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Flow cytometric evaluation of crude synaptosome preparation as a way to study synaptic alteration in neurodegenerative diseases

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Abstract

Neurodegenerative diseases, the most common among them Alzheimer's disease (AD) and Lewy body disease (LBD), are a group of progressive incurable illnesses. In both AD and LBD, abundant evidence points to the synapse as the critical and early focus of pathological changes. Here we present a method for the isolation and flow cytometric analysis of synaptosomes prepared from postmortem human brain tissue, which we also applied to animal models, including mice and nonhuman primates. The use of flow cytometry for analysis allows for relatively fast and efficient examination of thousands of synaptosome particles in a matter of minutes, and also makes it possible to use crude, rather than purified, synaptosomal preparation, thus conserving tissue resources. We have applied this method to study synaptic alteration in several brain regions in human research participants and animal models.

Keywords

flow cytometry; human neurodegeneration; postmortem; Alzheimer's; amyloid beta

1. INTRODUCTION

1.1. Neuro-degenerative Diseases

Neurodegenerative diseases are a heterogeneous group of debilitating illnesses that result in progressive degeneration of nerve cells. They affect millions of people worldwide, and no effective cure is available [1].

The most prevalent neurodegenerative disease is Alzheimer's disease (AD), which is also the most common cause of dementia in the elderly, affecting more than 40 million people worldwide (Alzheimer's Association, 2015). Pathological features of AD include neuronal loss, and two characteristic lesions: neuritic plaques, which predominantly accumulate fibrillary forms of amyloid beta (Aβ) peptides, and neurofibrillary tangles (NFTs), which are

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largely composed of a hyperphosphorylated form of a microtubule-associated protein τ (paired-helical filament-τ, or PHF-τ) [2].

Another widespread neurodegenerative disease is Lewy body disease (LBD), the second most common cause of dementia in the elderly, which includes dementia with Lewy bodies (DLB) and Parkinson's disease (PD). Pathological features of LBD include neuronal loss, and the formation of the Lewy bodies and Lewy neurites, intracellular protein inclusions, that can be seen in a portion of the surviving cells [3]. A principal component of these inclusions is α-synuclein, a protein that is ubiquitously expressed in neurons, but principally localized to the synaptic region [4, 5].

1.2. Synaptic Dysfunction Is Key to Pathophysiology of AD and LBD

Although neuronal loss and characteristic lesions are the most pronounced features of both AD and LBD, mounting evidence suggests that synaptic dysfunction and synapse loss are early events driving the pathologic process. In 2003, Li et al. [6] introduced the term "synaptopathy" and suggested that synaptic dysfunction may be the critical first step in the pathophysiology of several neurodegenerative diseases that have been traditionally explained by loss of neurons. Li and colleagues used this term to describe changes in the brain of patients with Huntington's disease, but the notion has been since extended to other diseases, such as AD and LBD.

There is evidence to suggest that in AD, synaptic dysfunction may preclude neuronal loss by many years or even decades [7, 8]. Both AD and mild cognitive impairment (MCI), which often precede AD, are characterized by significant synapse loss in frontal and temporal cortex, and also the dentate gyrus of hippocampus [9, 10, 11, 12, 13]. Moreover, there is a significant correlation between synapse loss and cognition in both AD and MCI [10, 14], with cortical synaptic density showing better correlation with psychometric indices than plaques and tangles [13].

Despite mounting evidence that synaptic dysfunction is integral to AD pathophysiology, and that it occurs early in the disease process, the exact mechanism leading to impaired synaptic function in AD is not known. In general, synaptic dysfunction can be caused by molecular alterations that begin inside the pre- or postsynaptic neuron or that result from pathological changes in the environment surrounding the synapse. It has been shown that loss of synapses in AD is most pronounced in the proximity of Aβ-containing plaques [15], and that many neural processes around plaques are dystrophic and contain aggregates of PHF-τ [16, 17, 18, 19, 20, 21]. It has also been demonstrated in vitro and in animal models that Aβ, both in fibrillar and oligomeric form, is toxic to neurons and can induce synaptic dysfunction [22], such as loss of dendritic spines, alteration of electrophysiological properties [23, 24, 25], and impaired memory formation [26, 27]. Oligomeric forms of Aβ have been shown to be significantly more toxic to synaptic function than fibrils [28, 29]. It is currently unclear whether Aβ's effect on the synapse is mediated by its interaction with a specific receptor [7]. Aβ is known to bind to many receptors, including NMDA, metabotropic glutamate receptors, and nicotinic acetylcholine receptors [30], but the relative importance of these interactions in the synaptic effects of Aβ has not been established.

The relationship between PHF-τ-containing neurofibrillary tangles and impairment in synaptic function is less clear. It has been demonstrated that in the brain of AD patients, surviving neurons containing neurofibrillary tangles receive fewer synapses onto their somata and express less synaptic proteins than neighboring neurons [31, 32, 33]. The most likely mechanism of PHF-τ toxicity is disruption in microtubule-associated cellular transport, which impairs transport of mitochondria and other essential cargo to pre- and postsynaptic terminals [34, 35]. In addition, recent evidence suggests that the effects of Aβ and PHF-τ at the synapse are most likely synergistic [36, 37, 38, 39, 40].

In PD and other disorders with Lewy body pathology, accumulating pathological forms of α-synuclein appear to be central to the synaptic dysfunction. The normal function of α-synuclein is not completely understood, but it has been demonstrated to be involved in regulating neurotransmitter release at the presynaptic terminal [4, 41]. α-Synuclein can undergo conformational changes to form oligomers, protofibrils, and fibrils, all of which can accumulate in cells [42, 43] and induce synaptic dysregulation [5, 44]. Results from in vitro and in vivo studies in rodents suggest that pathological forms of α-synuclein, such as oligomers or protofibrils, affect protein degradation, impair vesicle trafficking at the synapse, and compromise long-term potentiation [45, 46, 47]. In dopaminergic neurons, fibrillar α-synuclein can bind to and permeabilize the membrane of dopamine-containing synaptic vesicles, spilling dopamine into the cytosol [48, 49], which in turn can lead to oxidative stress and neurotoxicity [50, 51, 52]. In addition to α-synuclein, other proteins implicated in the pathogenesis of PD, such as parkin and LRRK2, have also recently been shown to impair synaptic vesicle recycling and autophagy, providing additional evidence for synaptic dysfunction as an early event in the disease process [53].

1.3. Challenges to Studies of Human Synapses

It appears that in both AD and LBD, the pathological process either begins at the synapse, or, at the very least, synaptic structure and function become affected very early in the course of the disease. Therefore, developing a better understanding of the pathological process at the synaptic level could provide important insights into the development of early therapeutic interventions. Our current understanding of mechanisms of synaptic dysregulation in AD and LBD came from in vitro studies and animal models, which can never fully re-create human disease process. But studying synapses in human brain tissue can be challenging. Traditional histochemical techniques were perfected for studying whole cells, and are not well suited for visualization of single-nerve terminals. High-resolution microscopy technique allows detailed examination of fine synaptic architecture, and can show association of pathological elements with synaptic structures, but is labor intensive and not practical for studying multiple brain regions in large-scale research cohorts [54]. Methods that involve quantifying proteins in tissue homogenates, such as Western blotting, allow for precise quantification of synaptic proteins, physiological and pathological, but don’t distinguish between proteins that are present at the synapse and the ones that are ultimately destined for the synapse, but are not there yet. In short, all of these methods have their limitations, with the general trade-off between quantification, precise localization of the pathological changes, and scalability.

1.4. Using Flow Cytometry to Study Synaptosomes

Preparing synaptosomes from brain tissue homogenates is a well-established technique, which has been extensively used in animal studies of synaptic physiology [55, 56]. Synaptosomes obtained from postmortem human tissue are not generally suitable for functional studies, because of their compromised electrochemical integrity, but can be a great resource for testing hypotheses about molecular alterations in human synapses.

A recent approach, pioneered by Karen Gylys's group [57], combines synaptosome preparations with flow cytometry methodologies in order to study synaptic alterations and associations of synapse subtypes with Aβ and tau species in AD in human subjects and animal models [57, 58, 59, 60, 61].

To better understand synaptic changes that occur in cerebral cortex and striatum in neurodegeneration, especially AD and LBD, we have adapted the method developed by Gylys and colleagues to probe the molecular composition of synaptic particles in multiple regions of human brain in several disease groups [62, 63]. Because the previous studies by both groups used autopsy material obtained from research cohorts, they may present a somewhat skewed view of the development and type of synaptic injury that occurs with aging. In addition, the effect of postmortem interval (PMI), unavoidable in human studies, may have systematically affected the changes that were observed. To address these issues, we examined the molecular composition of synaptosomal preparations obtained from several brain regions from aged nonhuman primates with no PMI (sacrificed as part of a separate study). We have also effectively used this method to study synaptic changes in transgenic mouse models, such as a model of dopaminergic degeneration [62] and mice with human *ApoE ε3* or *ApoE ε4* alleles [63].

2. MATERIALS AND METHODS

Our protocol for analyzing synaptosome preparation flow cytometry [62] was adapted from Gylys et al. [57] and includes the following steps:

1. Tissue collection (and storage if needed)
2. Preparation of the crude synaptosome fraction (and storage if needed)
3. Immunolabeling of the crude synaptosome fraction
4. Flow cytometry analysis of the labeled synaptosome fraction

2.1. Tissue Collection

Our protocol has been established for human tissue samples that are obtained at autopsy or during surgery, and animal tissue samples that are collected immediately after sacrifice. At autopsy, we aim for collecting approximately 0.5 g of tissue from each structure of interest, which provides enough material to be later divided into 20 experimental samples. We only collect tissue from regions that are free of macroscopic lesions (hemorrhage, etc.). For human cerebral cortex samples we focus on collecting gray matter. To achieve that, we dissect out cortical gray matter with about 1 mm of underlying white matter from a 5 mm thick coronal section taken from the region of interest. For human hippocampal samples, a

coronal section containing the hippocampus at the level of lateral geniculate nucleus is taken, and the whole hippocampus (dentate gyrus and cornu ammonis) is dissected out in one piece. Basal ganglia structures (putamen and head of the caudate nucleus) are collected from coronal slices at the level of anterior commissure. We carefully remove white matter surrounding the nuclei, to the extent that it can be done without compromising the structure of interest. For the caudate nucleus and putamen, it is usually necessary to use two adjacent coronal sections to provide enough material, because caudate nucleus samples are divided into equal dorsolateral and ventromedial subregions.

Animal tissue samples are obtained immediately after sacrifice. At collection, mouse cortex is split into anterior and posterior parts, striatum is collected as one piece, and hippocampus is also collected as one piece.

Upon collection, all tissue samples are placed in Petri dishes, and minced into smaller (approximately 2 mm × 2 mm × 2 mm) pieces using two razor blades. The samples are then transferred to cryotube vials and frozen in 1 mL of 0.32 M sucrose with 10% DMSO using a "Mr. Frosty™" freezing container (Sigma-Aldrich, MO), which is designed to achieve an optimal cooling rate of ~−1 °C/min, and later stored at −80 °C. We only collect human tissue from cases with a PMI of 8 h or less. To test if a PMI of this length resulted in significant degradation of synaptic material, we prepared synaptosomes from human cortex resected during surgery for intractable temporal lobe epilepsy, and labeled them with the antibody against glutamate transporter VGLUT1 [62]. The percentage of VGLUT1-positive particles in the surgically resected samples were not significantly different from those obtained at autopsy. In addition, we recently [63] compared relative levels of synaptosomes positive for several different markers—SNAP-25 (a component of the SNARE complex), VGLUT1 and VGLUT2 (markers for glutamatergic synapses), VGAT (vesicular GABA transporter, a marker for GABAergic synapses), SERT (serotonin transporter, a marker for serotoninergic synapses), $A\beta_{42}$, and PHF-τ—between autopsy samples (PMI ≤8 h) of human frontal cortex and striatum and samples from aged nonhuman primates (euthanized for a different study) immediately after sacrifice. The levels of SNAP-25, VGLUT1, VGLUT2, SERT, Aβ42, and PHF-τ were not decreased in the human samples, although levels of VGAT were lower in human frontal cortex (but not striatum), allowing us to conclude that a PMI of 8 h or less is unlikely to elicit enough degradation of synaptic material to significantly affect our results. We also concluded that agonal events, unavoidable in human cases, but eliminated in the monkey samples, did not have a significant effect on preparation. Moreover, longer PMIs may be acceptable under certain circumstances. In wild-type mice, we tested the effect of longer (12, 18, and 24 h) PMIs on VGLUT1, VGAT, and DAT level in cortex and striatum and found that levels of these proteins changed significantly only in the tissue collected 24 h after euthanasia.

2.2. Preparation of the Crude Synaptosome Fraction

Our protocol produces a crude synaptosome fraction—obtained from minced tissue samples by homogenization and a series of centrifugations, but not gradient purification. Although gradient purification is undoubtedly beneficial for removing non-synaptosome material, it

significantly reduces the overall yield. Instead, we use a strategy of gating on particle size during flow cytometry to restrict our analysis to particles of the correct size (Fig. 1).

To obtain this crude fraction, the samples are thawed by shaking in a 37 °C water bath for approximately 2 min. The contents of the tube are then added to 10 volumes of 0.32 M sucrose in 10 mM Tris buffer (pH 7.4) with cOmplete™ protease inhibitor cocktail (Roche, IN) and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich, MO). Tissue is then homogenized using a glass/Teflon homogenizer (clearance 0.1–0.15 mm) with 6 to 8 up-and-down strokes. The homogenate is transferred to a 15 mL conical tube and centrifuged at 1000 × *g* at 4 °C for 10 min. The supernatant is then collected, taking extra care to avoid collecting any pellet material, transferred into 1.75 mL centrifuge tubes (20 tubes for one 0.5 × *g* sample), and centrifuged again at 10,000 × *g* at 4 °C for 20 min. The resulting pellets are resuspended in 200 μL of a sucrose/Tris solution (to facilitate resuspension after thawing) and stored at −80 °C for future use.

2.3. Immunolabeling of the Crude Synaptosome Fraction

The synaptosome pellets are thawed by shaking the tubes in a 37 °C degree water bath. The pellets are then washed by adding 1 mL of Krebs-Ringer phosphate buffer (KRB, 118 mM NaCl, 5 mM KCl, 4 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM KH_2PO_4, 16 mM sodium phosphate buffer, and 10 mM glucose), and centrifuging at 8000 × *g* for 4 min at 4 °C. For each future incubation or wash, the synaptosome pellets are resuspended by gentle vortexing for about 5 s, and then collected by centrifugation (5000 × *g* for 4 min at 4 °C). After two washes with KRB, the pellets are fixed with 0.25% paraformaldehyde (PFA). To facilitate resuspension after fixation, the pellets are first resuspended in 200 μL phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4, 1.8 mM KH_2PO_4, pH 7.4), to which 250 μL 0.5% PFA solution is then added. After fixation with PFA for 1 h at 4 °C on a rocker, another 1 mL of PBS is added to the tubes, to further decrease the final concentration of PFA and facilitate further resuspension. The pellets are then collected by centrifugation and washed with 1 mL PBS. For labeling of intracellular antigens, the synaptosomal membranes are then permeabilized by incubation with 1 mL of 0.2% Tween 20 in PBS for 15 min at 37 °C. The permeabilization step can be skipped if the antigen of interest is located extracellularly. After permeabilization, the pellets are washed twice with PBS, and then with 500 μL of blocking buffer (2% fetal calf serum in PBS). Afterwards, the pellets are incubated with the appropriate primary antibodies—either specific to the antigen of interest or isotype control antibodies—in blocking buffer for 30 min at room temperature on a rocker. For each experimental sample labeled with a primary antibody selective to the protein of interest, an identical sample, processed simultaneously, is labeled with the respective isotype control antibody, conjugated to the same fluorophore as the primary antibody (when applicable), for use as a negative control during flow cytometric evaluation. After incubation, the pellets are washed with 1 mL 0.2% Tween 20 in PBS. If the primary antibody and the respective isotype control antibody were conjugated to a fluorophore, the pellets are washed again with 1 mL PBL, and resuspended in 1 mL PBS for flow cytometric analysis. When labeling with an unconjugated primary antibody (and isotype control antibody), the pellets are washed once more with 1 mL of 0.2% Tween 20 in PBS, and then incubated with 500 μL of the respective fluorophore-conjugated secondary antibody in

blocking buffer for 30 min at room temperature on a rocker. The pellets are then washed once with 1 mL of 0.2% Tween 20 in PBS, and once with 1 mL PBS, and resuspended in 1 mL PBS for flow cytometric analysis.

When more than one primary antibody is used, all of the primary antibodies are combined for the first incubation, and all of the secondary (when applicable) antibodies are combined for the second incubation. When a combination of conjugated and unconjugated primary antibodies is used, the fluorophore-conjugated primary antibodies are added to the fluorophore-conjugated secondary antibodies and used in the second incubation.

For experiments involving several fluorophores, a set of several synaptosome samples, all prepared from the same tissue sample, are used to estimate compensation values for overlapping fluorophores (e.g., FITC and PE). One subset of these samples is individually labeled with only one fluorescent primary antibody (or antibody pair if using unconjugated primary and fluorophore-conjugated secondary antibody) or an isotype control antibody, and another set of samples are labeled with the combination of all the antibodies of interest or isotype control antibodies involved.

2.4 Flow Cytometry Analysis of the Immunolabeled Synaptosome Fraction

The synaptosome suspension is then analyzed using an Apogee A50 (Apogee Flow Systems, Hertfordshire, UK) flow cytometer equipped with 15 mW 488 nm (blue) and 633 nm (red) lasers. For our initial experiments, we used a BD FACSCalibur Flow cytometer (BD Biosciences, CA) equipped with the same combination of lasers. The results obtained with the two machines were not significantly different, but Apogee A50, unlike most other flow cytometers, was developed specifically for the analysis of smaller particles and is thought to provide better discrimination for synaptosomes.

Because our synaptosomal preparation is not gradient-purified, it is likely to contain some large non-synaptosomal elements (such as pieces of myelin, parts of nuclei, extracellular matrix, membranes). To focus our analysis on the population that we believe to be mostly synaptosomal, we use gating on size (Fig. 1A). We use polystyrene (Polysciences Inc., PA) and silica (Spherotech Inc., IL) microspheres of varying diameter (0.5, 0.6, and 1.2 μm) as size standards to restrict all our analysis to particles that are approximately 1 μm. Because the refractory indices of both silica and polystyrene are significantly different from the refractory index of cell-derived particles, synthetic beads of 0.5 μm are expected to scatter approximately as much light as a 1.0 μm synaptosome [64]. Therefore, we use 0.5 and 0.6 μm beads to guide the placement of our size gate. Our size-gated population is then gated again based on the fluorescent signal of interest. This second gate is set on the negative control sample, which is the sample identical to the experimental sample but labeled with the respective isotype control antibody (Fig. 1B and 1C). This gate is drawn to include the most positive 1 ± 0.1% of the negative (isotype control) sample. The gate is then applied to the experimental sample, and the resulting percentage of positive particles is reported. We found that drawing the positive gate to include 1 ± 0.1% of the total size-gated particle population offers optimal discrimination between the positive and negative sample and provides the best reproducibility in repeated experiments. In our previous studies, we also

used a gate of 5 ± 0.1%. Others have previously reported using a similar or more inclusive size gate [65].

2.5. Select Applications

We have previously demonstrated the effectiveness of this technique by looking at the percentage of synaptic particles positive for dopamine transporter (DAT) labeling in the striatum of mice that underwent a toxin-mediated ablation of DAT-containing neurons [62]. Figure 2A shows unpublished data from a recent study in which we used flow cytometry analysis to compare synaptic DAT expression between two models of striatal dopamine loss: DAT-neuron-lesioned mice, in which DAT-expressing neurons are ablated [66], and a tyrosine hydroxylase (*Th*) knockout strain, in which dopamine is lost, but DAT-expressing neurons are spared [67]. Mice with lesioned DAT neurons had a significantly ($p < 0.01$) decreased percentage of DAT-positive particles in striatal synaptosomal preparation (2%), compared to controls (~50%) and to tyrosine hydroxylase knockout mice (40%).

We have also examined synaptosome preparations from postmortem human cortical samples for evidence of synaptosomal accumulation of Aβ [62]. We reported that samples from patients with the highest neuropathological AD burden, as assessed by either Braak stage (Braak stages V–VI), CERAD score (3, or “Frequent”), or Thal phase (Thal phases 4–5), had significantly more Aβ-positive particles than samples with the lowest neuropathologic burden of AD (each $p < 0.05$).

Finally, we used a combination of several antibodies to determine if pathological proteins, such as $A\beta_{42}$ and PHF-τ, co-localize with specific types of synapses, and whether the degree of co-labeling is affected by disease state. As shown in Fig. 2B and 2C, synaptosomes originating from cortical glutamatergic terminals expressing VGLUT1 also show positive signal for $A\beta_{42}$ and PHF-τ; this signal is more pronounced in the AD group.

3. CONCLUSIONS AND FUTURE DIRECTIONS

Synaptic pathophysiology is notoriously difficult to study in humans. At the same time, mounting evidence supports a crucial role for synaptic dysfunction in multiple neurodegenerative diseases, among them AD and LBD. Because subtle changes in synaptic function appear to happen early in the disease process, unravelling mechanisms underlying synaptic changes may offer new perspectives for early treatment of these debilitating diseases for which there is currently no cure. Available animal models of AD and LBD, although undoubtedly valuable, fail to fully recapitulate the disease process, making studies in human tissue a necessity.

We and others have previously demonstrated the utility of crude synaptosome preparation obtained from postmortem human brain tissue for studies of synaptic alteration in AD and LBD. Using flow cytometry to analyze the crude fraction makes it possible to omit the gradient purification step, thus significantly decreasing preparation time and, most importantly, conserving valuable tissue resources. Another important benefit of using flow cytometry is its high throughput, which allows for analysis of tens of thousands of particles in a matter of minutes, and the ability to multiplex with several fluorophores.

In addition to human postmortem samples, we have also analyzed synaptosome preparation from nonhuman primates and human surgical specimens, and concluded that the effect of postmortem interval and agonal state, unavoidable in studies involving autopsy tissue, does not significantly influence the results, at least for measurements of SNAP-25, VGLUT1, VGLUT2, SERT, Aβ, and PHF-τ.

One exciting future direction for this method is to use another flow cytometry technique, fluorescent-activated cell sorting (FACS), to isolate synaptosomal particles based on a combination of several characteristics, such as the neuron of origin or association with a particular pathological protein, and use the sorted population for molecular and genetic studies to answer questions about the relative susceptibility of different neuronal populations to pathological processes. Another is to use bead-based immunological isolation techniques to enrich synaptosomes and use the enriched population for molecular and genetic studies.

In summary, applying the techniques of flow cytometry to synaptosomal preparation provides an efficient tool for assessing relative abundance of different types of synapses in multiple regions of human and animal brain, as well as the degree of association of pathological proteins, such as Aβ, PHF-τ, and α-synuclein, with specific nerve terminals.

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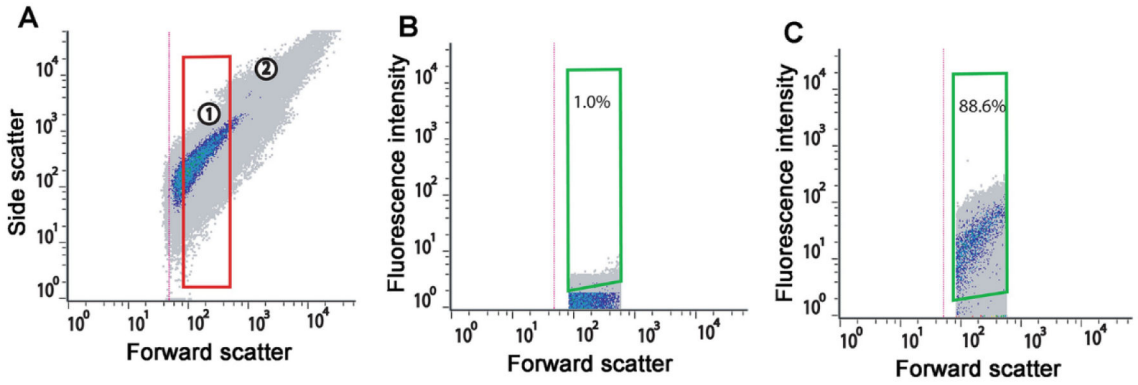

Figure 1. Flow cytometry of a synaptosomal preparation of human frontal cortex.

(A) Size gating of particles using silica bead standards (numbered circles): 1–0.6 μm beads, 2–1.2 μm beads. The size gate for synaptosomes is drawn in red. **(B)** Isotype control antibody labeling of preparations. The gate (drawn in green) is set to include 1 ± 0.1% of all size-gated positive particles in the isotype control sample, and then applied to the sample labeled with SNAP-25 antibody. **(C)** SNAP-25 antibody labeling of samples. Indicated is the percentage of positive particles within the gate (green).

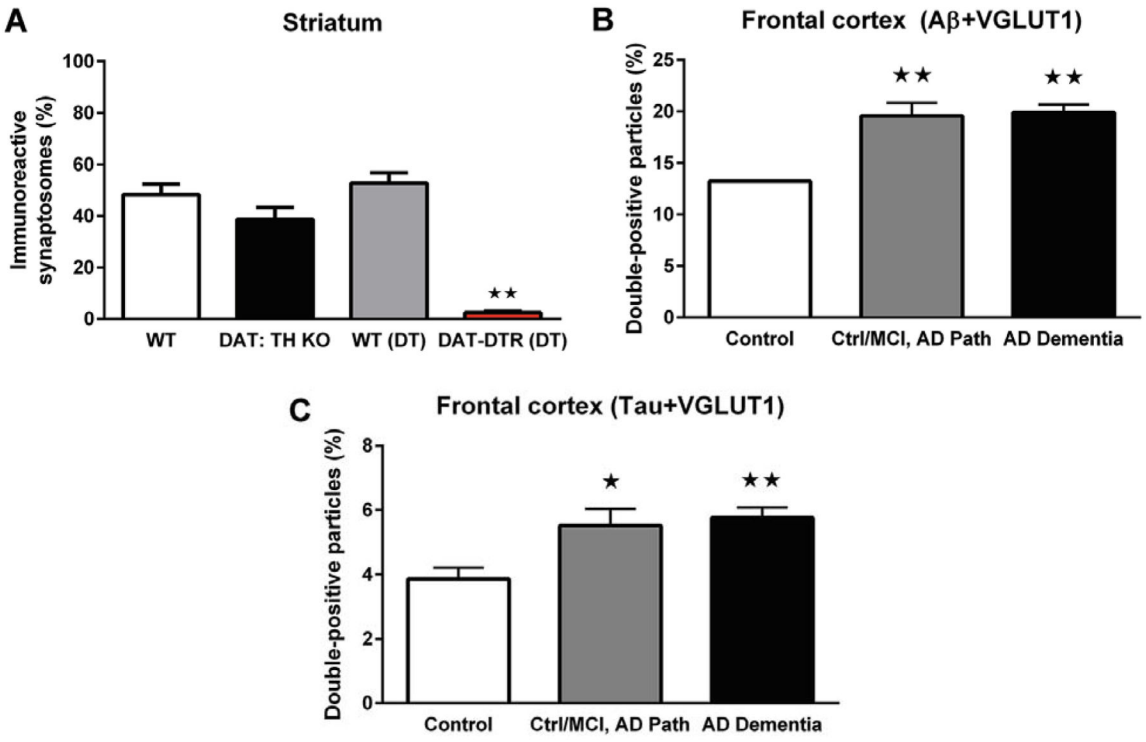

Figure 2. Flow cytometry analysis of synaptosomal preparations from mouse and human brain tissue.

(A) Percentage of synaptosomal preparations from mouse striatum immunolabeled with antibody against DAT. Tissue was collected from mice with tyrosine hydroxylase (TH) inactivation in dopamine active transporter (DAT) neurons (DAT: TH KO), mice with diphtheria toxin (DTR)-induced loss of DAT neurons (DAT-DTR), and respective wild-type (WT) control mice. Loss of DAT neurons in DAT-DTR mice resulted in significantly (by ANOVA and post hoc) reduced levels of DAT-positive synaptosomes, while the number of DAT-positive synaptic particles in TH inactivation mice was not significantly different from WT mice. **(B** and **C)** Percentage of synaptosomal preparations from human cortex co-immunolabeled with antibodies against (b) Aβ and VGLUT1, and (c) PHF-τ and VGLUT1. Cortical tissue was collected from individuals with no dementia and only low levels of AD pathology (control), MCI or normal cognition and moderate-to-high levels of AD pathology (Ctrl/MCI, AD Path), or clinical diagnosis of dementia and a high level of AD pathology (AD dementia). The number of double-positive particles was significantly higher in the AD dementia and the Ctrl/MCI, AD Path group than the control group (each by ANOVA and post hoc). Data are shown as group means ± standard error of mean. *$p < 0.05$ and **$p < 0.01$