diluted
633 1:500 in blocking buffer. Slices were washed four times in PBST, then four times in PBS for 5 minutes (5
634 minutes for each wash) before mounting with Floromount G (Thermo Fisher Scientific). Slices were
635 imaged an Olympus VS120 slide scanning microscope, including those housed in the Neuro Imaging
636 Facility.

637

638 **Electrophysiology** – Coverslips containing cultured neurons or acute brain slices were transferred into a
639 recording chamber mounted on an upright microscope (Olympus BX51WI) and continuously superfused
640 (2–3 ml min⁻¹) with ACSF containing (in mM) 125 NaCl, 2.5 KCl, 25 NaHCO₃, 2 CaCl₂, 1 MgCl₂, 1.25
641 NaH₂PO₄ and 25 glucose (295 mOsm kg⁻¹). ACSF was warmed to 32–34 °C by passing it through a
642 feedback-controlled in-line heater (SH-27B; Warner Instruments). Cells were visualized through a 60X
643 water-immersion objective with either infrared differential interference contrast optics or epifluorescence
644 to identify tdTomato⁺ cells. For whole cell voltage clamp recordings, patch pipettes (2–4 M Ω) pulled from
645 borosilicate glass (G150F-3, Warner Instruments) were filled with a Cs⁺-based low Cl⁻ internal solution
646 containing (in mM) 135 CsMeSO₃, 10 HEPES, 1 EGTA, 3.3 QX-314 (Cl⁻ salt), 4 Mg-ATP, 0.3 Na-GTP, 8
647 Na₂-phosphocreatine (pH 7.3 adjusted with CsOH; 295 mOsm·kg⁻¹) For voltage clamp recordings,
648 mEPSCs were recorded for 5 min in the presence of 1 μ M tetrodotoxin (Tocris), 10 μ M CPP (Tocris), and
649 10 μ M gabazine (Tocris) at a holding potential of -70 mV. Paired evoked EPSCs for probability of release
650 measurements were recorded as previously described (Jackman et al., 2016). Briefly, a cut was made
651 between CA3 and CA1 to prevent recurrent excitation. Extracellular stimulation was performed with a

652 stimulus isolation unit (Iso-flex). Bipolar electrodes (PlasticOne) were placed near CA3 proximal to the cut
653 and stimulation parameters were 20 Hz, 50-100uA. Paired evoked-EPSCs from CA1 cells were recorded
654 at a holding potential of -70mV with 10 μ M gabazine added to the bath.

655

656 **Acute brain slice preparation** – Brain slices were obtained from 2-4-month-old mice (both male and
657 female) using standard techniques. Mice were anaesthetized by isoflurane inhalation and perfused
658 cardiacly with ice-cold ACSF containing (in mM) 125 NaCl, 2.5 KCl, 25 NaHCO₃, 2 CaCl₂, 1 MgCl₂, 1.25
659 NaH₂PO₄ and 25 glucose (295 mOsm kg⁻¹). Brains were blocked and transferred into a slicing chamber
660 containing ice-cold ACSF. Coronal slices of striatum for amperometric recordings or transverse slices of
661 hippocampus (for probability of release measurements) were cut at 300 μ m thickness with a Leica
662 VT1000 s vibratome in ice-cold ACSF, transferred for 10 min to a holding chamber containing choline-
663 based solution (consisting of (in mM): 110 choline chloride, 25 NaHCO₃, 2.5 KCl, 7 MgCl₂, 0.5 CaCl₂, 1.25
664 NaH₂PO₄, 25 glucose, 11.6 ascorbic acid, and 3.1 pyruvic acid) at 34°C then transferred to a secondary
665 holding chamber containing ACSF at 34C for 10 min and subsequently maintained at room temperature
666 (20–22°C) until use. All recordings were obtained within 4 hr of slicing. Both choline solution and ACSF
667 were constantly bubbled with 95% O₂/5% CO₂.

668 **Amperometric recordings** – To deplete presynaptic terminals of dopamine, *Slc6a3*^{lRES-Cre} mice were
669 administered the VMAT2 antagonist tetrabenazine (Sigma, 30 mg kg⁻¹ intraperitoneally) 2 h before slicing.
670 Control mice were injected with a DMSO/Saline mixture containing the same proportion of both solvents
671 as would be used for a tetrabenazine injection. Constant-potential amperometry was performed as
672 previously described (Tritsch et al., 2012). Briefly, glass-encased carbon-fiber microelectrodes (CFE1011
673 from Kation scientific - 7 μ m diameter, 100 μ m length) were placed approximately 50-100 μ m within dorsal
674 striatum slices and held at a constant voltage of + 600 mV versus Ag/AgCl by a Multiclamp 700B amplifier
675 (Molecular Devices). Electrodes were calibrated with fresh 5 μ M dopamine standards in ACSF to
676 determine CFE sensitivity and to allow conversion of current amplitude to extracellular dopamine
677 concentration. Dopaminergic terminals surrounding the CFE were stimulated by Bipolar electrodes with
678 0.1 ms and 100-250 μ A delivered at 3 min intervals.

679 **Data acquisition and analysis** – Membrane currents were amplified and low-pass filtered at 3 kHz using
680 a Multiclamp 700B amplifier (Molecular Devices), digitized at 10 kHz and acquired using National
681 Instruments acquisition boards and a custom software (<https://github.com/bernardosabatini/SabalabAcq>)
682 written in MATLAB (Mathworks). Amperometry and electrophysiology were analyzed offline using Igor Pro
683 (Wavemetrics). Detection threshold for mEPSCs was set at 7 pA. Averaged waveforms were used to
684 obtain current latency, peak amplitude, 10–90% rise time and decay time. Current onset was measured
685 using a threshold set at three standard deviations of baseline noise. Peak amplitudes were calculated by
686 averaging over a 2 ms window around the peak. Data were compared statistically by unpaired two-tailed
687 Student's t-test. *P* values less than 0.05 were considered statistically significant.

688 **GC/MS** – GC-MS analysis was carried out and analyzed as described (Parker et al., 2017). In brief, dried,
689 extracted metabolites were derivatized using a MOX-tBDMCS method and analyzed by GC-MS using a
690 DB-35MS column (30m x 0.25mm i.d., 0.25 μ m) in an Agilent 7890B gas chromatograph interfaced with a
691 5977B mass spectrometer. Metabolites were identified by unique fragments and retention time in
692 comparison to known standards. Peaks were picked in OpenChrom and integrated and corrected for
693 natural isotopic abundance using in-house algorithms adapted from Fernandez et al (Fernandez et al.,
694 1996; Lewis et al., 2014; Wenig and Odermatt, 2010).

695

696 **LC-MS/MS with the hybrid metabolomics method** – Samples were subjected to an LCMS analysis to
697 detect and quantify known peaks. A metabolite extraction was carried out on each sample with a
698 previously described method (Pacold et al., 2016). The LC column was a MilliporeTM ZIC-pHILIC (2.1
699 x150 mm, 5 μ m) coupled to a Dionex Ultimate 3000TM system and the column oven temperature was set
700 to 25 $^{\circ}$ C for the gradient elution. A flow rate of 100 μ L/min was used with the following buffers; A) 10 mM
701 ammonium carbonate in water, pH 9.0, and B) neat acetonitrile. The gradient profile was as follows; 80-
702 20% B (0-30 min), 20-80% B (30-31 min), 80-80% B (31-42 min). Injection volume was set to 1 μ L for all
703 analyses (42 min total run time per injection). MS analyses were carried out by coupling the LC system to
704 a Thermo Q Exactive HFTM mass spectrometer operating in heated electrospray ionization mode (HESI).
705 Method duration was 30 min with a polarity switching data-dependent Top 5 method for both positive and
706 negative modes. Spray voltage for both positive and negative modes was 3.5kV and capillary
707 temperature was set to 320 $^{\circ}$ C with a sheath gas rate of 35, aux gas of 10, and max spray current of 100
708 μ A. The full MS scan for both polarities utilized 120,000 resolution with an AGC target of 3e6 and a
709 maximum IT of 100 ms, and the scan range was from 67-1000 *m/z*. Tandem MS spectra for both positive
710 and negative mode used a resolution of 15,000, AGC target of 1e5, maximum IT of 50 ms, isolation
711 window of 0.4 *m/z*, isolation offset of 0.1 *m/z*, fixed first mass of 50 *m/z*, and 3- way multiplexed
712 normalized collision energies (nCE) of 10, 35, 80. The minimum AGC target was 1e4 with an intensity
713 threshold of 2e5. All data were acquired in profile mode.

714 **Metabolomics Data Processing**

715 *Relative quantification of metabolites* – The resulting ThermoTM RAW files were converted to mzXML
716 format using ReAdW.exe version 4.3.1 to enable peak detection and quantification. The centroided data
717 were searched using an in-house python script Mighty_skeleton version 0.0.2 and peak heights were
718 extracted from the mzXML files based on a previously established library of metabolite retention times
719 and accurate masses adapted from the Whitehead Institute (Chen et al., 2016), and verified with
720 authentic standards and/or high resolution MS/MS spectral manually curated against the NIST14MS/MS
721 (Voge et al., 2016) and METLIN (2017) (Smith et al., 2005) tandem mass spectral libraries. Metabolite
722 peaks were extracted based on the theoretical *m/z* of the expected ion type e.g., [M+H]⁺, with a \pm 5 part-

723 per-million (ppm) tolerance, and a \pm 7.5 second peak apex retention time tolerance within an initial
724 retention time search window of \pm 0.5 min across the study samples.

725 *Detection of untargeted features* – The MS1 level data in both positive and negative mode were searched
726 for representative features across all study files using an in-house python script called Ungrid (version
727 0.5). The algorithm reduces all detected MS1 peak data into representative features by sorting intensity
728 from high to low (across all samples) and then applying an array bisection algorithm (python v3.0.1) on
729 the m/z and retention time values with custom tolerances (25 ppm m/z tolerance for peak discrimination,
730 0.5 min RT delta for chromatographic discrimination, 1e5 minimum intensity, 10X signal to noise (within
731 spectrum)). The output is a list of representative high intensity features of a defined m/z and retention
732 time. These feature intensities were then extracted across all samples using Mighty_skeleton (above) to
733 give the peak intensities for each feature in each sample.

734 *Metabolomics informatics* – The resulting data matrices of metabolite intensities for all samples and blank
735 controls (either retention time library data or untargeted data) was processed with an in-house statistical
736 pipeline Metabolyze version 1.0 and final peak detection was calculated based on a signal to noise ratio
737 (S/N) of 3X compared to blank controls, with a floor of 10,000 (arbitrary units). For samples where the
738 peak intensity was lower than the blank threshold, metabolites were annotated as not detected, and the
739 threshold value was imputed for any statistical comparisons to enable an estimate of the fold change as
740 applicable. The resulting blank corrected data matrix was then used for all group-wise comparisons, and
741 t-tests were performed with the Python SciPy (1.1.0) (Jones E, et al) library to test for differences and
742 generate statistics for downstream analyses. Any metabolite with p-value < 0.05 was considered
743 significantly regulated (up or down). Any outliers were omitted with the Grubb's outlier test. Values are
744 reported as log2 fold changes \pm standard error of the mean. In order to adjust for significant covariate
745 effects (as applicable) in the experimental design the R package, DESeq2 (1.24.0) (Love et al., 2014)
746 was used to test for significant differences. Data processing for this correction required the blank
747 corrected matrix to be imputed with zeroes for non-detected values instead of the blank threshold to avoid
748 false positives. This corrected matrix was then analyzed utilizing DESeq2 to calculate the adjusted p-
749 value in the covariate model.

750 **Targeted LC/MS for dopamine detection** – Metabolite profiling was conducted on a QExactive bench
751 top orbitrap mass spectrometer equipped with an Ion Max source and a HESI II probe, which was
752 coupled to a Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific, San Jose, CA). External
753 mass calibration was performed using the standard calibration mixture every 7 days. 5 μ L were injected
754 onto a SeQuant® ZIC®-pHILIC 150 x 2.1 mm analytical column equipped with a 2.1 x 20 mm guard
755 column (both 5 mm particle size; EMD Millipore). The following method was adapted from Tufi et al.
756 (2015): Buffer A was 10 mM ammonium formate with 0.2% formic acid in 90% acetonitrile; Buffer B was
757 water with 0.2% formic acid. The column oven and autosampler tray were held at 25°C and 4°C,

758 respectively. The chromatographic gradient was run at a flow rate of 0.300 mL/min as follows: 0-2 min:
759 hold at 0% B; 2.5-15 min.: linear gradient from 0-40% B; 15.5-16 min.: linear gradient from 40-0% B; 16-
760 21 min.: hold at 0% B. The mass spectrometer was operated in full-scan, polarity-switching mode, with
761 the spray voltage set to 3.0 kV, the heated capillary held at 275°C, and the HESI probe held at 350°C. The
762 sheath gas flow was set to 40 units, the auxiliary gas flow was set to 15 units, and the sweep gas flow
763 was set to 1 unit. MS data acquisition was performed in a range of m/z =70-1000, with the resolution set
764 at 70,000, the AGC targeted at 1×10^6 , and the maximum injection time was 20 msec. In addition, timed
765 targeted selected ion monitoring (tSIM) scans were included in positive mode to enhance detection of
766 Dopamine (m/z 154.08626). MS settings were as described above, with the AGC target set at 1×10^5 , the
767 maximum injection time was 200 msec, and the isolation window was 1.0 m/z .

768

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770

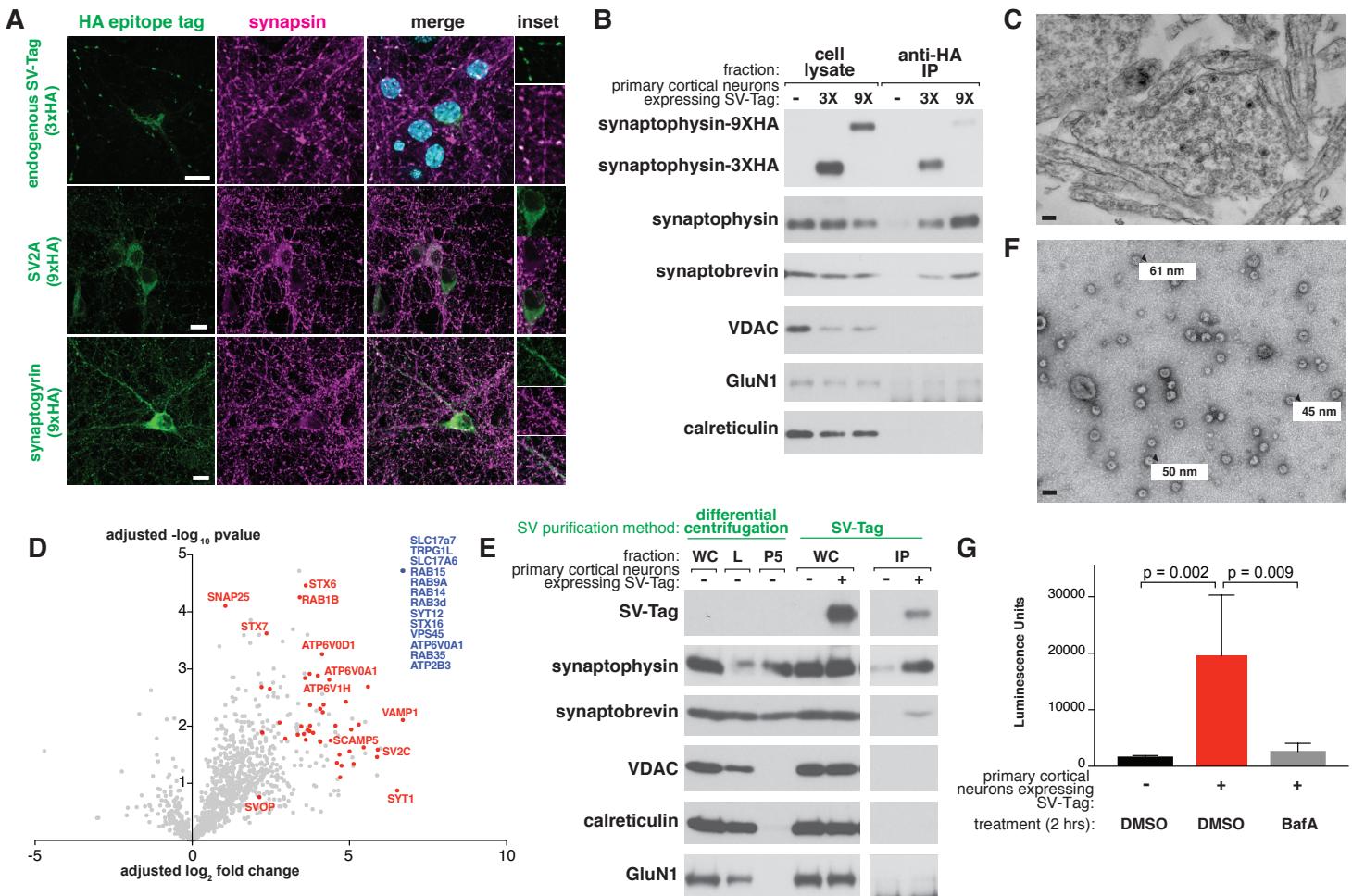
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784

785 **Declaration of Interests**

786

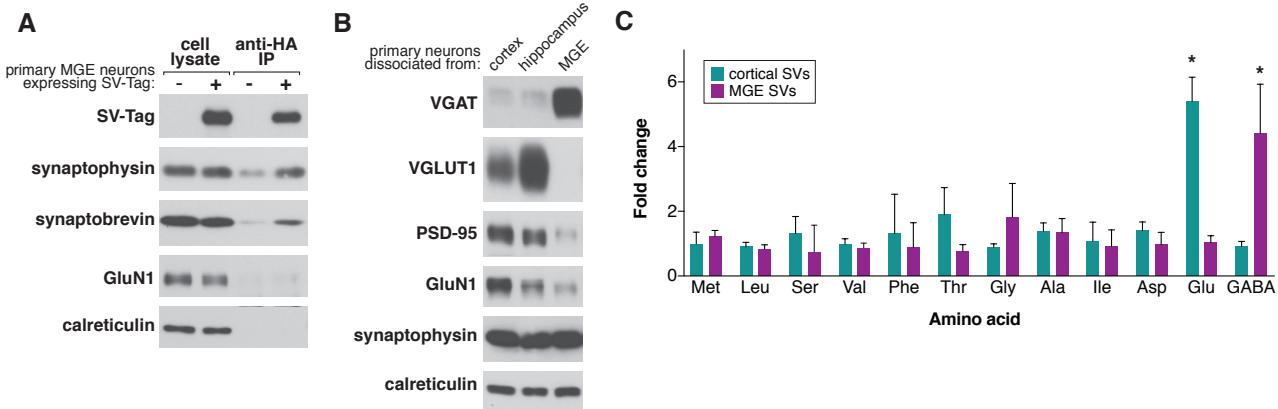
787 The authors declare no competing interests



Supplemental Figure 1:

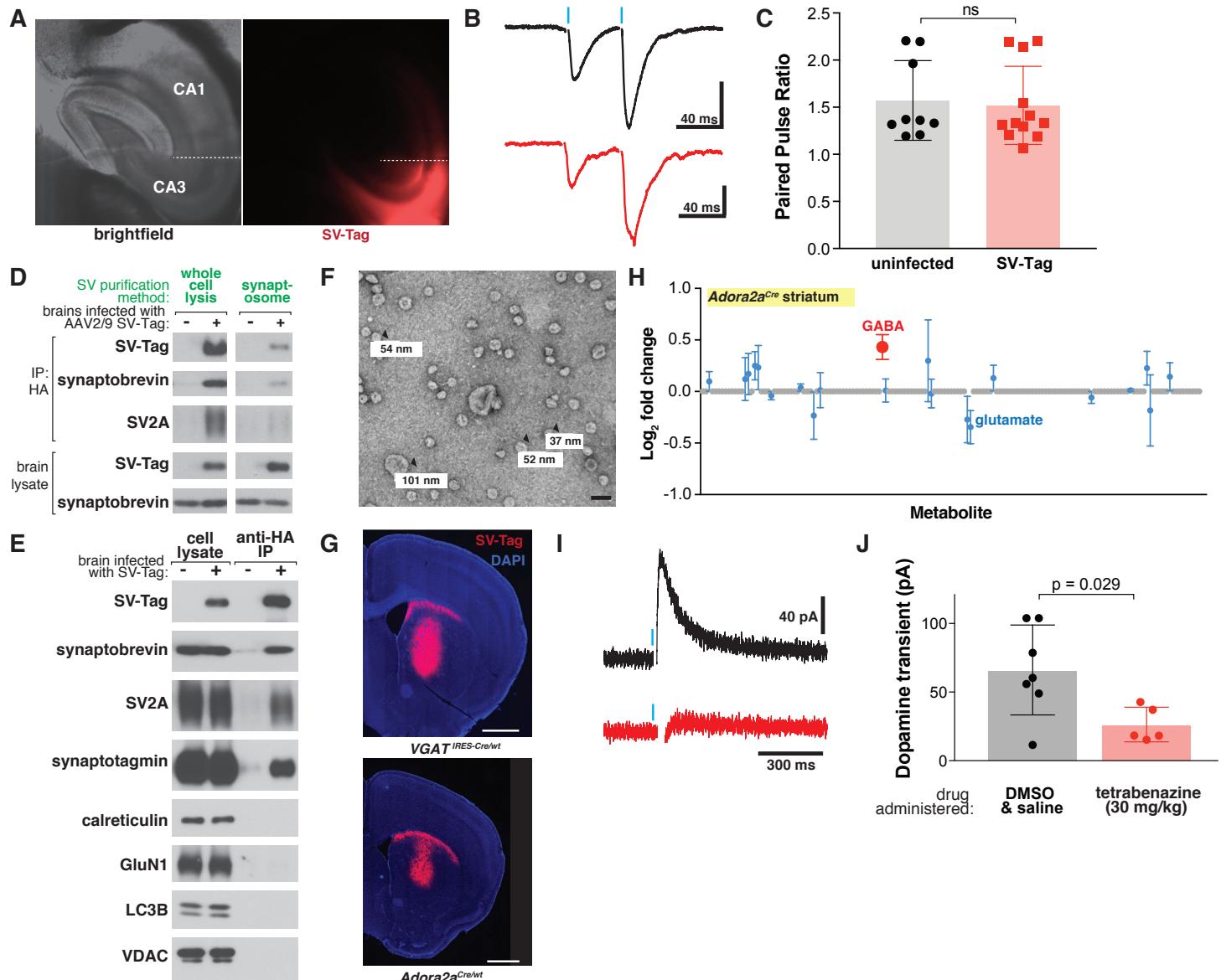
(A) Immunostaining of primary neurons expressing indicated HA-tagged proteins (green) and endogenous synapsin (magenta). Specifically, neurons are expressing endogenously tagged synaptophysin with a triple HA tag (top row), SV2A tagged at the N terminus with nine HA tags (9x HA) (middle row), and synaptogyrin tagged at the N terminus with a 9X HA tag (bottom row). Insets represent selected fields that were magnified 1.7X (top row) and 1.35X (middle and bottom row). Scale bars, 10 μ m.

(B) Immunoblot analysis of the effect of the number of HA tags on the efficiency of SV isolation. Lysates were prepared from neurons infected with lentivirus encoding for synaptophysin conjugated to three HA tags (3X) or nine HA tags (9X). **(C)** Electron microscope image of synaptosomes generated with the workflow. Scale bar, 100 nm **(D)** Proteomics analysis of isolated SVs versus control IP. The blue dot denotes proteins in which there were no detected peptides in the control IP ($n = 70$, 14 of which are validated synaptic vesicle proteins), and red dots represent established synaptic vesicle proteins based on literature. **(E)** Immunoblot analysis of SVs isolated with the SV-Tag based workflow compared to a previously established one based on differential centrifugation. WC: whole cell, L: whole cell lysate, P5: synaptic vesicle pellet, IP: HA immunoprecipitate **(F)** Electron microscope image of SVs generated with the differential centrifugation method used in S1E. Values denote diameter of indicated particles. Scale bar, 100 nm **(G)** Luminescence-based detection of glutamate in SVs isolated from neurons treated with DMSO or Bafilomycin A (500 nM) 2 hours prior to isolation.



Supplemental Figure 2:

(A) Immunoblot analysis of indicated proteins in whole cell lysates, HA immunoprecipitates from medial ganglionic eminence (MGE) cultures infected with SV-Tag, and control immunoprecipitates from uninfected MGE cultures. **(B)** Immunoblot analysis of whole cell lysates of primary cortical, hippocampal, and MGE neurons. Lysates were probed for the presence of indicated synaptic and pan neuronal markers. **(C)** Comparison of the relative abundances of specified amino acids in purified synaptic vesicles isolated from cortical versus MGE cultures and profiled with GC/MS (mean \pm SEM, n=3). Asterisk denotes statistical significance. Glutamate fold change p value = 7.5×10^{-5} , GABA fold change p value = 4.8×10^{-3}



Supplemental Figure 4:

(A) Immunofluorescence images of transverse sections containing hippocampal CA3 transduced with synaptophysin-9XHA. A cut was made between CA2 and CA1 (dashed white line) to prevent recurrent excitation of these synapses. **(B)** Representative traces of evoked current responses in hippocampus from electrical stimulation. Vertical blue bars indicate stimulation that occurred with an interstimulus interval of 50 ms of Schaffer collaterals with (red) and without (black) expression of SV-Tag. Vertical bar indicates 100 pA. Non-significant p value = n.s. **(C)** Summary of paired pulse ratios of electrically evoked synaptic responses from CA3 to CA1 synapses that were uninfected or expressing SV-Tag. **(D)** Immunoblot analysis of indicated proteins for synaptic vesicles isolated from the whole cell lysis method of brains compared to vesicles isolated with the synaptosome method. **(E)** Immunoblot analysis of synaptic vesicles isolated from mouse brains transduced with SV-Tag in the cortex. **(F)** Electron microscope image of synaptic vesicles generated with the workflow. Values denote diameter of indicated

particles. Scale bar: 100 nm (G) Coronal sections from *Slc32a1*^{IRES-Cre/wt} (*VGAT*^{IRES-Cre/wt}) and *Adora2a*^{Cre/wt} mice transduced with a Cre-dependent SV-Tag in the striatum. (H) LC/MS profile of synaptic vesicles isolated from *Adora2a*^{Cre/wt} mice brains infected with SV-Tag in striatum compared with uninfected brains. (mean ± SEM, n=4) (I) Representative traces of amperometry demonstrating the effects of DMSO (black) and tetrabenazine (black) on evoked dopamine. Blue indicates electrical stimulation. (J) Amperometry for dopamine in coronal slices prepared from mice administered intraperitoneally with tetrabenazine (30mg/kg) or vehicle control 2 hours before slicing.

Supplemental Table 1:

Proteomics profile of immunoprecipitates from primary cortical neurons expressing SV-Tag versus uninfected neurons, related to Figure S1D.

Supplemental Table 2:

List of the metabolites interrogated in the targeted LC/MS runs, related to Figure 2C, D and F, Figure 4D, E and G, and Figure S4H.

Supplemental Table 3:

Metabolites detected in SVs from cortical cultures via GC/MS and LC/MS and their associated calculations of fold changes, significance, and SEM, related to Figure 2.

Supplemental Table 4:

Metabolites detected via global LC/MS performed on cortical culture SVs, treated with DMSO or BafilomycinA, related to Figure 3.

Supplemental Table 5:

Metabolites detected in SVs from hippocampus and striatum via LC/MS and their associated calculations of fold changes, significance, and SEM, related to Figure 4.

788 **References**

789

790 Abu-Remaileh, M., Wyant, G.A., Kim, C., Laqtom, N.N., Abbasi, M., Chan, S.H., Freinkman, E., and
791 Sabatini, D.M. (2017). Lysosomal metabolomics reveals V-ATPase- and mTOR-dependent mechanisms
792 for the efflux of amino acids from lysosomes. *Science* (80-). 358, 4.

793 Ahmed, S., Holt, M., Riedel, D., and Jahn, R. (2013). Small-scale isolation of synaptic vesicles from
794 mammalian brain. *Nat. Protoc.* 8, 998–1009.

795 Atasoy, D., Aponte, Y., Su, H.H., and Sternson, S.M. (2008). A FLEX Switch Targets Channelrhodopsin-2
796 to Multiple Cell Types for Imaging and Long-Range Circuit Mapping. *J. Neurosci.* 28, 7025–7030.

797 Beale, D.J., Pinu, F.R., Kouremenos, K.A., Poojary, M.M., Narayana, V.K., Boughton, B.A., Kanjaria, K.,
798 Dayalan, S., Jones, O.A.H., and Dias, D.A. (2018). Review of recent developments in GC – MS
799 approaches to metabolomics-based research (Springer US).

800 Beaudoin III, G.M.J., Lee, S., Singh, D., Yuan, Y., Ng, Y., Reichardt, L.F., and Arikath, J. (2012).
801 Culturing pyramidal neurons from the early postnatal mouse hippocampus and cortex. *Nat. Protoc.* 7,
802 1741–1754.

803 Bowman, E.J., Sieberst, A., and Altendorf, K. (1988). Bafilomycins : A class of inhibitors of membrane
804 ATPases from microorganisms , animal cells , and plant cells. 85, 7972–7976.

805 Burger, P.M., Mehl, E., Cameron, P.L., Maycox, P.R., Baumert, M., Lottspeich, F., De Camilli, P., and
806 Jahn, R. (1989). Synaptic vesicles immunoisolated from rat cerebral cortex contain high levels of
807 glutamate. *Neuron* 3, 715–720.

808 Camilli, P.D.E., Harris, S.M., Huttner, W.B., and Greengard, P. (1983). Synapsin I (Protein I), a Nerve
809 Terminal-Specific Phosphoprotein . II . Its Specific Association with Synaptic Vesicles Demonstrated by
810 Immunocytochemistry in Agarose-embedded Synaptosomes. *J. Cell Biol.* 96, 1355–1373.

811 Cash, D. (1994). Gammahydroxybutyrate : An Overview of the Pros and Cons for it Being a
812 Neurotransmitter And / Or a Useful Therapeutic Agent. *Neurosci. Biobehav. Rev.* 18, 291–304.

813 Chantranupong, L., Scaria, S.M., Saxton, R.A., Harper, J.W., Gygi, S.P., Sabatini, D.M., Chantranupong,
814 L., Scaria, S.M., Saxton, R.A., Gygi, M.P., et al. (2016). The CASTOR Proteins Are Arginine Sensors for
815 the The CASTOR Proteins Are Arginine Sensors. *Cell* 1–12.

816 Chen, W.W., Freinkman, E., Wang, T., Birsoy, K., and Sabatini, D.M. (2016). Absolute Quantification of
817 Matrix Metabolites Reveals the Dynamics of Mitochondrial Metabolism. *Cell* 166, 1324-1337.e11.

818 Chen, W.W., Freinkman, E., and Sabatini, D.M. (2017). Rapid immunopurification of mitochondria for
819 metabolite profiling and absolute quantification of matrix metabolites. *Nat. Protoc.* 12, 2215–2231.

820 Clarke, M.C., and Haig, D.A. (1971). Taurine-a Possible Neurotransmitter? *Nature* 234, 107–108.

821 Cox, J., and Witten, I.B. (2019). Striatal circuits for reward learning and decision-making. *Nat. Rev.*
822 *Neurosci.* 20, 482–494.

823 Craige, B., Salazar, G., and Faundez, V. (2004). Isolation of Synaptic Vesicles (John Wiley & Sons, Inc.).

824 Damier, P., Hirsch, E.C., Agid, Y., and Graybiel, A.M. (1999). The substantia nigra of the human brain II .

825 Patterns of loss of dopamine-containing neurons in Parkinson 's disease. *Brain* 122, 1437–1448.

826 Daubner, S.C., Le, T., and Wang, S. (2011). Tyrosine hydroxylase and regulation of dopamine synthesis. *Arch. Biochem. Biophys.* 508, 1–12.

827 Dulcis, D., Jamshidi, P., Leutgeb, S., and Spitzer, N.C. (2013). Neurotransmitter switching in the adult
829 brain regulates behavior. *Science* (80-.). 340, 449–453.

830 Durieux, P.F., Bearzatto, B., Guiducci, S., Buch, T., Waisman, A., Zoli, M., Schiffmann, S.N., and
831 Kerchove, A. De (2009). D2R striatopallidal neurons inhibit both locomotor and drug reward processes. *Nat. Neurosci.* 12, 2008–2010.

832 Edwards, R.H. (1992). The transport of neurotransmitters into synaptic vesicles. *Curr. Opin. Neurobiol.* 2,
833 586–594.

834 Elsworth, J.D., and Roth, R.H. (1997). Dopamine Synthesis, Uptake, Metabolism, and Receptors :
835 Relevance to Gene Therapy of Parkinson's Disease. *Exp. Neurol.* 9, 4–9.

836 Eng, J.K., Mccormack, A.L., and Yates, J.R. (1994). An Approach to Correlate Tandem Mass Spectral
837 Data of Peptides with Amino Acid Sequences in a Protein Database. *Am. Soc. Mass Spectrom.* 5, 976–
838 989.

839 Erickson, J.D., Schxfert, M.K., Bonner, T.O.M.I., Eiden, L.E.E.E., and Weihet, E. (1996). Distinct
840 pharmacological properties and distribution in neurons and endocrine cells of two isoforms of the human
841 vesicular monoamine transporter. *Proc. Natl. Acad. Sci.* 93, 5166–5171.

842 Eshkind, L.G., and Leube, R.E. (1995). Mice lacking synaptophysin reproduce and form typical synaptie
843 vesicles. *Cell Tissue Res.* 282, 423–433.

844 Fernandez, C.A., Rosiers, C. Des, Previs, S.F., David, F., and Brunengrabert, H. (1996). Correction of 3C
845 Mass Isotopomer Distributions for Natural Stable Isotope Abundance. *J. Mass Spectrom.* 31, 255–262.

846 Fleck, M.W., and Palmerv, A.M. (1993). Aspartate and Glutamate Mediate in Area CA1 of the
847 Hippocampus Excitatory Synaptic Transmission. *J. Neurosci.* 13, 3944–3955.

848 Franchi, S.A., Macco, R., Astro, V., Tonoli, D., and Savino, E. (2018). A Method to Culture GABAergic
849 Interneurons Derived from the Medial Ganglionic Eminence. *Front. Cell. Neurosci.* 11, 1–21.

850 Fremeau Jr, R.T., Troyer, M.D., Pahner, I., Nygaard, G.O., Tran, C.H., Reimer, R.J., Bellocchio, E.E.,
851 Fortin, D., Storm-mathisen, J., and Edwards, R.H. (2001). The Expression of Vesicular Glutamate
852 Transporters Defines Two Classes of Excitatory Synapse University of Oslo. *Neuron* 31, 247–260.

853 Gondré-lewis, M.C., Park, J.J., and Loh, Y.P. (2012). Cellular Mechanisms for the Biogenesis and
854 Transport of Synaptic and Dense-Core Vesicles (Elsevier).

855 Gronborg, M., Pavlos, N.J., Brunk, I., Chua, J.J.E., Munster-Wandowski, A., Riedel, D., Ahnert-Hilger, G.,
856 Urlaub, H., and Jahn, R. (2010). Quantitative Comparison of Glutamatergic and GABAergic Synaptic
857 Vesicles Unveils Selectivity for Few Proteins Including MAL2, a Novel Synaptic Vesicle Protein. *J.*
858 *Neurosci.* 30, 2–12.

859 Gundersen, R.Y., Vaagene, P., Breivik, T., Fonnum, F., and Opstad, P. (2005). Glycine - an important
860 neurotransmitter and cytoprotective agent. *Acta Anaesthesiol Scand* 49, 1108–1116.

862 Herring, B.E., Silm, K., Edwards, R.H., and Nicoll, R.A. (2015). Is Aspartate an Excitatory
863 Neurotransmitter? *J. Neurosci.* 35, 10168–10171.

864 Hnasko, T.S., and Edwards, R.H. (2012). Neurotransmitter corelease: mechanism and physiological role.
865 *Annu. Rev. Physiol.* 74, 225–243.

866 Huang, K.W., Ochandarena, N.E., Philson, A.C., Hyun, M., Birnbaum, J.E., Cicconet, M., and Sabatini,
867 B.L. (2019). Molecular and anatomical organization of the dorsal raphe nucleus. *Elife* 8, 1–34.

868 Huttlin, E.L., Ting, L., Raphael, J., Paulo, J.A., Wade, J., Gygi, S.P., Huttlin, E.L., Ting, L., Bruckner, R.J.,
869 Gebreab, F., et al. (2015). The BioPlex Network : A Systematic Exploration of the Human Interactome.
870 *Cell* 162, 425–440.

871 Huttner, W.B., Schiebler, W., Greengard, P., and De Camilli, P. (1983). Synapsin I (Protein I), a Nerve
872 Terminal-Specific Phosphoprotein. III. Its Association with Synaptic Vesicles Studied in a Highly Purified
873 Synaptic Vesicle Preparation. *J. Cell Biol.* 96, 1374–1388.

874 Jackman, S.L., Beneduce, B.M., Drew, I.R., and Regehr, W.G. (2014). Achieving High-Frequency Optical
875 Control of Synaptic Transmission. *J. Neurosci.* 34, 7704–7714.

876 Jackman, S.L., Turecek, J., Belinsky, J.E., and Regehr, W.G. (2016). The calcium sensor synaptotagmin
877 7 is required for synaptic facilitation. *Nature* 529, 88–91.

878 Jahn, R., and Sudhof, T.C. (1994). Synaptic vesicles and exocytosis. *Annu. Rev. Neurosci.* 17, 219–246.

879 Jonas, P., Bischofberger, J., and Sandkühler, J. (1998). Corelease of two fast neurotransmitters at a
880 central synapse. *Science* 281, 419–424.

881 Jones E, Oliphant E, Peterson P, et al. SciPy: Open Source Scientific Tools for Python.

882 Kanazawa, H., and Wada, Y.O.H. (2000). Luminal acidification of diverse organelles by V-ATPase in
883 animal cells. *J. Exp. Biol.* 203, 107–116.

884 Kilb, W., and Fukuda, A. (2017). Taurine as an Essential Neuromodulator during Perinatal Cortical
885 Development. *Front. Cell. Neurosci.* 11, 1–13.

886 Kim, J.-I., Ganesan, S., Luo, S.X., Wu, Y.-W., Park, E., Huang, E.J., Chen, L., and Ding, J.B. (2015).
887 Aldehyde dehydrogenase 1a1 mediates a GABA synthesis pathway in midbrain dopaminergic neurons.
888 *Science* (80-). 350, 102–106.

889 Kugler, S., Kilic, E., and Bahr, M. (2003). Human synapsin 1 gene promoter confers highly neuron-
890 specific long-term transgene expression from an adenoviral vector in the adult rat brain depending on the
891 transduced area. *Gene Ther.* 10, 337–347.

892 Lammel, S., Elizabeth, E., Luo, L., Robert, C., Wall, N.R., Beier, K., and Luo, L. (2015). Diversity of
893 Transgenic Mouse Models for Selective Targeting of Midbrain Dopamine Neurons. *Neuron* 85, 429–438.

894 Lei, Z., Huhman, D. V, and Sumner, L.W. (2011). Mass Spectrometry Strategies in Metabolomics. *J. Biol.*
895 *Chem.* 286, 25435–25442.

896 Lewis, C.A., Parker, S.J., Fiske, B.P., Mccloskey, D., Gui, D.Y., Green, C.R., Vokes, N.I., Feist, A.M.,
897 Heiden, M.G. Vander, and Metallo, C.M. (2014). Tracing Compartmentalized NADPH Metabolism in the
898 Cytosol and Mitochondria of Mammalian Cells. *Mol. Cell* 55, 253–263.

899 Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for
900 RNA-seq data with DESeq2. *Genome Biol.* **15**, 1–21.

901 Lynch, B.A., Lambeng, N., Nocka, K., Kensem-Hammes, P., Bajjalieh, S.M., Matagne, A., and Fuks, B.
902 (2004). The synaptic vesicle protein SV2A is the binding site for the antiepileptic drug levetiracetam. *Proc.
903 Natl. Acad. Sci. U. S. A.* **101**, 9861–9866.

904 Macdonald, R.L. (1994). GABA A receptor channels. *Annu. Rev. Neurosci.* **17**, 569–602.

905 Mariani, M., Camagna, M., Tarditi, L., and Seccamani, E. (1991). A new enzymatic method to obtain high-
906 yield F(ab)2 suitable for clinical use from mouse IgG1. *Mol. Immunol.* **28**, 69–77.

907 Mathews, G.C., and Diamond, J.S. (2003). Neuronal Glutamate Uptake Contributes to GABA Synthesis
908 and Inhibitory Synaptic Strength. *J. Neurosci.* **23**, 2040–2048.

909 Mickelsen, L.E., Iv, F.W.K., Chimileski, B.R., Fujita, A., Norris, C., Nelson, C.E., and Jackson, A.C.
910 (2017). Neurochemical Heterogeneity Among Lateral Hypothalamic Hypocretin / Orexin and Melanin-
911 Concentrating Hormone Neurons Identified Through Single-Cell Gene Expression Analysis. *ENeuro* **4**, 1–
912 24.

913 Nagy, A., Baker, R.R., Morris, S.J., and Whittaker, V.P. (1976). The preparation and characterization of
914 synaptic vesicles of high purity. *Brain Res.* **109**, 285–309.

915 Nicoll, A., Malenka, C., Kauer, A., and Francisco, S. (1990). Functional Comparison of Neurotransmitter
916 Receptor Subtypes in Mammalian Central Nervous System. *Physiol. Rev.* **70**, 514–549.

917 Nishiyama, J., Mikuni, T., and Yasuda, R. (2017). Virus-Mediated Genome Editing via Homology-Directed
918 Repair in Mitotic and Postmitotic Cells in Mammalian Brain. *Neuron* **96**, 755–768.e5.

919 Olbrich, H., and Braak, H. (1985). Anatomy and Embryology Ratio of pyramidal cells versus non-
920 pyramidal cells in sector CA1 of the human Ammon's horn. *Anat Embryol* **173**, 105–110.

921 Pacold, M.E., Brimacombe, K.R., Chan, S.H., Rohde, J.M., Lewis, C.A., Swier, L.J.Y.M., Possemato, R.,
922 Chen, W.W., Sullivan, L.B., Fiske, B.P., et al. (2016). A PHGDH inhibitor reveals coordination of serine
923 synthesis and one-carbon unit fate. *Nat. Chem. Biol.* **12**, 452–462.

924 Parker, S.J., Svensson, R.U., Divakaruni, A.S., Lefebvre, A.E., Murphy, A.N., Shaw, R.J., and Metallo,
925 C.M. (2017). LKB1 promotes metabolic flexibility in response to energy stress. *Metab. Eng.* **43**, 208–217.

926 Patneau, K., and Mayer, L. (1990). Structure-Activity Relationships for Amino Candidates Acting at N-
927 Methyl-D-Aspartate Receptors Acid Transmitter and Quisqualate Receptors. *J. Neurosci.* **10**, 2385–2399.

928 Patti, G., Yanes, O., and Siuzdak, G. (2012). Innovation: Metabolomics: the apogee of the omics trilogy.
929 *Nat. Publ. Gr.* **13**, 263–269.

930 Peng, J., and Gygi, S.P. (2001). Proteomics : the move to mixtures. *J. Mass Spectrom.* **36**, 1083–1091.

931 Pines, G., Danbolt, N.C., Bj, M., Zhang, Y., Bendahan, A., Eide, L., Koepsell, H., Storm-mathisen, J.,
932 Seeberg, E., and Kanner, B.I. (1992). Cloning and expression of a rat brain L-glutamate transporter.
933 *Nature* **360**, 464–467.

934 Pisoni, R.L., and Thoene, J.G. (1991). The transport systems of mammalian lysosomes. *Biochim.
935 Biophys. Acta* **1071**, 351–373.

936 Ray, G.J., Boydston, E.A., Shortt, E., Wyant, G.A., Chen, W.W., and Sabatini, D.M. (2020). A PEROXO-
937 Tag enables rapid isolation of peroxisomes from human cells. *ISCIENCE* 101109.

938 Reis, D.J., and Regunathan, S. (2000). Is agmatine a novel neurotransmitter in brain? *TiPS* 21, 31–34.

939 Rizzoli, S.O., and Betz, W.J. (2005). Synaptic vesicle pools. *Nat. Rev. Neurosci.* 6, 57–69.

940 Root, D.H., Mejias-Aponte, C.A., Zhang, S., Wang, H.-L., Hoffman, A.F., Lupica, C.R., and Morales, M.
941 (2014). Single rodent mesohabenular axons release glutamate and GABA. *Nat. Neurosci.* 17, 1543–
942 1551.

943 Saunders, A., Granger, A.J., Sabatini, B.L., and Nelson, S.B. (2015). Corelease of acetylcholine and
944 GABA from cholinergic forebrain neurons. *Elife* 4, e06412.

945 Scherman, D., Jaudon, P., and P, H.J. (1983). Characterization of the monoamine carrier of chromaffin
946 granule membrane by binding of [2-3H]dihydrotetraabenazine. *Proc. Natl. Acad. Sci.* 80, 584–588.

947 Schousboe, A., Bak, L.K., and Waagepetersen, H.S. (2013). Astrocytic control of biosynthesis and
948 turnover of the neurotransmitters glutamate and GABA. *Front. Endocrinol. (Lausanne)*. 4, 1–11.

949 Sciarretta, C., and Minichiello, L. (2010). The Preparation of Primary Cortical Neuron Cultures and a
950 Practical Application Using Immunofluorescent Cytochemistry. In *Mouse Cell Culture: Methods and
951 Protocols*, A. Ward, and D. Tosh, eds. (Totowa, NJ: Humana Press), pp. 221–231.

952 Shabel, S.J., Proulx, C.D., Piriz, J., and Malinow, R. (2014). Mood regulation. GABA/glutamate co-release
953 controls habenula output and is modified by antidepressant treatment. *Science* (80-.). 345, 1494–1498.

954 Smith, C.A., Maille, G.O., Want, E.J., Qin, C., Trauger, S.A., Brandon, T.R., Custodio, D.E., Abagyan, R.,
955 and Siuzdak, G. (2005). METLIN: A Metabolite Mass Spectral Database. *Ther. Drug Monit.* 27.

956 Sobolevsky, T., Revelsky, A., Miller, B., Oreido, V., Chernetsova, E.S., and Revelsky, I. (2003).
957 Comparison of silylation and esterification / acylation procedures in GC-MS analysis of amino acids. *J.
958 Sep. Sci.* 26, 1474–1478.

959 Spitzer, N.C. (2012). Activity-dependent neurotransmitter respecification. *Nat. Rev. Neurosci.* 13, 94–106.

960 Stenerson, K. (2011). The Derivatization and Analysis of Amino Acids by GC-MS. *Sigma-Aldrich* 25, 9–
961 11.

962 Strata, P., and Harvey, R. (1999). Dale's principle. *Brain Res. Bull.* 50, 349–350.

963 Südhof, T.C. (2013). A molecular machine for neurotransmitter release: synaptotagmin and beyond. *Nat.
964 Publ. Gr.* 19, 1227–1231.

965 Takamori, S., Rhee, J.S., Rosenmund, C., and Jahn, R. (2001). Identification of Differentiation-Associated
966 Brain-Specific Phosphate Transporter as a Second Vesicular Glutamate Transporter (VGLUT2). *J.
967 Neurosci.* 21, 1–6.

968 Takamori, S., Holt, M., Stenius, K., Lemke, E.A., Grønborg, M., Riedel, D., Urlaub, H., Schenck, S.,
969 Brügger, B., Ringler, P., et al. (2006). Molecular Anatomy of a Trafficking Organelle. *Cell* 127, 831–846.

970 Tiedje, K.E., Stevens, K., Barnes, S., and Weaver, D.F. (2010). Beta-Alanine as a small molecule
971 neurotransmitter. *Neurochem. Int.* 57, 177–188.

972 Trapp, X.S., and Cork, S.C. (2020). PPG neurons of the lower brain stem and their role in brain GLP-1

973 receptor activation. *Am J Physiol Regul Integr Comp Physiol* 309 309, 795–804.

974 Traynelis, S.F., Wollmuth, L.P., McBain, C.J., Menniti, F.S., Vance, K.M., Ogden, K.K., Hansen, K.B.,

975 Yuan, H., Myers, S.J., and Dingledine, R. (2014). Glutamate Receptor Ion Channels : Structure,

976 Regulation, and Function. *Pharmacol. Rev.* 10, 405–496.

977 Tritsch, N.X., Ding, J.B., and Sabatini, B.L. (2012). Dopaminergic neurons inhibit striatal output through

978 non-canonical release of GABA. *Nature* 490, 262–266.

979 Tritsch, N.X., Granger, A.J., and Sabatini, B.L. (2016). Mechanisms and functions of GABA co - release.

980 *Nat. Rev. Neurosci.* 1–7.

981 Vergnano, A.M., Rebola, N., Savtchenko, L.P., Pinheiro, P.S., Casado, M., Kieffer, B.L., Rusakov, D.A.,

982 Mulle, C., and Paoletti, P. (2014). Zinc dynamics and action at excitatory synapses. *Neuron* 82, 1101–

983 1114.

984 Voge, N. V, Perera, R., Mahapatra, S., Gresh, L., Balmaseda, A., Loro, M.A., Hopf-jannasch, A.S.,

985 Belisle, J.T., Harris, E., Blair, C.D., et al. (2016). Metabolomics-Based Discovery of Small Molecule

986 Biomarkers in Serum Associated with Dengue Virus Infections and Disease Outcomes. *Plos* 1–27.

987 Vong, L., Ye, C., Yang, Z., Choi, B., Chua, S., and Lowell, B.B. (2011). Leptin Action on GABAergic

988 Neurons Prevents Obesity and Reduces Inhibitory Tone to POMC Neurons. *Neuron* 71, 142–154.

989 Wenig, P., and Odermatt, J. (2010). OpenChrom : a cross-platform open source software for the mass

990 spectrometric analysis of chromatographic data.

991 Wojcik, S.M., Katsurabayashi, S., Guillemin, I., Friauf, E., Rosenmund, C., and Brose, N. (2006). A

992 Shared Vesicular Carrier Allows Synaptic Corelease of GABA and Glycine. *Neuron* 4, 575–587.

993 Yelin, R., and Schuldiner, S. (1995). The pharmacological profile of the vesicular monoamine transporter

994 resembles that of multidrug transporters. *FEBS* 377, 201–207.

995 Yoshida, M., and Precht, W. (1971). Monosynaptic inhibition of neurons of the substantia nigra by

996 caudatonigral fibers. *Brain* 32, 225–228.

997 Zhang, S., Qi, J., Li, X., Wang, H.-L., Britt, J.P., Hoffman, A.F., Bonci, A., Lupica, C.R., and Morales, M.

998 (2015). Dopaminergic and glutamatergic microdomains in a subset of rodent mesoaccumbens axons.

999 *Nat. Neurosci.* 18, 386–392.

1000 Zhou, F., Wilson, C.J., and Dani, J.A. (2002). Cholinergic Interneuron Characteristics and Nicotinic

1001 Properties in the Striatum. *J Neurobiol* 53, 590–605.

1002