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Mass Synaptometry: High-Dimensional Multi Parametric Assay for Single Synapses

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Abstract

Background: Synaptic alterations, especially presynaptic changes, are cardinal features of neurodegenerative diseases and strongly correlate with cognitive decline.

New Method: We report “Mass Synaptometry” for the high-dimensional analysis of individual human synaptosomes, enriched nerve terminals from brain. This method was adapted from cytometry by time-of-flight mass spectrometry (CyTOF), which is commonly used for single-cell analysis of immune and blood cells.

Result: Here we overcome challenges for single synapse analysis by optimizing synaptosome preparations, generating a ‘SynTOF panel,’ recalibrating acquisition settings, and applying computational analyses. Through the analysis of 390,000 individual synaptosomes, we also provide proof-of principle validation by characterizing changes in synaptic diversity in Lewy Body Disease (LBD), Alzheimer’s disease and normal brain.

Comparison with Existing Method(s): Current imaging methods to study synapses in humans are capable of analyzing a limited number of synapses, and conventional flow cytometric techniques are typically restricted to fewer than 6 parameters. Our method allows for the simultaneous detection of 34 parameters from tens of thousands of individual synapses.

Conclusion: We applied Mass Synaptometry to analyze 34 parameters simultaneously on more than 390,000 synaptosomes from 13 human brain samples. This new approach revealed regional

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AUTHOR CONTRIBUTIONS

CG and TM designed the study. CG and RF performed CyTOF experiments. CG and NP prepared synaptosomes. CG and TM generated mass synaptometry antibody panel. CG optimized and validated method and protocol. CK and NP performed the rapid autopsies and provided pathologic evaluations. MA and SB provided expertise in CyTOF operation and data analysis. CG, RF, MA, SB, DT, and EF interpreted and analyzed the data. CG, KM, and TM wrote the manuscript. All co-authors critically revised the manuscript. TM supervised all aspects of the work.

COMPETING FINANCIAL INTERESTS

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and disease-specific changes in synaptic phenotypes, including validation of this method with the expected changes in the molecular composition of striatal dopaminergic synapses in Lewy body disease and Alzheimer's disease. Mass synaptometry enables highly parallel molecular profiling of individual synaptic terminals.

Keywords

Mass cytometry; CyTOF; Synapse; Alzheimer's disease; Parkinson's disease

1. INTRODUCTION

Alzheimer's disease (AD) and Lewy body disease (LBD), which includes Dementia with Lewy Bodies (DLB) and Parkinson's disease (PD), are proposed to initiate with regional synaptic injury and degeneration (Bellucci et al., 2012; Braak and Braak, 1991; McKeith et al., 2017; Overk and Masliah, 2014; Scott et al., 2010; Wishart et al., 2006). Synaptic alterations, especially presynaptic changes, are cardinal feature of neurodegenerative diseases, including LBD, and AD, and strongly correlate with cognitive decline (DeKosky and Scheff, 1990; Dickson et al., 1995; Masliah et al., 1991; Scheff and Price, 2003; Sze et al., 1997; Terry, 1996; Terry et al., 1991). Although there is substantial support for this hypothesis from animal models (Dietrich et al., 2018; Duyckaerts et al., 2008; Galli et al., 2014; Jucker, 2010; Zhu et al., 2017), data on human synaptic changes derive mostly from electron microscopy, Golgi stains, or tissue homogenates. Thus a major gap exists regarding comprehensive molecular characterization of individual synapses from human brain.

One approach to this challenge is multiplex analysis of synaptosomes: pinched-off, re-sealed, mostly presynaptic terminals used widely in neurophysiology and neuropharmacology for decades (Hebb and Whittaker, 1958). Like extracellular vesicles, such as exosomes and microvesicles, synaptosomes are smaller than a cell and bound by the cell of origin's lipid bilayer. Several labs, including ours, have adapted conventional flow cytometric analysis of synaptosomes from human cerebral cortex (Sokolow et al., 2012), hippocampus (Bilousova et al., 2016; Gyls and Bilousova, 2017), and striatum (Postupna et al., 2017; Postupna et al., 2014) and demonstrated, despite using different samples, probes, and instruments, that cytometry of human synaptosomes is a robust and reproducible technique. Importantly, comparison to non-human primate brain collected with no agonal state and no post mortem interval demonstrated that protein in human synaptosomes from rapid brain autopsy is intact (Postupna et al., 2014). However, despite this advance of analyzing thousands of individual synaptic particles in a single run, to date multiplexing has involved a limited number of probes (Bilousova et al., 2016; Gyls and Bilousova, 2017; Postupna et al., 2017; Postupna et al., 2014; Sokolow et al., 2012). As with cytometry in immunology and cancer biology, the repertoire of probes needed to assess comprehensively the type of synapse, pathologic proteins (including protein products of risk genes discovered in GWAS), and markers of stress and injury far exceeds what is achievable with conventional flow cytometry.

In contrast to fluorescence-based cytometry, mass cytometry—also known as cytometry by time-of-flight mass spectrometry (CyTOF)—uses antibody probes conjugated to metal ions

coupled with time-of-flight mass spectrometry (Amir el et al., 2013; Anchang et al., 2016; Bandura et al., 2009; Bendall et al., 2012), overcoming many of the multiplexing limitations of conventional flow cytometry. Indeed, CyTOF has been used by us and others to great effect in oncology and immunology research to reveal tissue and cellular diversity (Bendall et al., 2011; Chevrier et al., 2017; Gonzalez et al., 2018; Hamers et al., 2017; Korin et al., 2017; Lavin et al., 2017; Mrdjen et al., 2018; Simmons et al., 2015; Spitzer et al., 2015; Spitzer and Nolan, 2016; Wong et al., 2015). Here we describe a novel technique, termed mass synaptometry, which adapts mass cytometry and synaptosome preparation to achieve high throughput molecular characterization of individual synapses. Although we have applied our novel approach to the investigation of individual human synapses, our work also serves as a template for others focused on mass cytometry of subcellular individual events such as platelets or extracellular vesicles.

2. MATERIALS AND METHODS

2.1. Collection and preservation of brain tissue

Human samples were obtained from rapid (post mortem interval < 8 hrs.) brain autopsy by the University of Washington Alzheimer's Disease Research Center or the Pacific Udall Center. Brain regions (caudate, cerebral cortex and cerebellum) approximately 0.5 cm³ in size were collected, minced with two razor blades, transferred to cryo tubes containing 1 ml of 0.32 M sucrose with 10% DMSO, and mixed by shaking. Following gradual cooling to -80°C the samples were stored in liquid nitrogen, or at -80°C. Samples were shipped on dry ice.

2.2. Preparation of synaptosomes

The synaptosome preparation protocol was adapted and modified from established protocol from our lab and other labs (Gyls and Bilousova, 2017; Gyls et al., 2000; Postupna et al., 2017; Postupna et al., 2014; Weiler et al., 1981; Weiler et al., 1978). Samples of cryopreserved minced tissue were thawed rapidly in a 37°C water bath. Thawed tissue was supplemented with 10 vol ice- cold homogenization buffer (HB), and spun at 4,000 g for 4 min at 4°C. The supernatant was then discarded. HB composition: 0.32 M sucrose in 10 mM Tris buffer (pH 7.4) with EDTA-free, mass spectroscopy-grade protease and phosphatase inhibitor cocktail (Sigma # MSSAFE). 1X HB was made by mixing 20 ml of 0.32 M sucrose in 10 mM Tris buffer (pH 7.4) to 1 vial of MSSAFE.

The minced tissue pellet was resuspended in 10 volumes of ice cold HB and homogenized in a Teflon/glass homogenizer (clearance 0.1–0.15 mm) with 8 up-and-down strokes at 800 RPM. The homogenate was transferred to pre-chilled centrifuge tubes and the homogenate sedimented at 1,000g for 10 min at 4°C to remove nuclei and cell debris. The supernatant (S1) was transferred into new centrifuge tubes. After discarding the pellet, the supernatant was divided, 1 ml per tube, and sedimented at 10,000 g for 20 min at 4°C to obtain the crude synaptosome pellet (P2). The supernatant was again discarded, and P2 resuspended in 200 µL of 0.32 M sucrose in 10 mM Tris buffer (pH 7.4) with 10% DMSO. Samples were cryopreserved as above and stored in liquid nitrogen, or 80°C.

2.3. Quality control for signal from conjugated antibodies:

As detailed in the Results section, we took four independent steps to ensure quality of signal from our new neuroscience panel. One of these quality control steps involved western blots with conjugated antibodies. Only conjugated antibodies with Western blot banding pattern expected from the literature and unchanged from non-conjugated antibody were included. 34 of 36 antibodies passed this quality control (Table S1).

Appropriate 'positive' samples were homogenized in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.2 by sonication (sonic dismembrator, Model F60, Supplier Fisher Scientific, 4+4 second at setting 15, with intermittent incubation on ice for 5 min). Protein lysates were separated in denaturing gels by electrophoresis transferred onto Immune-Blot PVDF membranes (Bio-Rad Cat#1620174) and blotted with metal-conjugated antibodies. Blots were developed by using ECL (Thermo Scientific, Cat # 1856135/6, 34094). We used P2 as the positive control for the CD11b signal because 5 micron filtration of P2 yielded synaptosomes without detectable microglia-derived particles.

2.4. Staining for mass synaptometry:

The original synaptosome preparation and staining protocols were adapted, modified conjoined, optimized and streamlined for suitability of analyzing synaptosomes using our SynTOF panel (Bandura et al., 2009; Gyls and Bilousova, 2017; Gyls et al., 2000; Postupna et al., 2017; Postupna et al., 2014). We optimized conjoined and streamlined method with the least number of steps that work for most antibodies.

Cryopreserved resuspended P2 was thawed in a 37°C water bath. The thawed synaptosome suspension was topped up with 1.5 ml Krebs-Ringer phosphate buffer (KRP: 118 mM NaCl, 5 mM KCl, 4 mM MgSO₄, 1 mM CaCl₂, 1 mM KH₂PO₄, 16 mM sodium phosphate buffer (pH 7.4), and 10 mM glucose). Following sedimentation at 8,000g for 4 min at 4°C to remove supernatant, the pellet was washed twice with 1.5ml of KRP and passed through a 5 µm centrifuge filter. All subsequent centrifugation steps were performed at 8,000g for 4 min. The resuspended synaptosome was incubated with benzonase for 30 min at 37°C (10µl benzonase (250U/µl) in 200ul KRP) to reduce viscosity, and then washed thrice with PBS. The synaptosomes were then fixed with 1 ml of 1x Fix I buffer in PBS (Fluidigm), at room temperature (RT), for 15 min and again washed thrice with PBS. The fixed synaptosomes were divided into multiple tubes per experiment as needed. Pause point (optional): the synaptosomes can be stored in 200µl PBS, 4°C, overnight. Next, the synaptosomes were washed twice with 1x Perm buffer (Fluidigm) and incubated with 1ml 1x Perm buffer, RT, 30 min. Following two washes twice with cell staining buffer, the synaptosomes were kept in fresh cell staining buffer until cocktail of antibodies was prepared. Stocks of individually labeled antibodies were stored in separate tubes. A cocktail of 34 antibodies was made on the day of staining in a single tube and cleared of aggregates by 0.1 micron filtration. There were no substantial differences in signal when using antibodies individually or combined in the 34-member cocktail.

Synaptosomes were incubated with 100µl of antibody cocktail in cell staining buffer at RT, 1.5 hours. The stained pellet was washed once with cell staining buffer, and then washed twice with PBS. The stained synaptosomes were fixed in 500 µl of 1%PFA in PBS, 4°C, ON. The following day the synaptosomes were wash thrice with PBS, incubated with 200µl of 1x Iridium 191/193, for 20 min, on ice. Following a final wash with 1X with PBS and three washes with Milli Q water, the synaptosome pellet was kept in 100 µl of MilliQ water. Synaptosomes stained with metal conjugated antibody were diluted in MilliQ water spiked with 10 fold diluted EQ four element calibration Beads (Fluidigm # 201078). Note: the most critical steps are incubation of the synaptosomes with benzonase, filtration, and post-fixation of stained synaptosomes.

2.6. Acquisition settings for mass synaptometry data:

Mass synaptometry data was acquired on CyTOF (Model: Helios, with workstation equipped with CyTOF software version 6.5.358 by Fluidigm, south San Francisco, CA). Initiation and tuning of machine was performed according to manufacturer's recommendation. We processed raw Helios .IMD files modifying minimum event length, maximum event length, lower convolution threshold to determine settings optimal for synaptosomes rather than cells. Post acquisition processing of .IMD file was done using on computer equipped with CyTOF software version 6.7.1014. Settings used for final analysis were the same as cells for minimum event length (10); maximum event length was set at 100 (rather than 150 for cells) and lower convolution threshold was set to 200 (rather than 400 for cells) because synaptosomes are smaller than cells and therefore have some low abundance markers.

2.7. Processing and analysis of mass synaptometry data:

Mass synaptometry data was normalized to beads using Fluidigm CyTOF software version 6.7.1014. Gating and analysis were done using Flowjo- or Cytobank, a cloud-based computational and machine-learning platform with advanced tools and functionality that enables algorithms to analyze and visualize multiple single-event data sets simultaneously. Standard steps that are used for single cell analysis were modified and adapted for synaptosome data processing. Gating strategy was adapted and aligned with panel design for analysis of synapses using multiple markers, i.e., first filter out normalizing beads and nucleus; second filter out non-neuronal events using CD11b and MBP; third select for neuronal events using CD56 or CD 298; fourth select for presynaptic events using CD47 and SNAP25; fifth by gating out presynaptic events that are attached with post-synaptic membrane, often known as snowman using PSD95.

SPADE and viSNE visualization and analysis includes careful consideration of number of input events, number of nodes, and number of markers used for clustering to generate SPADE. For proof of principle demonstration for mass synaptometry, we empirically tested by changing nodes from 3 to 500. Choosing less nodes would lead to loss of diversity visualization of synaptic events, whereas choosing too many nodes for given number of input events would lead to complex tree with near empty nodes. To balance these factors, we selected 50 nodes for 30,000 events per sample for optimal visualization of DAT+ events in

caudate. For in-depth analysis and interrogation of further marker changes in DAT+ cluster, we exported events from the DAT+ cluster.

2.8. Statistics

No statistical methods were used to predetermine sample sizes. When comparing LBD versus control groups P values were determined by one-tailed unpaired t-test as an asymmetric distribution is expected or one-tailed unpaired Wilcoxon–Mann–Whitney test for two-group comparison. We used Wilcoxon rank sum test (two-tailed) with Bonferroni-corrected alpha of $p < 0.001$ ($0.05/34=0.0015$), Note: SynTOF Panel has 34 markers. The investigators were not blinded to allocation during experiments and outcome assessment. Graphs were generated by the ggplot2 package in R.

3. RESULTS

3.1. Overview of Mass Synaptometry

As illustrated in Figures 1 and 2, we pursued a multi-step approach to enable analysis of single synapses. Briefly, we optimized synaptosome preparation for CyTOF; compiled a “synapse by time-of-flight (SynTOF)” panel of 34 conjugated antibodies that probed cell type, synapse type, protein products of several AD or LBD risk genes, and markers of injury/response to injury (Tables 1 and S1); established acquisition parameters (Figure S1); and applied bioinformatics tools to visualize and analyze the large number of single-event parameters generated by mass synaptometry (Figures 2 and 3).

We used cryopreserved regions of human brain from rapid autopsies as described previously (Postupna et al., 2017; Postupna et al., 2014), and prepared synaptosomes from the P2 pellet with 5 micron filtration (Hollingsworth et al., 1985); we reduced viscosity with a protease-free, high purity nuclease (Benzonase at 37°C for 30 min) (Figure 2). Human P2 events, synaptosomes, and platelets averaged 9.8 ± 0.9 fL ($n=39$), 7.9 ± 0.9 fL ($n=43$), and 9.5 ± 0.4 fL ($n=33$) (Figure 3F). Preparation of conjugated antibody probes used purified, carrier protein-free antibody (100 µg) that was commercially available, custom ordered, or prepared in-house as indicated (Table S1). Synaptosomes were stabilized by fixation (1% PFA, o/n, 4°C) prior to suspension in distilled water for loading. Optimal minimum and maximum event lengths were determined to be 10 and 100, and the optimal lower convolution threshold was 200 (Figure S1).

3.2. Design of SynTOF panel

3.2.1. Considerations for choosing markers in SynTOF panel: Synaptosome fraction (P2) is enriched in presynaptic terminals but also contains some post synaptic elements as well as homogenate debris. We used stepwise gating based on a series of six non-neuronal, neuronal, and presynaptic markers to identify synaptosomes and discard non-synaptic debris, e.g., CD11b as microglial marker; MBP as myelin marker; CD56 and CD298 as neuronal markers; and SNAP25 and CD47 as presynaptic markers. (Figure 4A; Table S1).

Next we focused on typing synaptosomes using antibodies against specific neurotransmitter transporters: dopamine transporter (DAT), norepinephrine transporter (NET), serotonin transporter (SERT), vesicular glutamate transporters (vGLUT1 and vGLUT2), and vesicular GABA transporter (VGAT). Since our “positive control” is loss of striatal dopaminergic terminals in Lewy body disease (LBD), we also included an antibody to tyrosine hydroxylase (TH) as validation. Synaptosomes often maintain contact with astrocytic elements, so we included GFAP. A small fraction of synaptosomes maintain contact between pre- and postsynaptic elements, structures called snowmen, and for this reason we included an antibody to PSD95. Additionally we included antibodies to markers of injury or response to injury proposed to be important in brain aging, Alzheimer’s disease (AD), and Parkinson’s disease (PD): 3-Nitrotyrosine (3NT), Lysine-48 ubiquitin (K48), Lysine-63 ubiquitin (K63), 8-hydroxyguanosine, cleaved caspase 3, LC3B, and C1q.

3.2.2. Selection of specific antibody for chosen markers in SynTOF panel: If multiple antibodies to a target were available, we selected a specific antibody using the following criteria: (i) monoclonal preferred over polyclonal antibodies, (ii) antibodies already extensively used in immunohistochemistry by NIA ADRC Centers, (iii) antibodies already validated by immunofluorescence and flow cytometry, (iv) antibodies validated with knockout mice or by western blot with positive and negative controls, (v) antibodies available as purified and carrier protein free formulations, and (vi) antibodies available from vendors having CyTOF products. Each selected antibody must pass our in-house quality control steps before inclusion in panel (Table S1).

As SynTOF is a novel synaptic rather than immunological or cell (CyTOF) panel, all but two antibodies (GFAP and CD56) needed to be procured, conjugated, and validated. Some antibodies were also custom ordered or processed by us to remove carrier protein. In a few cases, vendors produced the same clone in a carrier-free formulation. For the panel used here, 28 of 34 antibodies are monoclonal, and 32 of 34 antibodies are available as carrier-free formulation (exceptions being CD298 and SERT). Table S1 summarizes all antibodies tested, quality control steps, and final selection of antibody for inclusion in the SynTOF panel.

3.2.3. Selection of metal and conjugation of metal with SynTOF panel antibody: We used stable, non-radioactive lanthanide series metals and conjugation reagents as previously described by us (Bandura et al., 2009; Bendall et al., 2012; Bendall et al., 2011). Metals and conjugation reagents were procured from Fluidigm (South San Francisco, USA). Some metal conjugates are known to provide stronger signal than others in CyTOF, and it is preferable to conjugate these with an antibodies against targets with low expression. Since the relative expression of most of our targets on synaptic terminals was not known, we performed pilot experiments to guide pairing of antibodies with metals

3.3 Quality control of SynTOF Panel

We undertook four quality control steps to ensure the sensitivity and specificity of our SynTOF panel. First, only antibodies with Western blot banding pattern unaltered by conjugation were included. Second, we used metal minus (MM) controls, in which

synaptosomes were incubated without conjugated antibody to determine background signal in the omitted channel; MM controls also were used to set gates (Figure S2 and S3). Third, isotype antibodies were obtained from the same supplier as the primary antibody whenever possible, always from the same host species and Ig subclass, and conjugated with the same metal ion and used at the same concentration as the primary antibody conjugate. Any signal overlapping with isotype control was excluded as non-specific. Lastly, we used regional variation in dopaminergic innervation between striatum and cerebellum to validate signal for antibodies to two key proteins: DAT and TH (Figure S3).

We assessed 55 antibodies (Table S1): two commercially available as metal conjugates (cluster of differentiation (CD) 56 and glial fibrillary acidic protein (GFAP)); the remainder conjugated as described by Fluidigm (South San Francisco, USA). Forty-seven conjugated antibodies passed MM quality control. Of these, 11 were excluded because they were polyclonal or repetitious with an alternate antibody that had stronger signal. Only two conjugated antibodies failed Western blot quality control because they did not give clear signals; each of the remaining conjugated antibodies gave reliable signal. Our final panel of 34 conjugated antibodies (plus 2 channels for DNA markers and 1 channel for normalization beads) included markers of (i) different brain cell types, e.g., CD56, GFAP, myelin basic protein (MBP), CD11b, CD298; (ii) pre- and postsynaptic markers, e.g., synaptosomal associated protein (SNAP) 25, CD47, and postsynaptic density (PSD) 95, as well as markers that distinguish types of synapses, e.g., serotonin transporter (SERT), dopamine transporter (DAT), tyrosine hydroxylase (TH), norepinephrine transporter (NET), vesicular glutamate transporter (VGLUT) 1, VGLUT2, and vesicular GABA transporter (VGAT); (iii) markers of injury or response to injury, e.g., light chain (LC) 3B, cleaved caspase 3, 3-nitrotyrosine, 8-hydroxyguanosine, C1q, K48-ubiquitin, and K63-ubiquitin, and (iv) our AD module: amyloid (A) β_{40} , A β_{42} , paired helical filament (PHF)-Tau, T22 (anti-tau oligomer antibody), bridging integrator (BIN) 1, Inositol Polyphosphate-5-Phosphatase D (INPP5D), Protein Tyrosine Kinase 2 Beta (PTK2B), apolipoprotein (Apo) E, ApoJ, CD33, CD36, and pTDP43. We termed these 34 conjugated antibodies as Synapse by Time Of Flight mass spectrometry (SynTOF) panel (Tables 1 and S1).

3.4. Computational analysis framework of high dimensional multiparametric data reveals synapse and marker diversity in brain

We applied well-established and reported mass cytometry bioinformatics tools to mass synaptometry (!!! INVALID CITATION !!! (Amir el et al., 2013; Anchang et al., 2016)). Figure 4A shows results of a typical SynTOF run; data consisted of >120,000 total events, of which 67% expressed high level of two pan-neuronal proteins (CD56+CD298+) to the exclusion of microglia (CD11b-) and oligodendrocytes (MBP-). Of these neuronal derived events, the vast majority (66% of total events) had high expression of presynaptic proteins (SNAP25+CD47+). Of these SNAP25+CD47+ events, 12% expressed high levels of PSD-95, consistent with ultrastructural data revealing occasional synaptosomes with connection between pre- and postsynaptic elements (Postupna et al., 2014). All subsequent analyses focused on presynaptic (SNAP25+CD47+PSD95-) events.

SPADE is a well-established computational approach to infer hierarchy among large-scale single event data, and is commonly used to analyze CyTOF data. Indeed, SPADE organizes multiplex single cell data into a hierarchy of cellular phenotypes that partially recapitulated well-described patterns of hematopoiesis (Anchang et al., 2016; Qiu et al., 2011). Figure 4B shows a SPADE analysis of all 34 SynTOF probes, color-coded for the expression of the dopamine transporter DAT (size of node indicates number of events), and includes a cluster of 10 nodes with highly restricted DAT expression (also see Figure 5A). Figure 4C shows SPADE trees for the SynTOF panel from normal caudate coded for expression of several markers proteins revealing synaptic and marker diversity in brain; no other marker demonstrated expression as restricted as DAT.

We included 10 synaptic markers (CD47, DAT, NET, PSD-95, SERT, SNAP-25, TH, VGAT, VGLUT1, and VGLUT2; listed in Table 1) in the SynTOF panel to investigate synaptic diversity as shown in Figures 4 and 5A. Our clustering analyses demonstrated unexpected individual synapse variation in the relative concentration of these markers among controls, as well as expected changes in DAT and TH between disease states. While this last finding at the individual synapse level validates tissue based assessments, our data are unique in showing diversity of DAT and TH loss among striatal synapses in LBD.

3.5. Validation by regional and disease-specific synaptic alterations

For validation, we focused on the caudate nucleus from autopsy verified age-matched individuals without clinical or pathologic evidence of brain disease (normal), or patients with clinical diagnosis of PD or DLB and high pathologic level of LBD. This patient population was chosen for validation because abundant autopsy and neuroimaging data show loss of dopaminergic terminals and DAT in LBD (Postupna et al., 2017; Postupna et al., 2014; Toulorge et al., 2016).

SPADE trees in Figure 5A are coded for DAT expression in (i) caudate and (ii) cerebral cortex (low dopaminergic innervation) from the same normal cases (regional negative control), (iii) caudates from two LBD samples, and (iv) caudates from two patients with AD (mildly impacts the dopaminergic system). SPADE trees for four normal and four LBD caudates are shown in Figure S4. Figure 5B is a violin and scatter plot showing greater DAT expression among the 26,383 presynaptic events from normal samples (n=4) compared to the 38,571 presynaptic events from LBD samples (n=4) in the DAT+ cluster (one-tailed t test, $p < 2 \times 10^{-16}$); superimposed are red box-and-whisker plots and black violin plots (green bars are medians). Figure 5C graphs the median DAT expression in the DAT+ cluster for each of the four normal and four LBD cases (bars are group medians; one-tailed t test, $p = 0.007$). We screened for additional differences between normal and LBD caudates among the SynTOF panel in the DAT+ cluster, excluding probes used for gating or those with low expression (average < 0.15) (Table S2); we observed reduced expression of VGLUT1+, TH+, DAT+, and VGAT+, and increased expression of GFAP+, cleaved caspase 3+, and 3-nitrotyrosine+ events in LBD cases compared to normal (two-tailed t test with Bonferroni-corrected $\alpha = 0.001$ ($0.05/34$), each $p < 2 \times 10^{-16}$). These findings extend, to the individual presynaptic level, discoveries made previously at the tissue and/or cellular level for LBD, i.e., dopaminergic degeneration, remodeling of glutamatergic and GABA-ergic innervation,

gliosis, pro-apoptotic signaling, and oxidative injury (Toulorge et al., 2016). Moreover, our multiplex data provide unique insight into the coincidence of these changes in DAT+ presynaptic particles rather than simply in regions of brain with abundant dopaminergic innervation but many other synapse types.

The advantages of visualizing hierarchical nodes with SPADE are balanced by the disadvantages of losing individual event data and the possibility of obscuring rare populations. In contrast, viSNE enables visualization of multiplex data on individual events in two dimensions (Amir el et al., 2013). Together, SPADE and viSNE provide a complementary approach to infer synaptic hierarchy from high-dimensional single event data. We used viSNE to plot the high dimensional data from the SynTOF panel for all human caudate presynaptic (SNAP25+CD47+PSD95-) events (Figure 5D, and Figure S5) and for the DAT+ cluster (Figures 4E and S6). The viSNE plot of all presynaptic events was similar for normal and LBD samples, and similar to DAT+ cluster events from normal samples; principal component analysis (PCA) of both normal sets ranked the top three elements as SNAP25, CD47, and CD56. The viSNE plot and PCA for presynaptic events in the DAT+ cluster for LBD was the most variable, with the top PCA rankings being BIN1, CD56, and SNAP25. These novel multiplex data suggest broader and more variable changes in the molecular composition of caudate synaptic terminals in LBD, perhaps deriving from the altered environment created by dopaminergic degeneration, reaction to injury, and/or exposure to medication.

DISCUSSION

Tissue heterogeneity has presented a significant challenge to the study of biomarkers, proteostasis, and the dissection of the regulatory programs in development and diseases. With the advent of single cell technologies, significant progress has been made in immunology and oncology in resolving tissue heterogeneity and the continuum of cellular diversity. CyTOF based technologies, in particular, have allowed the generation of single cell multi-parametric high-dimensional data. However, such approaches in neurobiology have been limited to immune cell compartments. Systematic use of CyTOF has not been reported for synaptic diversity and heterogeneity. We report mass synaptometry as a general approach, a SynTOF panel, and a computational framework that can be used to visualize and analyze high-dimensional data from single synapses. The major strength of mass synaptometry is quantitative, high level multiplexing of individual synapses without the high background inherent to fluorescence-based technologies in adult human brain. In other tissue types not limited by high autofluorescence, like immune cells, fluorescence-based approaches to high level multiplexing have revealed a striking diversity of cell types over the last few decades (Altman et al., 1996; Bendall et al., 2011; Bonner et al., 1972; Heath et al., 2016; Maecker et al., 2012; Mitra et al., 1999; Ornatsky et al., 2010; Parks et al., 1979; Silverstein, 2004; Tonegawa et al., 1974). We envision future versions of SynTOF panels will reveal rich, regional synaptic diversity in aging and disease that cannot be evaluated by other technologies.

We selected human brain regions from clinically and pathologically unaffected individuals and from patients with either LBD or AD, and used these to validate mass synaptometry by

focusing on the expected loss of dopaminergic terminals in LBD only. We deliberately assembled our SynTOF panel to validate at the single synapse level the many existing bulk tissue analyses of human synapses, including western blots, proteomics, and molecular imaging studies (Berezcki et al., 2018; Caudle et al., 2008; Datta et al., 2017; Galasko et al., 1996; Hansen et al., 1998; Jellinger, 2008; Kraybill et al., 2005; Leverenz et al., 2007; Liao et al., 2009; Mak et al., 2014; McKeith et al., 2007; McKeith et al., 2017; McKeith et al., 1996; O'Brien et al., 2004; Piggott et al., 1999; Ping et al., 2018; Revuelta et al., 2008; Samuel et al., 1997; Xia et al., 2008; Zaja-Milatovic et al., 2006; Zaja-Milatovic et al., 2005). Critically, our results localized these known bulk tissue changes to specific pre-synaptic populations, and revealed a diversity of change among affected synaptic subsets. In addition, our clustering algorithms revealed variation in expression level of proteins among subsets of individual synapses, such as synaptophysin and CD47, highlighting an unexpectedly rich pre-synaptic diversity. We stress, however, that our SynTOF panel was designed to be modular and versatile, and can be modified easily to focus on other diseases in brain regions from humans as well as rodent and non-human primate models, as we have done previously with conventional flow cytometry (Postupna et al., 2017; Postupna et al., 2014).

We used multiple markers to confirm the authenticity of events: CD56 and CD298 for neuronal; and SNAP25 and CD47 for presynaptic. One unexpected finding was the relatively low level of CD298 signal compared to CD56 signal, which may be due to lower affinity of the CD298 antibody or perhaps differences in preferential localization of CD298 to regions of neurons other than the presynaptic element. Another unexpected finding was restricted, low-level expression of CD33, classically thought of as a microglial protein. We hasten to add that another microglial antibody (CD11b) yielded no signal in presynaptic events, although there was a small positive signal in the P2 fraction (Fig S2). Together, these results may indicate internalization of some microglial proteins into synapses, or, as noted for GFAP, microglial processes may envelop or be closely associated with synapses. We expected SNAP25 to be uniformly expressed in presynaptic particles and were impressed by the variability of its expression across SPADE nodes (Figure 4A). Finally, the coincident detection of GFAP with only some pre-synaptic particles is intriguing. If this outcome was an artifact of homogenization, then why were highly variable levels detected among pre-synaptic events? GFAP can be expressed by hippocampal neurons in brain in AD (Hol et al., 2003) and one interpretation of our data is that we may have supporting evidence for caudate synapses in LBD. Another possibility is that astroglial elements are variably affixed to presynaptic particles through as yet unclear molecular mechanisms and that these are increased in caudate in LBD.

We adapted the tools of mass cytometry to analysis of synaptosomes, generated a SynTOF panel, and validated mass synaptometry against the expected loss of dopaminergic synapses in LBD. Mass synaptometry enables high-dimensional molecular profiling of human synaptic terminals. Our approach has the potential to serve as an innovative tool for discovery of synaptic changes in brain development, aging, and disease, as well as a template for molecular characterization of other subcellular vesicles at the single event level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- Mass synaptometry method for single synapse multiplexed molecular profiling.
- Synapse by Time Of Flight (SynTOF) panel of 34 antibodies for wide application.
- Optimized synaptosome preparation and CyTOF acquisition parameters.
- Proof of principle validation in 4 Lewy body disease and 2 Alzheimer's disease cases.

Figure 1. Overview of mass synaptometry and Development of mass synaptometry for multiparametric analysis of single synapses.

(A) Synaptosomes are prepared from a specific region of brain. (B) Each antibody in the antibody panel (see Table 1) is conjugated with a unique heavy metal having atomic mass ranging from 141 to 176. (C) Synaptosomes from healthy and diseased brain regions are incubated with the antibody panel. Labeled synaptosomes are then assayed by CyTOF. (D) Multi-parametric single event data can be analyzed by principal component analysis (PCA) and visualized by clustering algorithms such as Spanning-tree Progression Analysis of

Density-normalized Events (SPADE) and Barnes-Hut implementation of the t-Distributed Stochastic Neighbor Embedding (ViSNE).

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Figure 2. Detail work-flow diagram of mass synaptometry procedure.

Schematic showing the stepwise procedure with optimized experimental parameters, pause point, and critical steps

Figure 3. Filtration enriches synaptosomes from P2.

(A) Biaxial plot of P2, crude and unfiltered synaptosomes, shows high levels of DNA detected using Iridium (Ir) 191/193. Dashed oval shows P2 events with DNA signal, typical for events from nucleated cells, such as peripheral blood mononuclear cells (PBMC) (B), Platelets (small non-nucleated events) are shown for comparison (C). Biaxial plots of synaptosomes, after 5 micron (D) and 1 micron (E) cutoff filtration of P2, demonstrates the enhanced removal of nuclear artifacts. (F) Scatter plot showing average volume of platelets, P2 particles, and synaptosomes. Human platelets averaged 9.5 ± 0.4 fL ($n=33$), human P2 events averaged 9.8 ± 0.9 fL ($n=39$), and synaptosomes (5 micron filtration) averaged 7.9 ± 0.9 fL ($n=43$) ($P < 0.0001$ for size of P2 particles vs. synaptosomes). Filtration removed incompletely homogenized tissue fragments from P2 and yielded a population of particles significantly smaller in size, helping prevent clogging of CyTOF fluidics, as well as removing nuclear artifacts.

Figure 4. Mass synaptometry reveals synapse diversity.

(A) Population sunburst diagram describes the gating scheme used and shows the proportion of acquired events from normal human caudate synaptosomes. (B) Overview of population hierarchy for normal caudate pre-synaptic (SNAP25+CD47+PSD95-) events using Spanning-tree Progression Analysis of Density-normalized Events (SPADE). In SPADE, the size of each node represents the relative number of events contained in it. Here, the number of presynaptic (SNAP25+CD47+PSD95-) events per node varied from 22 to >4,500. The color of each node represents the relative level of expression across nodes in the SPADE

tree; here, color encodes relative DAT expression. The dopaminergic transporter (DAT)+ cluster (circled) is characterized further in Fig. 5. (C) SPADE tree for normal human caudate presynaptic (SNAP25+CD47+PSD95-) events showing the diversity and distribution of expression of numerous synapse and neuropathologic markers.

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Figure 5. Validation of mass synaptometry in human samples.

(A) Unsupervised organization of single event data by SPADE identified highly restricted DAT expression (DAT+ cluster) in normal caudate samples (n = 4). DAT expression in this cluster is low in the cerebral cortex (n = 3) from the same normal cases, reduced in the caudate of patients with Lewy body disease (LBD) cases (n=4), and was relatively unchanged in the caudate from Alzheimer's disease (AD) cases (n=2); two representative samples are shown for each group. We exported multiplex data from DAT+ cluster events for more in-depth analysis. (B) Scatter plot of DAT expression (log scale) by presynaptic (SNAP25+CD47+PSD95-) events in the DAT+ cluster from 4 normal (n=26383) and 4 LBD (n=38571) caudate samples ($P = < 2 \times 10^{-16}$). Superimposed boxplot (red lines) shows median DAT expression (green bar) and violin plot shows wider base for LBD compared to normal cases. LBD caudate samples had fewer synapses expressing DAT and those synapses which did express DAT, expressed it at a reduced level. (C) Scatter plot showing median DAT expression for DAT+ cluster in each of 4 normal caudate and 4 LBD caudate samples (bar is group median, $P < 0.01$). (D) viSNE maps high -dimensional data onto a two-dimensional plot while conserving the original high-dimensional structure. Each dot represents a single event. viSNE plots of all presynaptic (SNAP25+CD47+PSD95-) events from a normal case and an LBD case show no obvious derangement. (E) viSNE plot of DAT+ cluster events

from a normal caudate and LBD caudate shows broad derangement of presynaptic (SNAP25+CD47+PSD95-) events. See also Figures S4, S5, S6, and Table S2.

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Table 1.**SynTOF Panel.**

Synapse by Time of Flight (SynTOF) panel contains 34 conjugated antibodies used to probe cell type, synapse type, protein products of several AD or PD risk genes, and markers of injury/response to injury, as well as DNA and normalization controls. Included are brief descriptions of each antibody/marker. See also Table S1.

SynTOF Panel		
Category	Antibody/ Marker	Description
AD risk gene products	ApoE	Lipid and A β transport, inflammation
	BIN1	Endocytic recycling
	CD33	A β clearance, microglia activation
	CD36	Scavenger receptor
	CLU/ Clusterin (aka Apo J)	Lipid and A β transport, inflammation
	INPP5D (aka SHIP1)	Inflammation
	p-Tau (Ser202, Thr205)	Pathologic tau
	p-TDP-43	RNA and DNA binding protein, pathologic
	PTK2B (aka PYK2)	Synaptic modulation
	T22	Soluble tau oligomer
	β -Amyloid, x-40	Senile plaques
	β -Amyloid, x-42	Senile plaques
Synapse type	CD47	Presynaptic protein
	DAT	Dopamine transporter
	NET	Noradrenaline transporter
	PSD-95	Postsynaptic density protein
	SERT	Serotonin transporter
	SNAP-25	SNARE complex protein
	TH	Catecholamine biosynthesis
	VGAT	GABA transporter
	VGLUT1	Glutamate transporter 1
	VGLUT2	Glutamate transporter 2
Cell type	CD11b	Microglia
	CD298 (Na ⁺ K ⁺ ATPase)	Neuron
	CD56 (aka NCAM)	Neuron
	GFAP	Astrocytes
	MBP	Oligodendrocytes
Injury and response	3-Nitrotyrosine	Oxidative damage
	8-OH-Guanosine	Oxidative damage
	C1q	Aging, pruning and inflammation
	Caspase 3, cleaved Asp175	Apoptosis
	K48-ubiquitin	Proteasome
	K63-ubiquitin	Endocytic trafficking, inflammation

SynTOF Panel		
Category	Antibody/ Marker	Description
	LC3B	Autophagy
DNA Control/QC	Cell-ID Intercalator/ DNA I	Pan nuclear marker
	Cell-ID Intercalator/ DNA II	Pan nuclear marker
Normalizing control	Beads	Calibration

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