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RESEARCH ARTICLE

SV2A is expressed in synapse subpopulations in mouse and human brain: implications for PET radiotracer studies

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Theresa Wong[1], Zhen Qiu[2], Beverley Notman[1], Adriana Tavares[3,4], Colin Smith[1], Seth G. N. Grant[1,5]

[1]Institute for Neuroscience and Cardiovascular Research, University of Edinburgh, Edinburgh, EH16 4SB, UK
[2]Department of Biomedical Engineering, University of Strathclyde, Glasgow, G4 0NW, UK
[3]BHF/University of Edinburgh Centre for Cardiovascular Science, Queen's Medical Research Institute, Edinburgh, EH16 4TJ, UK
[4]Edinburgh Imaging, Queen's Medical Research Institute, Edinburgh, EH16 4TJ, UK
[5]Simons Initiative for the Developing Brain (SIDB), University of Edinburgh, Edinburgh, EH8 9XD, UK

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Abstract

Synapse pathology is a feature of most brain diseases and there is a pressing need to monitor the onset and progression of this pathology using brain imaging in living patients. A major step toward this goal has been the development of small-molecule radiotracers that bind to synaptic vesicle glycoprotein 2A (SV2A) for use in positron emission tomography (PET). Changes in SV2A radiotracer binding in PET are widely interpreted to report differences in the density of all synapses throughout brain region However, the expression of SV2A at single-synapse level across regions of adult mouse and human brain has not been comprehensively characterised.

Here, We employed high-resolution synaptome mapping in adult mouse (n=7) and human (n=3) brain tissue. Synaptic proteins were labelled using fluorescent immunohistochemistry, imaged using confocal microscopy and quantified using image analysis tools. Brain-wide SV2A expression was assessed in presynaptic and postsynaptic terminals.

Plain language summary

Many brain diseases and the process of ageing are linked to the loss of synapses—the connections between nerve cells. To better understand and address these conditions, it is important to develop ways to measure synapse numbers in clinical settings. One promising approach involves specialized imaging agents, called radiotracers, that bind to the synapse protein SV2A. These radiotracers enable brain imaging with positron emission tomography (PET), a tool that can be used in living humans and animals. Previous research has

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1. **Sridhar Natesan**, King's College London, London, UK

2. **Bong-Kiun Kaang**, Institute for Basic Science (IBS), Daejeon, South Korea

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suggested that SV2A PET imaging provides a reliable way to measure all synapses in the brain. However, our study shows that SV2A is not present in all synapses. Instead, it is found in specific subsets of synapses, and the proportion of synapses with SV2A varies across different brain regions. These findings have significant implications for interpreting SV2A PET imaging studies in the context of monitoring human health. We offer recommendations for interpreting these studies and highlight the need for more research into how synapses are affected in brain disorders and ageing.

Keywords
Positron emission tomography, synaptome, presynaptic terminal, synapse diversity

Corresponding author: Seth G. N. Grant (seth.grant@ed.ac.uk)

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Introduction

Synapses - the points of communication between neurons - are present in vast numbers throughout the brain. The proteome of mammalian brain synapses comprises thousands of protein types, and mutations in their cognate genes are associated with over 130 brain disorders[1]. These encompass a wide range of neurological and psychiatric disorders affecting individuals across the lifespan. Synapse pathology also arises from exogenous injury including stroke, trauma, inflammation, drug effects and chemical toxicity. Damage to synapses has major consequences for all aspects of brain function and commonly results in cognitive, mood, motoric and behavioural disorders. It is therefore of great importance to develop methods for monitoring synapse pathology in living humans.

Until recently, it was thought that there were comparatively few varieties of synapses in the mammalian brain. The principal classes are defined by their neurotransmitters and include excitatory (glutamatergic) and inhibitory (GABAergic), and several major classes of modulatory synapses releasing acetylcholine, dopamine, serotonin and norepinephrine, among others. However, molecular analysis has revealed an astonishing diversity of excitatory synapses arising from the differential expression of their constituent proteins[2,3]. These molecular 'types' of excitatory synapses, which differ in their protein composition, can be further classified into 'subtypes' by virtue of differences in the levels of expression, synaptic punctum morphology and nanoarchitecture[2,4]. Importantly, these excitatory synapse types and subtypes are differentially, and characteristically, distributed such that each brain region has a compositional 'signature'. The diversity of types and subtypes of excitatory synapses is referred to as the synaptome and their spatial distribution as the synaptome architecture[2].

The transmembrane protein SV2A is found on synaptic vesicles in the presynaptic terminal of mammalian synapses[5]. The development of SV2A radioligands for PET imaging has garnered considerable excitement as a tool for detecting changes in synapses in humans, non-human primates and rodents[6–9]. There are now over 100 clinical studies using SV2A PET radiotracers in a wide range of disorders, including neurodegeneration (e.g. Alzheimer's disease, frontotemporal dementia, supranuclear palsy), psychiatric disorders (e.g. schizophrenia), epilepsy and stroke.

Many publications on SV2A PET radiotracers refer to SV2A as being "ubiquitously expressed" or "ubiquitously and homogeneously expressed" in synaptic vesicles, synapses, neurons and regions of the brain[5,10–13]. As such, SV2A might be assumed a general reporter of changes in synapse density in pathology. In the present study we have employed synaptome mapping, a powerful technique that uncovers protein expression at single-synapse resolution and allows the measurement of synapse molecular diversity on a whole-brain scale. Synaptome mapping provides a first opportunity for a systematic and comprehensive examination of whether SV2A is truly ubiquitously or homogenously expressed at the level of individual excitatory and inhibitory synapses throughout regions of the mouse and human brain. The findings have important implications for the interpretation of SV2A PET imaging studies in a clinical setting and highlight the need for studies of synaptome architecture in brain disorders where SV2A imaging is being applied.

Methods

Preparation of mouse brain tissue

Male and female C57BL/6 adult mice (n=7; 3–6 months of age) were housed at a controlled temperature with ad libitum access to food and water. Animals were anaesthetised by intraperitoneal injection of 0.1 ml pentobarbital sodium BP 20% (Dolethal, Vetoquinol UK) and transcardially perfused with 10 ml phosphate buffered saline (PBS, Oxoid), followed by 10 ml 4% paraformaldehyde (Alfa Aesar 16%, diluted 1:4 in PBS). The brains were dissected and immediately post-fixed in same fixative for 3–4 hours at 4°C. Fixed samples were cryoprotected by incubating in 5 ml 30% sucrose (VWR International) in PBS for a minimum of 48 hours at 4°C or until they sank. Brains were frozen in Optimal Cutting Temperature (OCT, CellPath) using a beaker containing isopentane (VWR Chemicals) submerged into liquid nitrogen and stored at -80°C.

Sectioning of mouse brain tissue

Parasagittal and coronal tissue sections (18 µm thickness) were cut using a Leica CM3050S cryostat at -20°C. PBS was used as adhesive to collect tissue onto SuperFrost Plus slides (Thermo Scientific, J1800AMNZ). Slides were left to dry overnight at room temperature and stored at -20°C until use.

Immunolabelling of synaptic proteins in mouse brain tissue

Frozen mouse brain sections were defrosted to room temperature and rinsed with Tris buffer solution (1X TBS pH 7.4, Sigma) for 5 minutes before addition of blocking solution containing 5% bovine serum albumin (BSA, Sigma), 0.5% Triton X-100 (Sigma) in 1X TBS. Slides were incubated at room temperature for 2 hours in 100 µl blocking solution prior to overnight incubation at 4°C in 100 µl primary antibody (Table 1) and diluent solution (3% BSA, 0.2% Triton X-100 in 1X TBS). Sections were then washed three times for 10 minutes each before secondary antibody incubation (Table 1). The washing step was repeated, followed by mounting with a glass coverslip (VWR, 1.5 thickness, 18 mm diameter) using Mowiol mounting reagent (Sigma-Aldrich) with 1,4-diazabicyclo [2.2.2]octane (DABCO). Slides were left to curate overnight in a dry staining chamber before imaging.

Different approaches were employed to determine optimal immunofluorescence labelling conditions. First, we utilized C57BL/6J mouse brain tissue, which involved imaging tissue after labelling with or without primary and secondary antibodies (Figure 1). The SV2A antibodies produced strong punctate staining when labelled with secondary antibodies (Figure 1A), but signal was absent when either the primary (Figure 1B) or secondary antibodies were omitted. Second, we controlled for the blocking step, incubation times of antibodies and imaging parameters and derived optimal conditions based on

Table 1. Primary and secondary antibodies used in this study. AF488, Alexa Fluor 488; AF647, Alexa Fluor 647; CY5, Cyanine5; M, Mouse; H, Human.

Antibody	Species	Source	Catalogue Number	Dilution	Concentration	Tested in	RRID
SV2A	Rabbit	Abcam	ab32942	1:500, 1:250	1 mg/mL	M,H	AB_778192
SYP	Mouse	Synaptic Systems	101 111	1:500, 1:250	1 mg/mL	M,H	AB_2782972
SYN	Mouse	Synaptic Systems	106 011	1:500	1 mg/mL	M	AB_2619772
VGLUT1	Mouse	Neuromab	75-066	1:500	1 mg/mL	M	AB_2187693
VGAT	Guinea pig	Synaptic Systems	131 005	1:500	1 mg/mL	M	AB_1106810
PSD95	Mouse	Synaptic Systems	124 011	1:250	1 mg/mL	H	AB_10804286
GPHN	Mouse	Neuromab	75-465	1:500, 1:250	1 mg/mL	M,H	AB_2716264
Anti-rabbit AF488	Goat	Thermo Fisher Scientific	A-11008	1:1000, 1:500	2 mg/mL	M,H	AB_143165
Anti-rabbit AF647	Goat	Thermo Fisher Scientific	A-21244	1:1000, 1:500	2 mg/mL	M,H	AB_2535812
Anti-mouse CY5	Goat	Jackson ImmunoResearch Laboratories	115-175-205	1:1000, 1:500	1.2 mg/mL	M,H	AB_2338715
Anti-guinea pig AF647	Goat	Thermo Fisher Scientific	A-21450	1:1000	2 mg/mL	M	AB_2735091

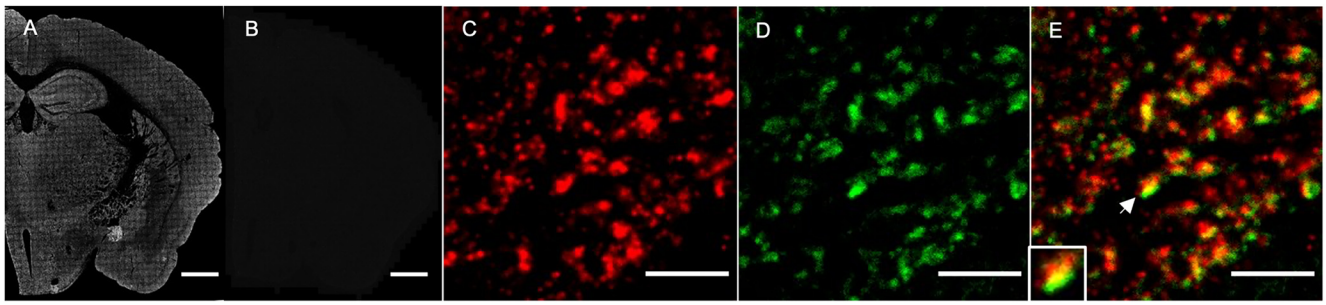

Figure 1. Optimisation of SV2A immunofluorescence staining on adult mouse brain. (**A**,**B**) Low-magnification image of coronal section of mouse brain stained with anti-SV2A rabbit polyclonal antibody and secondary antibody labelled with Alexa Fluor 647 (**A**). An adjacent brain section was stained without primary or secondary antibody as negative control (**B**). Scale bars, 1 mm. (**C**–**E**) High-magnification image (100x objective) of CA1sr regions of mouse hippocampal formation labelled with antibody to SV2A (**C**), SYN (**D**) and merged (**E**). Scale bars, 5 µm. An example of marker colocalization (arrow) is enlarged in the inset.

visual inspection and signal-to-noise ratio (SNR) analysis. Lastly, to test that the SV2A-positive puncta observed (Figure 1C) were presynaptic terminals, we immunolabelled with a second antibody to the presynaptic protein[14] synapsin 1 (Figure 1D) and observed colocalization in a subset of synapses (Figure 1E, arrow, inset).

Preparation of human brain tissue

Human brain tissue from three control subjects (1 male, 2 female; mean age 66 ± 4.4 years) was used in this study. Control subjects did not have a history of dementia, neurological, or psychiatric disorders at time of death. The brain tissue underwent processing in accordance with EBB departmental protocols[15]. In summary, 6 µm formalin-fixed paraffin-embedded (FFPE) tissue sections were stained with haematoxylin and eosin for routine neuroanatomical and neuropathological assessments. Examination of the brain tissue conducted by expert neuropathologist revealed minimal neuropathological changes in these control brains. Demographic and clinical characteristics are summarised in Table 2.

During post-mortem examination, brains were macroscopically dissected into 1 cm thick coronal slices and sampled following the established EBB protocol[15]. Tissue blocks sourced

Table 2. Demographic and clinical characteristics of human subjects. BMI, body mass index; CAT, coronary artery atherosclerosis; CIEDs, cardiac implantable electronic devices; DCM, dilated cardiomyopathy; HCL, hypercholesterolemia; HHD, hypertensive heart disease; HTN, hypertension; IHD, ischaemic heart disease; LBBB, left bundle branch block; MI, myocardial infarction; PE, pulmonary embolism; PMD, post-mortem delay; PMH, past medical history; T1D, type 1 diabetes mellitus.

Case ID	SD014/13	SD046/17	SD003/14
Age (years)	74	65	59
Gender	Female	Female	Male
Height (m)	1.63	1.63	1.75
Weight (kg)	82	57	83
BMI	50.3	35	47.4
Medication	No	No	No
Significant PMH	HTN	HTN, DCM, CIEDs, LBBB	HCL, T1D
Cause of death	PE	IHD, HHD	MI, CAT, T1D
Brain weight (g)	1520	1150	-
PMD (hr)	41	76	50
pH	6.3	6.35	6.1

from inferior temporal gyrus of the neocortex, defined as Brodmann area (BA) 20/21, were fixed in 10% formaldehyde (Genta Medical, UN2209) for 24–72 hours before tissue processing and paraffin wax embedding. Selection of anatomical region was influenced by the availability of blocks for the three control cases. Experienced pathologists conducted the macroscopic dissection of tissue blocks, closely adhering to topographical brain anatomy through the examination of sulci and gyri patterns, with assistance of a human brain atlas[16]. Adjacent Nissl-stained sections were also examined to confirm the integrity of the region of interest through assessment of distinctive cytoarchitectural features. A circular 6 mm biopsy corer (World Precision Instruments) was used to isolate smaller regions of interest from several different tissue blocks and re-embedded into a new block.

Sectioning of human brain tissue

Coronal tissue sections (8 µm thickness) were cut using a Leica RM2125 RTS microtome at room temperature. Sections were placed into a Paraffin Section Flotation Bath (Electrothermal, MH8517) before being collected onto SuperFrost Plus slides. Slides were left to dry in an incubator (GenLab, MINO/18/SS) overnight at 40°C and stored at room temperature until use.

Immunolabelling of synaptic proteins in human brain tissue

Human brain sections were dewaxed in two 3-minute xylene washes (Genta Medical, UN1307) and dehydrated by three 3-minute graded alcohol washes, comprising two 74°OP (IMS99%) alcohol washes and one 90% alcohol wash. To remove any formalin sedimentation, slides were washed in picric acid (TCS Biosciences, HS660-500) for 15 minutes followed by a final wash in lukewarm running water for 15 minutes. Antigen retrieval techniques were employed to improve cell surface staining, unmask antigen epitopes, and minimise non-specific background staining. The slides were then placed in 250 ml freshly made antigen retrieval solution (0.1 M sodium citrate buffer pH 6.0, Fisher Scientific) and pressure cooked (Biocare Medical, Decloaking Chamber NxGen) at 110°C for 15 minutes. Once cooled in water, human sections followed the same immunolabelling procedure as mouse protocol except for incubation time of 1 hour in blocking solution. The antibodies used to stain human brain tissue sections and their labelling conditions are described in Table 1.

Image acquisition

A high-resolution confocal spinning disk microscope (Nikon ECLIPSE Ti2) was used to image individual synaptic puncta in immunostained tissue (100X, NA=1.45). Obtained images measured 942 x 920 pixels in 16-bit depth with each pixel having 68 nm x 68 nm dimensions. Images were obtained through mosaic tiling whereby adjacent tiles are scanned without overlap using NIS-Elements (Nikon) software and saved in 12-bit tiff format.

Image analysis

The individual tiles were stitched together to create a montage image using an in-house developed MATLAB script. A TrackMate plugin from Fiji software was then utilized to detect and quantify synaptic puncta from immunofluorescence images.

After manually establishing the best parameters (punctum radius; mean quality threshold; threshold) for puncta detection of each antibody marker, they were used for batch processing of puncta detection in the MATLAB implementation of the TrackMate algorithm codified in-house, whereby puncta from each channel were automatically detected and their centroid coordinates and intensity exported in text files. Subsequently, they were imported in Python scripts developed in-house to calculate puncta density and average intensity for each brain region. In total, 34,678 synapses were examined across the mouse brain (n = 7), and 2,353,809 synapses were examined across the human brain (n = 3).

Statistical analysis

All quantifications were collected in Excel (Microsoft Office 2016 for Windows version). Density measurements represented the number of synaptic puncta per 100 μm^2 and intensity was quantified as arbitrary units (a.u.) representing greyscale values. All depicted values represent mean ± SEM.

Results

We took two approaches to establishing whether SV2A is expressed ubiquitously or in subpopulations of synapses. First, we immunolabelled brain tissue sections with antibodies to SV2A and compared the density of synaptic puncta with that of synapses expressing other synaptic proteins. Second, we performed double-immunolabelling experiments using antibodies to SV2A and another synaptic protein to determine whether the two proteins colocalize in the same or distinct populations of synapses.

Density of SV2A and other synaptic protein puncta in regions of the mouse brain

Because SV2A is a presynaptic protein, we compared its expression with four other well-characterised presynaptic proteins: synapsin 1 (SYN), synaptophysin (SYP), vesicular glutamate transporter 1 (VGLUT1) and the vesicular GABA transporter (VGAT). SYN and SYP are expressed in most presynaptic terminals, including both excitatory and inhibitory synapses[14]. To distinguish excitatory from inhibitory presynaptic terminals we used VGLUT1 and VGAT antibodies, which label each of these classes of synapse, respectively. To compare the number of SV2A presynaptic terminals with excitatory and inhibitory postsynaptic terminals, we used postsynaptic density protein 95 (PSD95) and gephyrin (GPHN) labelling, respectively. We utilised mice expressing green fluorescent protein (eGFP) fused to endogenous PSD95, which have been used extensively for mapping the diversity of excitatory synapses[2,17,18].

We chose nine brain regions known to show high levels of synapse diversity[2]. These comprise four regions of the hippocampal formation (HPF), three regions of the neocortex, and one region each from the striatum and the thalamus. The HPF regions comprise CA1 stratum radiatum (CA1sr), CA2 stratum radiatum (CA2sr), CA3 stratum radiatum (CA3sr) and dentate gyrus polymorphic layer (DGpo). The cortical regions included layer 2/3 and 5 of the somatosensory cortex (SMSL2/3, SMSL5) and layer 4/5 of the entorhinal cortex (ENTIL4/5). The striatal caudoputamen region (STRcp) and posterior complex of the thalamus (THpo) were also examined.

Representative images from the nine mouse brain regions labelled with seven antibodies (Figure 2) show regional variation in marker density. SV2A puncta were present at high density in CA1sr, CA2sr, CA3sr, SMSL5 and ENTIL4/5 but at low density in THpo and DGpo. Strikingly, THpo and DGpo contained very large SV2A puncta compared with the other regions. The SV2A images were similar to those for SYP, SYN and VGLUT1, but quite distinct from the VGAT puncta which were large in all brain regions. The postsynaptic markers PSD95 and GPHN showed a higher density of puncta than SV2A.

We quantified the density of each marker in each brain region (Table 3) and displayed the data in a heatmap (Figure 3A), which assists in comparing the patterns of expression between markers and regions. If SV2A were truly a universal marker for synapses, the density of SV2A should be equal to, or higher than, the combined density of the excitatory and inhibitory presynaptic markers VGLUT1 and VGAT. As shown in Table 3, the density of SV2A puncta was similar to that of VGLUT1 and greater than that of VGAT in each brain region. However, the SV2A puncta density accounts for 60–83% of the density of both VGLUT1 and VGAT (Table 4). This indicates that SV2A is consistently present below the combined density of VGLUT1 and VGAT, suggesting that SV2A is not expressed in all synapses, at least at the presynaptic level. In regions other than DGpo, the puncta density of the postsynaptic excitatory marker PSD95 was approximately double that of SV2A, and the puncta density of the postsynaptic inhibitory marker GPHN was higher than that of SV2A in all regions (Table 3). SV2A puncta density accounted for only 29–40% of the density observed for both PSD95 and GPHN (Table 5). These findings indicate that SV2A is expressed in subpopulations of excitatory and inhibitory synapses in the mouse brain.

The normalised heatmap (Figure 3A) reveals that SV2A has a similar regional distribution to SYP, SYN, VGLUT1, PSD95 and GPHN, with the highest puncta density in the CA1sr and CA2sr regions. By contrast, VGAT exhibited the highest density in ENTIL4/5.

Colocalization of SV2A with other synaptic markers in mouse brain

The results above show that SV2A puncta density is lower than that of the sum of excitatory and inhibitory markers at both the presynaptic and postsynaptic levels. From this we would expect SV2A to be present in subsets of excitatory and inhibitory synapses. To test this hypothesis, we conducted dual-labelling experiments using SV2A with the excitatory or inhibitory presynaptic marker VGLUT1 or VGAT. Representative images (Figure 4) clearly demonstrate the existence of synapses expressing either protein, as well as those expressing both markers. Quantification of these data shows that in the regions examined SV2A was present in 57–84% of VGLUT1 synapses and in 52–92% of VGAT synapses (Table 6). Furthermore, we investigated the juxtaposition of SV2A with the

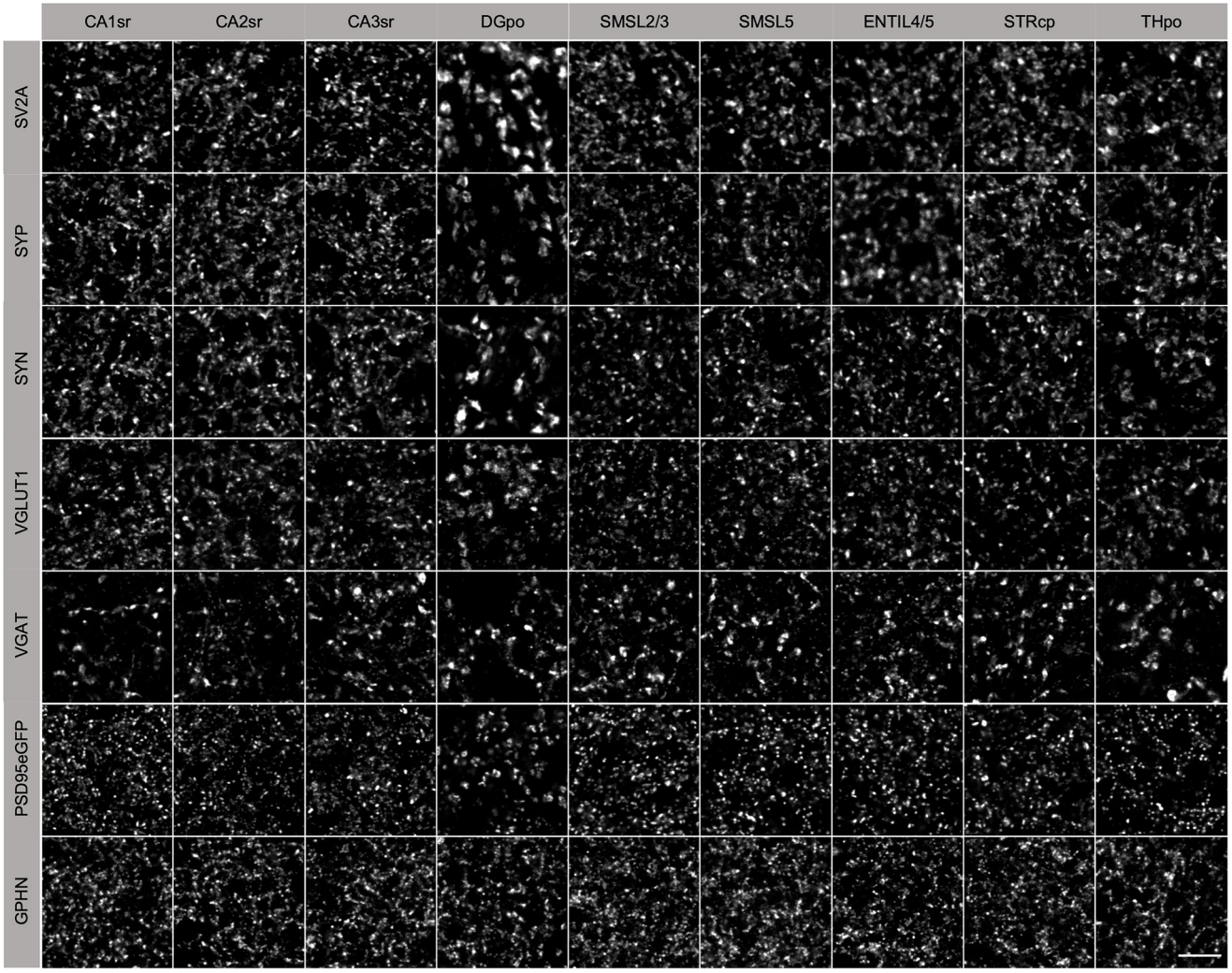

Figure 2. Synaptic puncta labelling in adult mouse brain regions. Nine brain regions (columns) were examined for five presynaptic (SV2A, SYP, SYN, VGLUT1, VGAT) and two postsynaptic (PSD95eGFP, GPHN) markers (rows). Scale bar, 5 μm.

Table 3. Quantification of synaptic puncta density of excitatory and inhibitory protein markers across regions the mouse brain. Measurements normalised to puncta/100 μm^2. Mean ± SEM. ROI, region of interest.

Marker/ROI	CA1sr	CA2sr	CA3sr	DGpo	SMS L2/3	SMS L5	ENTI L4/5	STRcp	THpo	Mean
SV2A	19±1	18±1	18±1	16±1	18±1	17±1	18±1	16±1	15±1	17±1
SYP	17±3	15±3	14±3	12±1	13±1	13±1	9±6	13±4	9±4	13±3
SYN	17±3	14±3	11±2	11±1	12±1	12±1	14±4	13±4	8±4	12±2
VGLUT1	19±1	19±3	17±3	16±3	16±2	18±3	14±1	15±1	12±1	16±2
VGAT	10±3	10±4	11±3	5±2	12±3	9±1	16±3	9±1	6±2	10±2
PSD95	39±1	37±2	28±3	18±3	34±1	33±1	35±1	29±4	27±1	31±2
GPHN	27±1	25±1	22±2	22±1	23±1	22±1	21±3	18±2	22±1	22±1

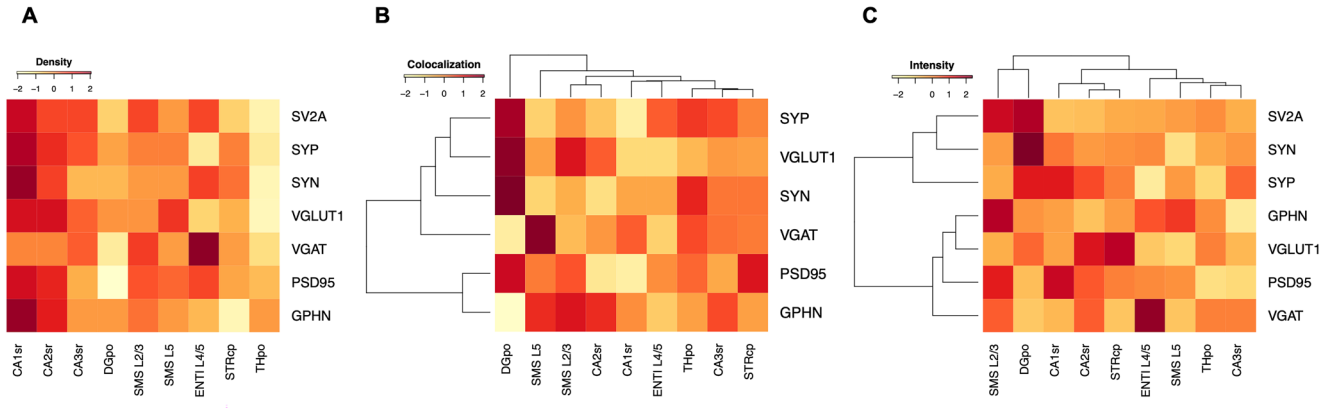

Figure 3. Comparison of synaptic puncta features in mouse brain regions. (**A**) Comparison of density (puncta/100 μm^2) of synaptic puncta labelled with markers (rows) across regions of the mouse brain (columns). (**B**) Percentage colocalization of presynaptic and postsynaptic puncta with SV2A across regions of the mouse brain clustered by similarity. (**C**) Average puncta intensity (a.u.) of different synaptic proteins across regions of the mouse brain clustered by similarity. Colours are normalised values for each marker (**A**–**C**, row).

Table 4. Comparison of SV2A, VGLUT1 and VGAT puncta density in mouse brain regions. SV2A puncta density (top row), summed density of VGLUT1 and VGAT (middle row), and the percentage of SV2A puncta compared with VGLUT1 and VGAT (bottom row).

Marker/ROI	CA1sr	CA2sr	CA3sr	DGpo	SMS L2/3	SMS L5	ENTI L4/5	STRcp	THpo	Mean
SV2A	19	18	18	16	18	17	18	16	15	17
VGLUT1 + VGAT	29	29	28	21	28	27	30	24	18	26
% SV2A	66	62	64	76	64	63	60	67	83	65

Table 5. Percentage of SV2A-positive puncta compared with postsynaptic markers in mouse brain regions. SV2A puncta density (top row), summed density of PSD95 and GPHN (middle row), and the percentage of SV2A puncta compared with PSD95 and GPHN (bottom row).

Marker/ROI	CA1sr	CA2sr	CA3sr	DGpo	SMS L2/3	SMS L5	ENTI L4/5	STRcp	THpo	Mean
SV2A	19	18	18	16	18	17	18	16	15	17
PSD95 + GPHN	66	62	50	40	57	55	56	47	49	53
% SV2A	29	29	36	40	32	31	32	34	31	32

postsynaptic markers PSD95 and GPHN (Figure 4, Table 6). In the brain regions examined, 32–49% (mean = 41%) of excitatory postsynaptic terminals and 38–45% (mean = 41%) of inhibitory postsynaptic terminals were found adjacent to presynaptic terminals positive for SV2A. Together, these findings provide strong evidence that SV2A is present in specific subsets of excitatory and inhibitory synapses.

We next asked if SV2A preferentially colocalizes with any particular synaptic markers in the different brain regions using a hierarchically clustered heatmap (Figure 3B). There was no single region where SV2A exhibited high colocalization with all markers. Instead, a dichotomy was evident: within the DGpo (which contains the largest SV2A puncta) SV2A had the highest colocalization with VGLUT1 and lowest colocalization with VGAT. The opposite was observed in SMSL5. These findings indicate that SV2A does not consistently represent excitatory and inhibitory synapses in all brain regions.

Variation in the amount of SV2A protein in individual synapses

The visual examination of synaptic puncta labelled with SV2A and other markers revealed a wide range of sizes and intensities (Figure 2). Some brain regions, such as the DGpo, consistently

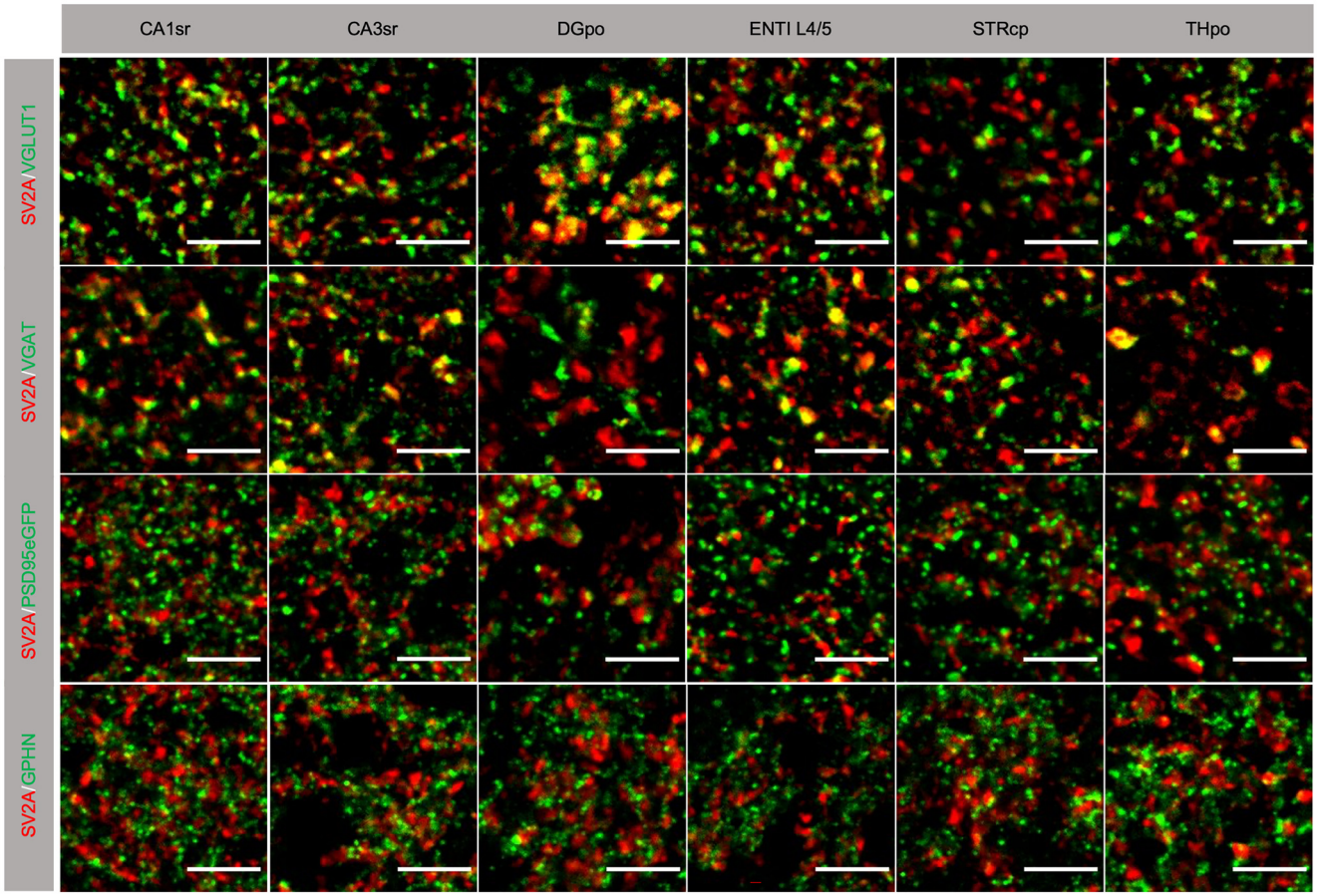

Figure 4. Synaptic colocalization of SV2A with presynaptic and postsynaptic markers in adult mouse brain. Images of individual synaptic puncta labelled with SV2A (red) and excitatory (VGLUT1, PSD95eGFP) and inhibitory (VGAT, GPHN) synaptic markers (green). Yellow puncta indicate colocalization of SV2A with the other markers. Scale bars, 5 μm.

Table 6. Colocalization of SV2A with excitatory and inhibitory synaptic markers across regions of mouse brain. Values (mean ± SEM) represent percentage of marker puncta that contain SV2A-positive puncta.

Marker/ROI	CA1sr	CA2sr	CA3sr	DGpo	SMS L2/3	SMS L5	ENTI L4/5	STRcp	THpo	Mean
SYP	65±1	70±8	78±5	85±3	74±1	69±2	77±7	75±3	79±4	75±4
SYN	57±10	49±11	62±12	80±6	56±19	51±19	57±13	62±13	69±11	60±13
VGLUT1	57±2	70±5	65±3	84±6	77±6	64±4	57±6	64±1	61±8	67±5
VGAT	74±3	68±7	72±7	52±8	62±5	92±12	59±9	71±7	76±6	69±7
PSD95	32±9	33±5	39±3	49±6	44±2	42±1	41±7	48±3	43±3	41±4
GPHN	41±2	44±3	43±3	34±1	45±2	44±1	38±3	40±6	40±3	41±3

contained larger and more intensely labelled presynaptic terminals. To analyse the synapse populations in each brain region in a quantitative manner, we calculated the mean puncta intensity for each marker in each region (Table 7) and, to assist in comparing these data, we created a hierarchically clustered heatmap (Figure 3C).

As shown in Figure 3C, SV2A puncta exhibited highest mean intensity in SMSL2/3 and DGpo, as compared with other brain regions. Comparing the cortical layers shows that SV2A puncta have higher intensity in the superficial (SMSL2/3) than deeper (SMSL5, ENTIL4/5) layers, a distribution pattern that was not observed for any of the other markers (Figure 3C). SYN and

SYP clustered with SV2A, whereas the other markers formed a separate cluster. It is worth noting that like SV2A, SYN and SYP are markers that do not distinguish between inhibitory and excitatory synapses. By contrast, markers specific to either excitatory or inhibitory synapses formed a distinct cluster (Table 7, Figure 3C).

SV2A is expressed in subsets of human brain synapses

To investigate whether SV2A is also expressed in subsets of synapses in the human brain, sections from the inferior temporal gyrus (BA20/21) of the neocortex (layers 1 to 6) were immunolabelled for SV2A, SYP, PSD95 or GPHN (Figure 5). Punctate staining was observed for all markers, with SV2A and

Table 7. Mean intensity expression of protein markers in individual synapses in mouse brain regions. Intensity values (a.u.) shown as mean ± SEM.

Marker/ROI	CA1sr	CA2sr	CA3sr	DGpo	SMS L2/3	SMS L5	ENTI L4/5	STRcp	THpo	Mean
SV2A	328±69	331±76	346±80	519±109	494±92	367±69	353±58	350±92	384±77	386±80
SYP	378±74	358±41	349±38	377±93	321±36	330±22	279±7	341±73	298±26	337±46
SYN	395±76	375±68	378±94	487±111	373±39	324±24	385±99	381±89	366±101	385±78
VGLUT1	232±22	250±21	229±11	240±19	230±14	224±13	226±14	255±30	238±22	236±18
VGAT	276±37	301±51	293±41	270±27	301±36	271±15	338±71	272±20	293±20	291±35
PSD95	325±55	283±59	214±29	231±34	309±65	259±44	264±50	266±42	208±20	262±44
GPHN	257±5	252±9	243±3	260±1	280±2	270±2	267±8	258±6	260±7	261±5

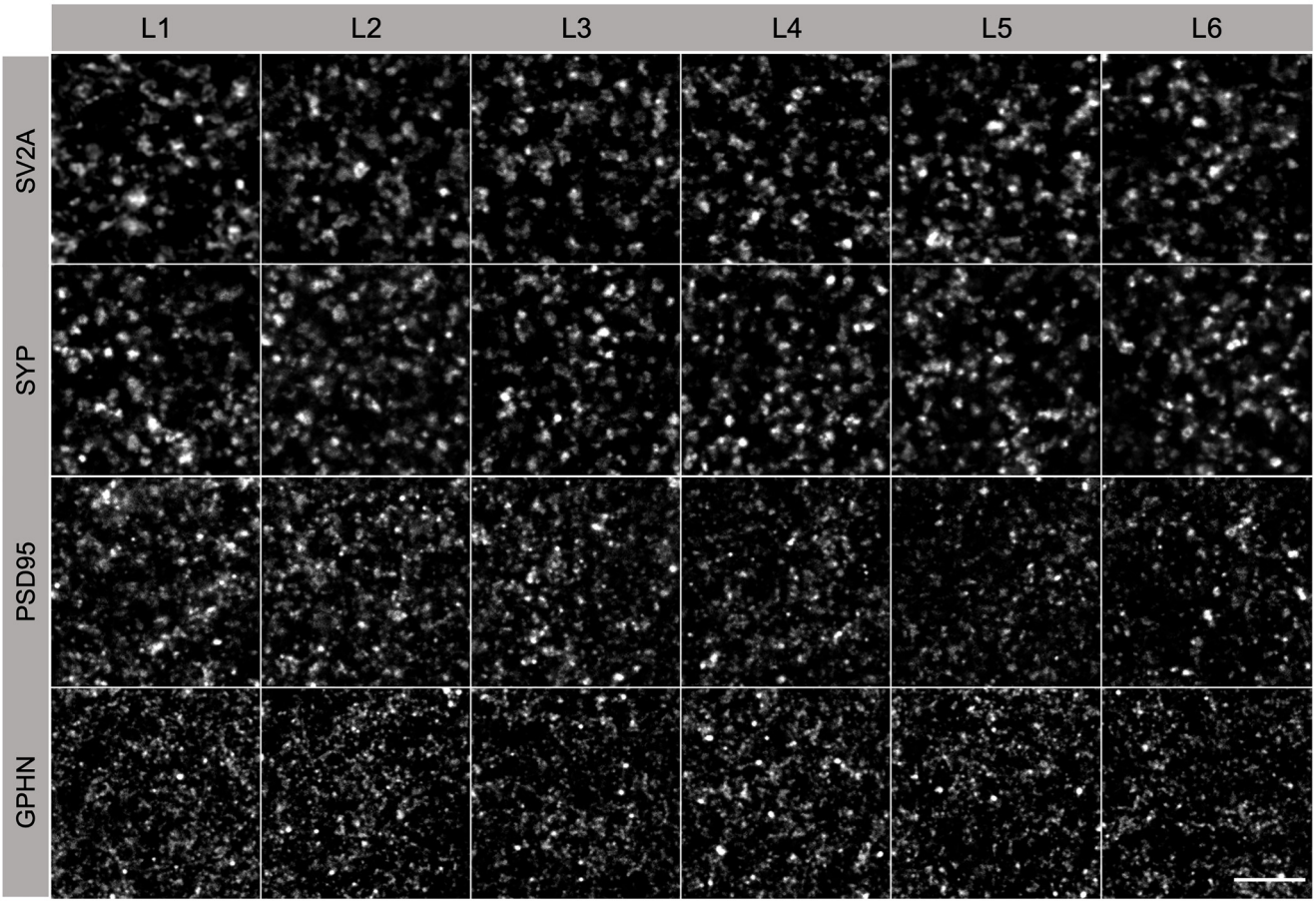

Figure 5. Antibody labelling of synapses in human temporal cortex. Individual synaptic puncta in the six layers (L1-L6) of the human temporal cortex labelled with presynaptic (SV2A, SYP) and postsynaptic (PSD95, GPHN) markers. Scale bar, 5 μm.

SYP labelling larger puncta than PSD95 and GPHN. Variation in puncta shape, size and intensity was observed across cortical layers.

We first compared the density of the four markers in each of the six layers. The density of SV2A and SYP puncta was approximately half that of PSD95 or GPHN in each layer (Table 8). The density of SV2A and SYP puncta was similar in all layers, in contrast to PSD95 and GPHN which showed a gradient, with the higher puncta density in superficial layers and lower density in deeper layers (Table 8). Comparing the density of SV2A puncta with the total (sum of PSD95 and GPHN) density revealed that SV2A accounted for 23–27% across layers and 25% mean overall (Table 9).

We next examined the colocalization of SV2A with SYP, PSD95 or GPHN (Figure 6, Table 10). Although SV2A and SYP have a similar density in each cortical layer (Table 8), overall across all layers 67% of SYP synapses express SV2A (Table 10). Only 30% of PSD95 synapses and 23% of GPHN synapses were labelled with SV2A (Table 10).

Finally, we examined the amount of each marker in synapses by measuring the mean intensity of individual puncta in each brain region (Figure 7, Table 11). As shown in the heatmap, layer 1 contains synapses with the most intense puncta. SV2A intensity was lowest in layer 4, SYP in layer 2, PSD95 and GPHN in layer 6.

Differences in SV2A synaptic expression between human and mouse brain

Comparing L2 in the mouse cortex (SMSL2/3) with L2 in human temporal cortex, the density of SYP, PSD95 and GPHN puncta was consistently higher in human, whereas the density of SV2A was similar in the two species (Table 3, Table 8). The percentage colocalization of pairs of markers also differed between mouse cortex (SMSL2/3) and L2 in human temporal cortex (Table 6, Table 10). Averaging all regions, the mean percentage colocalization of SV2A with either PSD95 or GPHN for all regions examined was higher in the mouse (PSD95: mouse 41%, human, 30%; GPHN: mouse 41%, human 23%) (Table 6, Table 10). Although the exact same regions are not being compared in the two species, the current findings nonetheless suggest that there are differences in the composition of the synaptome between mouse and human brain regions, likely due to evolutionary divergence[19].

Discussion

The presynaptic protein SV2A has gained clinical importance because it is the target of PET radiotracers used to measure synapse density in the brain of living patients. It is also the target of anti-epileptic drugs. Understanding how widely SV2A is expressed in the brain, and whether it reports all or only subpopulations of synapses, are clearly of relevance to its clinical applications. Using high-resolution, large-scale spinning disk confocal microscopy we have quantified the number of synapses expressing SV2A, and the levels of expression in

Table 8. Quantification of synaptic puncta density of excitatory and inhibitory markers across layers of human temporal cortex. Measurements (mean ± SEM) normalised to puncta/100 μm².

Marker/ROI	L1	L2	L3	L4	L5	L6	Mean
SV2A	18±1	19±1	19±1	18±1	18±1	17±1	18±1
SYP	20±5	21±2	22±2	20±4	20±3	19±3	21±3
PSD95	41±3	44±3	42±4	40±2	36±3	32±1	39±3
GPHN	37±8	34±4	36±3	35±5	34±9	32±8	35±6

Table 9. SV2A puncta represent a minor fraction of the total number of synaptic puncta in human temporal cortex layers. SV2A puncta density (top row), summed density of PSD95 and GPHN (middle row), and the percentage of SV2A puncta compared with PSD95 and GPHN (bottom row).

Marker/ROI	L1	L2	L3	L4	L5	L6	Mean
SV2A	18	19	19	18	18	17	18
PSD95 + GPHN	78	78	78	75	71	64	74
% SV2A	23	25	24	24	25	27	25

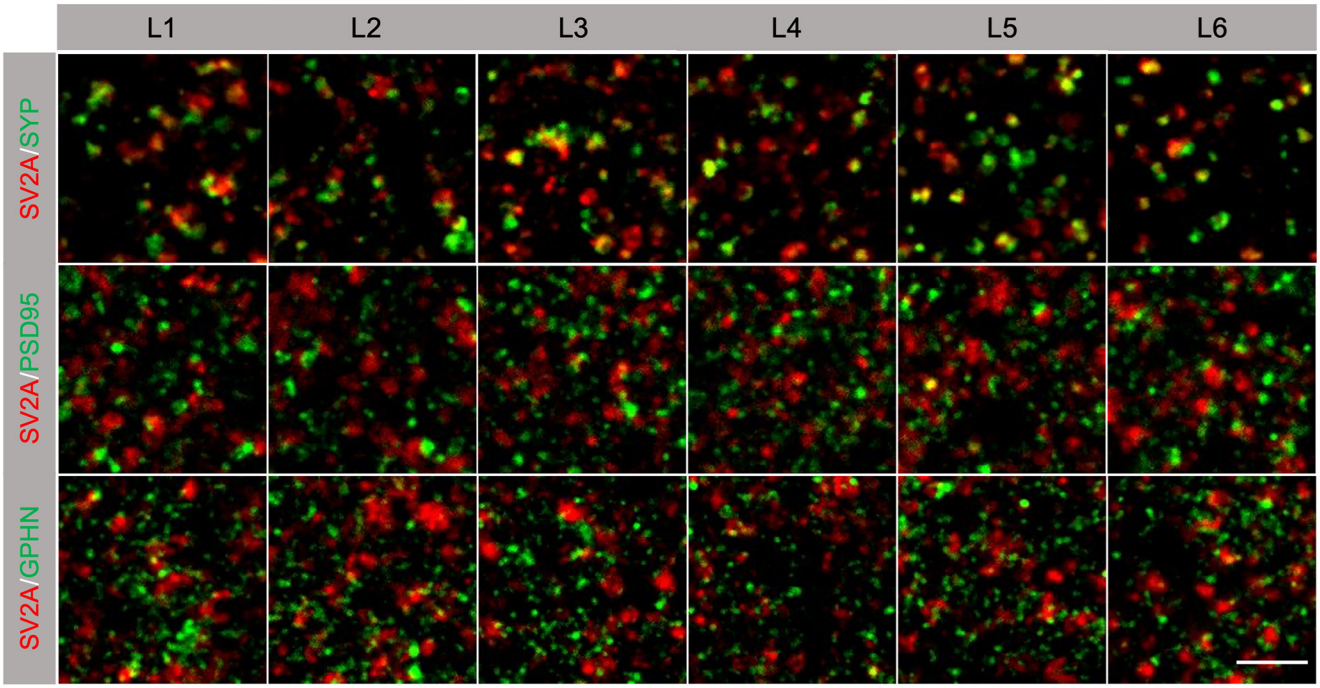

Figure 6. Colocalization of SV2A with presynaptic and postsynaptic markers in human brain. Images of individual synaptic puncta in layers (L1-L6) of human temporal cortex labelled with SV2A (red) and presynaptic marker SYP, postsynaptic excitatory synapse marker PSD95, and postsynaptic inhibitory marker GPHN (all green). In SV2A/SYP labelling (top row), yellow indicates colocalization of the two markers. In SV2A/PSD95 (middle row) and SV2A/GPHN (bottom row) labelling, the juxtaposition of red and green puncta indicates synaptic colocalization. Scale bar, 5 μm.

Table 10. Percentage of SV2A colocalization with other synaptic markers in layers of human temporal cortex. Values (mean ± SEM) represent percentage of marker puncta that contain SV2A-positive puncta.

Marker/ROI	L1	L2	L3	L4	L5	L6	Mean
SYP	68±2	68±1	65±3	65±3	67±3	71±1	67±2
PSD95	27±4	26±2	30±2	29±1	32±1	34±4	30±2
GPHN	21±6	26±7	25±6	22±7	22±8	22±7	23±7

individual synapses, in conjunction with other presynaptic and postsynaptic proteins in mouse and human brain. These studies reveal that SV2A is expressed in subpopulations of excitatory and inhibitory synapses, the density of which varies across mammalian brain regions, and that there is high diversity among individual SV2A-positive synapses.

In the temporal cortex of adult humans, we found that SV2A is expressed in 67% of SYP-positive presynaptic terminals across cortical layers. The percentage of SV2A that colocalized with postsynaptic markers was lower than that observed with SYP: only 30% of excitatory (PSD95) and 23% of inhibitory (GPHN) synapses colocalized with SV2A and these fractions varied across the layers of the cortex.

We observed similar results in the mouse brain, where 65–85% of SYP-positive synapses expressed SV2A. Examination of SV2A colocalization with other presynaptic markers showed that 57–84% of VGLUT1 synapses and 52–92% of VGAT synapses expressed SV2A in different regions. The percentage of SV2A colocalization with postsynaptic markers was lower than for presynaptic colocalization: SV2A colocalized with 32–49% of excitatory (PSD95) and 38–45% of inhibitory (GPHN) postsynaptic terminals in different brain regions.

Together, these results indicate that in the mammalian brain a major fraction of presynaptic terminals do not express SV2A and an even greater fraction (>50%) of postsynaptic terminals are not associated with SV2A-positive presynaptic terminals.

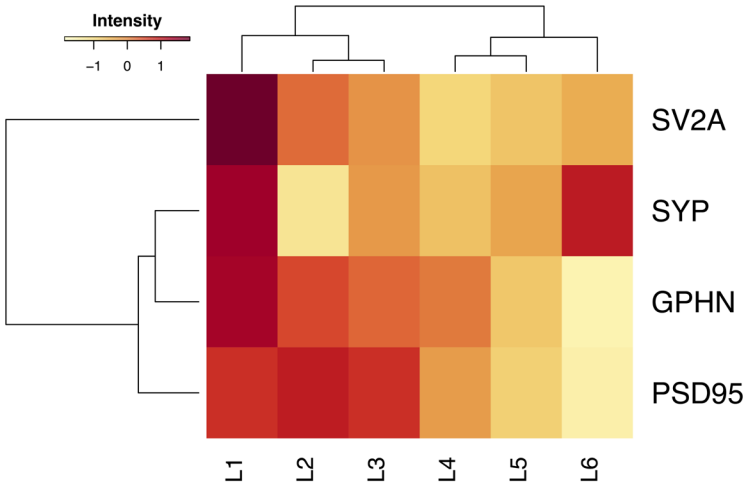

Figure 7. Comparison of intensity of presynaptic and postsynaptic puncta across layers of human temporal cortex. Average puncta intensity (a.u.) of four synaptic proteins across layers (L1-L6) of the human temporal cortex clustered by similarity. Colours are normalised values for each marker (row).

Table 11. Mean intensity of synaptic puncta in layers of the human temporal cortex. Intensity values (a.u.) are shown as mean ± SEM.

Marker/ROI	L1	L2	L3	L4	L5	L6	Mean
SV2A	442±54	403±37	396±44	376±46	382±44	388±47	397±45
SYP	300±47	270±23	282±25	276±41	280±55	296±75	284±44
PSD95	321±29	327±42	321±19	292±16	275±23	256±16	299±24
GPHN	277±71	266±26	262±11	259±22	247±67	235±61	258±43

Moreover, this fraction varies between brain regions and between excitatory and inhibitory synapses. We also observed considerable heterogeneity of SV2A-positive synapses with respect to their size and protein content. These findings are in line with previous investigations showing that a diversity of synapse types can be distinguished by the combinations of proteins they express and that these synapse types can be further divided into subtypes according to punctum size, shape, nanoarchitecture, protein lifetime and level of expression[2,4,17,18]. Future studies could explore why more postsynaptic than presynaptic terminals lack SV2A and determine whether this might reflect the maturity or turnover of dendritic spines.

SV2A PET radiotracers as reporters of synapse density

SV2A is commonly described as being expressed in all synapses at homogenous levels and in all brain regions. To understand how this view arose and evolved in the PET literature, we examined over 60 studies that refer to its expression as ubiquitous. The vast majority of this literature refers to two papers[6,13]. The earliest is from Bajjelieh and colleagues, who used mRNA in situ hybridisation on rat brain tissue sections (imaged at low resolution) and describe "ubiquitous expression" in the context of brain regions and suggest that their results are "consistent" with expression of the gene in all neurons. They do not show SV2A protein expression at single-synapse resolution. The latter paper from Finnema and colleagues refers to SV2A as "ubiquitously and homogeneously located in synapses across the brain" and cites the Bajjelieh study. It is notable that in Figure 1K of their paper they show immunofluorescent label-ling of SV2A and SYP synaptic puncta in a region of grey mat-ter and it is clear from this image that the two proteins have differential and overlapping expression, consistent with our find-ings. To our knowledge, studies that directly correlate brain regional or pathological single-synapse resolution quantification of synapse density with PET binding data remain absent from the published literature.

Implications of synapse diversity for the clinical use of PET imaging

In considering the impact of our findings on the interpretation of PET imaging data it is useful to contrast two models: the 'standard model' where SV2A is expressed ubiquitously and homogeneously, and the 'synaptome model' of the present study in which SV2A is expressed in as few as 25% of synapses. Our findings point to five relevant factors:

1. SV2A radiotracers may bind to a minority of the total number of brain synapses;

2. SV2A is present in different proportions of excitatory and inhibitory synapses, with a higher fraction of inhibitory synapses expressing SV2A;

3. The fraction of all synapses expressing SV2A differs between brain regions, as does the fraction of excitatory and inhibitory synapses;

4. The amount of SV2A varies between individual synapses;

5. The average amount of SV2A in the populations of synapses in brain regions differs.

The design of most clinical studies with SV2A PET imaging involves a comparison of a set of cases (e.g. patients with a brain disorder) with controls. We will consider a hypothetical study that reports a 20% reduction in SV2A PET radiotracer binding in two brain regions (A and B) of patients. First, the SV2A radiotracer tells us nothing about the 75% of brain synapses that do not express SV2A. Instead, the synaptome model interprets the 20% reduction in signal as a 5% reduction in the total number of synapses, as only 25% of synapses express SV2A. Furthermore, the synaptome model tells us that this might differentially impact excitatory versus inhibitory synapses, and region A versus region B (i.e. the extent of pathology may differ between those regions), especially if synapses in these two regions express different levels of SV2A.

Factors 4 and 5 are important because the capacity to detect the loss of a synapse is affected by the amount of protein in that synapse. PET radiotracers occupy a small fraction of available binding sites, hence synapses that express small amounts of protein will be more likely to escape detection in PET imaging. For example, if region A is composed of large SV2A synapses with high-intensity expression and region B has small low-intensity synapses, then a 20% reduction in binding in region B will reflect a greater loss of synapses.

It is important to recognise that disease processes may interfere with any of the steps (expression of the gene, translation of the protein in soma or axon, transport of the protein to the synapse, assembly into the synaptic vesicle) that determine the amount of SV2A in a synapse. For example, Pazalar and colleagues found that the level of *SV2A* mRNA in single neurons isolated from patients with epilepsy was reduced in many types of neurons[20]. Importantly, the level of SV2A mRNA and/or protein has no impact on the number of synapses, as mice lacking SV2A show no loss of synapse density or change in synapse morphology[21]. This finding, together with our present study, emphasises that a change in SV2A radiotracer binding does not necessarily imply a change in synapse density, as it could equally reflect a (pathological) change in the level of the protein.

A further factor to be considered is that confocal microscopy detects (synaptic) puncta because of the clustering of single molecules which escape detection when dispersed. A recent study using single-molecule detection shows that PSD95, a protein that is typically observed as synaptic puncta in confocal microscopy, exists as a large population in the extra-synaptic compartment as individual proteins or dimers, which remain undetected by confocal microscopy[22]. Although the proportion of SV2A found in the extra-synaptic compartment (including the axon) is unknown, radiotracers would be expected to report on this population.

Recommendations for interpretation of SV2A PET imaging studies

We argue that changes in SV2A PET radiotracer binding might not reflect a change in synapse density, or may greatly overestimate it, or may fail to detect major losses of SV2A-negative synapses. Consequently, in studies that employ PET radiotracers to study human brain disease, we caution against the conclusion that altered binding be interpreted as a direct reporter of pathological change in synapse density. Instead, changes in SV2A PET radiotracer binding should be interpreted as changes in SV2A-positive synapses. Similar reservations may impact the interpretation of SV2A radiotracer binding studies undertaken for the normal brain. A comprehensive brain-wide, single-synapse resolution examination of the distribution of SV2A is called for, incorporating further synaptic markers that enable any change in synaptic populations to be quantified with confidence. Synaptome mapping technology is ideally suited to this objective as it has been demonstrated that brain-wide synaptome analysis of PSD95-positive synapses is possible in human brain[23].

Such a detailed knowledge of the synaptome architecture of SV2A across the brain, and lifespan, could greatly enhance the discovery value of SV2A PET imaging. For example, in Alzheimer's disease, which is characterised by synapse loss during the early stages, it would be informative to determine whether and how SV2A-positive synapse types and subtypes are affected at the different stages of disease progression. One of the key principles that has emerged from synaptomic theory is that synaptic proteins that are encoded by disease-risk genes are expressed in subpopulations of synapses[24,25]. Indeed, *SV2A* is recognised as a schizophrenia risk gene[26] and thus SV2A-positive synapses are candidates for the underlying pathology of this disorder. Thus, it would be valuable to characterise expression of other proteins in SV2A-positive synapses, as this might indicate that SV2A PET studies could be particularly useful in these disorders. It is also noteworthy that SV2A is the target of a highly effective anti-epileptic drug and the identification of the SV2A-positive subpopulations of synapses may inform on its mechanism of action and lead to new therapies.

In conclusion, detailed synaptome analysis of SV2A in the human brain in health and disease will greatly enhance the value of SV2A PET radiotracers and advance their application in clinical settings.

Ethics statement

The animal experiments underwent review by the University of Edinburgh Animal Welfare and Ethical Review Body (AWERB). They were subsequently approved (PPL PF3F251A9) by the UK Animals in Science Regulation Unit (ASRU) under the Animals (Scientific Procedures) Act 1986 in strict accordance

with the Home Office Code of Practice. The experiments were conducted following an authorised experimental protocol endorsed by both the AWERB and the Bioresearch and Veterinary Services (BVS) department at the University of Edinburgh.

Human brain tissue from deceased individuals was acquired from the University of Edinburgh Brain Bank (EBB), funded by the Medical Research Council UK. All activities involving the use of post-mortem human brain tissue received approval from the East of Scotland Research Ethics Service (21/ES/0087). Informed written consent was secured from each participant.

Data availability

The BioImage Archive[27] contains all data (under the title "SV2A synapse diversity project") and the ARRIVE guidelines checklist (under the title "SV2A diversity project ARRIVE 2.0 checklist") at https://doi.org/10.6019/S-BSST2108 (DOI: 10.6019/S-BSST2108). The project contains the following underlying data: Raw experimental files. Data are available under the terms of the Creative Commons Zero (CC0) license.

Author contributions

Laboratory experiments: TW, BN. Image analysis: TW, ZQ. Human brain samples: CS. Supervision: AT, CS, SGNG. Writing: TW, SGNG.

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✔ **Bong-Kiun Kaang**
Institute for Basic Science (IBS), Daejeon, South Korea

The authors investigated whether SV2A, the target molecule of SV2A-based PET that is widely used as a means to detect brain disorders by identifying synaptic pathology, is indeed ubiquitously expressed across synapses. They employed single synapse synaptome mapping based on a well-designed immunohistochemistry to brain tissues from both mice and humans to analyze SV2A expression at the synaptic level. Their results show that SV2A is not expressed in all synapses; rather, its expression varies between excitatory and inhibitory synapses, as well as across different brain regions. These findings strongly suggest that the diversity of synaptic SV2A expression should be carefully considered when interpreting clinical data obtained from SV2A PET imaging. The experimental design and data interpretation are sound, and the manuscript is well written. I only have minor suggestions to improve the manuscript:

1. The functional role of SV2A and the rationale or historical reason for its use as a PET synaptic marker would be further described in the Introduction for general readers.
2. I wonder whether the authors analyzed neuronal or nuclear RNA-seq data published elsewhere to compare the expression levels of SV2A and other excitatory and inhibitory synaptic markers at the single-cell transcriptomic level. If so, they may discuss it.
3. Minor copy-editing is needed (e.g., label-ling, mat-ter, find-ings, etc.).

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Synapse biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 06 October 2025

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Sridhar Natesan
King's College London, London, England, UK

The authors have employed high-resolution synaptome mapping in adult mouse and human brain tissue sections capturing the expression of SV2A protein. SV2A is protein known for its expression in synaptic vesicles and of lately has been labelled as a surrogate marker for all synapses especially in context of brain PET imaging using radiotracers that selectively bind to it.

The result of this study shows that SV2A is not present in all synapses but is found in specific subsets and its expression varies across different brain regions. This finding is very important as interpretation of SV2A PET imaging studies in human clinical cases have assumed that SV2A is expressed in all synapses and in a homogenous manner.

The authors have validated their methodology in an adequate manner especially to determine optimal immunofluorescence labelling conditions of target proteins in the mouse brain. They have also taken adequate steps in the case of human brain tissues.

In the mouse brain they have carefully chosen regions with high levels of synaptic diversity and have measured SV2A density along with well-characterized presynaptic/postsynaptic proteins. Their results indicate that SV2A is expressed in subpopulations of excitatory and inhibitory synapses across the mouse brain. This has been extended to the human brain sections with similar findings.

I have a few observations to make:

1. In terms of methodology fluorescence is the key measure in this study. In the case of SV2A in mice brain sections, the secondary Ab is Anti rabbit and they are compared to fluorescence generated from Anti-mouse/guinea pig albeit all generated from Goats. In this context how rigorous is the methodology to compare SV2A puncta density to summed density of PSD95 and GPHN to determine fractional expression based on fluorescence read outs.
2. It is interesting to note that co-localization of SV2A with postsynaptic proteins markers in a smaller proportion also means that it is not uniquely presynaptic as commonly mentioned in the literature.
3. In PET most studies for SV2A compare large ROIs and though it is not right for authors to have claimed “ubiquitously and homogeneously located in synapses across the brain”, findings from PET studies could still hold but need to be carefully interpreted. More research on this is needed to qualify its relationship to pathology.
4. There was no disease models of synaptic loss included in the study and that could have been more interesting.
5. The human brains were from an elderly group but nevertheless helped in understanding SV2A expression in human brains.

Overall, it is well written and adequately referenced.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neuroimaging and Psychopharmacology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.