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Enrichment of Presynaptic and Postsynaptic Markers by Size-Based Gating Analysis of Synaptosome Preparations From Rat and Human Cortex

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Background: Synapse regions in the brain are difficult to isolate and study; resealed nerve terminals (synaptosomes) are a widely used in vitro system for the study of neurotransmission, but nonsynaptosomal elements in the homogenate complicate data interpretation. With the goal of quantitative analysis of pathways leading to synapse loss in neurodegenerative disease, we have developed a method that allows focus on the intact synaptosomes within a crude synaptosomal preparation by gating the largest particles based on forward angle light scatter (FSC).

Methods: Crude synaptosomal fractions (P-2) were prepared and labeled with a viability dye (calcein AM), a presynaptic marker (SNAP-25), and a postsynaptic marker (PSD-95). Forward scatter gates based on size standards were drawn to identify the large population (1.4–4.5 μm), and the enrichment of each marker was quantified in preparations from fresh rat homogenates and from cryopreserved human cortex.

Results: Gating on forward scatter resulted in an increase that was highly significant ($P < 0.001$) for all three markers examined. The calcein-AM–positive fraction in the large synaptosomes was 98% ± 0.8, and 75% ± 9.8 for rat and human, respectively. Of large particles, 90% ± 2.7 in rat and 82% ± 2.6 in human were positive for SNAP-25, indicating a relatively pure population of intact synaptosomes. A total of 76% ± 2.9 of the large particles were positive for PSD-95 in rat. This compared to 36% ± 3.0 in human tissue, and indicates that both presynaptic and postsynaptic elements may be analyzed with this methodology.

Conclusions: Most nonsynaptosomal elements can be excluded and the intact subpopulation of interest within the P-2 can be identified based on size. Size-based gating analysis provides a simple and cost-effective method to monitor fluorescence changes in synapse regions. © 2004 Wiley-Liss, Inc.

Key terms: synaptosome; SNAP-25; PSD-95; calcein AM; Alzheimer's disease; flow cytometry

A model system of resealed nerve terminals, or synaptosomes, was first described in 1960 (1); since that time, this in vitro preparation has been widely used to examine neurotransmitter synthesis and release. When brain tissue is homogenized in sucrose, the neck of the axon terminal pinches off and the terminal regions reseal into functional spheres that contain mitochondria and possess a transmembrane potential. The synaptosome preparation has enabled detailed study of the brain's functional unit, the synapse, which is technically difficult to isolate and examine. Synaptosomes are considered a primarily presynaptic preparation (2), consistent with the vesicle-filled profiles most commonly observed using EM (3,4). However, the postsynaptic element may remain attached opposite the postsynaptic density as an intact "neurosome" (2,5).

Synaptosomes are usually purified by centrifugation on a sucrose or Percoll density gradient, although many studies have used the crude synaptosomal pellet (P-2) because of its faster preparation time and higher yield. Interpretation of data has historically been complicated by nonsynaptosomal elements, and electron microscopy studies confirm significant amounts of free mitochondria, myelin, and membranous fragments within both the P-2 and purified synaptosomes (4,6). With the aim of studying mechanisms underlying synapse loss in Alzheimer's disease, we have been interested in the advantages offered by the use of the crude synaptosomal pellet (P-2), particularly the improved function that results from the shorter preparation time.

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Our initial characterization of the P-2 fraction by flow cytometry demonstrated that, of the total number of particles, 66% were positive for a synaptic marker found only in neurons, SNAP-25 (7). This compares to 84% for flow cytometric analysis of biochemically purified synaptosomes (8). Light scattering analysis for purified synaptosomes and the P-2 fraction reveal particles ranging from 0.2 to 5 μm based on size standards, with the greatest density being small particles less than 1 μm in size. This is consistent with EM observations, in which synaptosomes containing mitochondria are surrounded by free mitochondria, vesicles, and membranous fragments.

Because the largest particles in the P-2 fraction represent the population of interest, we reasoned that gating based on particle size would result in focus on the population of interest and remove the need for biochemical purification. We have examined a viability marker (calcein AM), and neuronal markers for presynaptic (SNAP-25) and postsynaptic (PSD-95) elements in two preparations: a P-2 from freshly homogenized rat brain, and a P-2 from cryopreserved human cortex. In the present experiments, size-based gating analysis (from ≈1–5 μm) resulted in synaptosomal enrichment superior to biochemical purification, and revealed that a postsynaptic component is detected on a majority of the synaptosomes in the size-gated population.

MATERIALS AND METHODS

Materials

Calcein acetoxymethyl ester (calcein AM) was purchased from Molecular Probes (San Diego, CA), and mouse $IgG_{1\alpha}$ was purchased from Pharmingen (La Jolla, CA). Anti-SNAP-25 antibody was purchased from Sternberger Monoclonals Inc. (Lutherville, MD). The monoclonal anti-PSD 95 antibody was obtained from Upstate Biotechnology (Lake Placid, NY). Polystyrene microsphere size standards were purchased from Polysciences, Inc. (Warrington, PA).

P-2 Preparation

Human A7 and A9 cortex samples (aged normal controls, 69–91 years of age) were obtained from the University of Southern California (USC; Los Angeles, CA) Alzheimer's disease centers. Samples approximately 1 gm each, were frozen in 10% DMSO 0.32 M sucrose until homogenization. Human and rat samples were obtained under approvals issued by the Office for Protection of Research Subjects (OPRS) at UCLA. The P-2 fraction was prepared as described previously (9); briefly, adult male Sprague-Dawley rats were decapitated and brains (minus cerebellum) were rapidly removed and placed in 10 volumes of ice-cold 0.32 M sucrose in 10 mM Tris buffer (pH 7.4) with protease inhibitors (2 mM ethylene glycol-bis[β-aminoethyl ether]N,N,N′N′-tetraacetic acid (EGTA), 2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 4 μg/ml leupeptin, 4 μg/ml pepstatin, 5 μg/ml aprotinin, and 20 μg/ml trypsin I). The tissue was homogenized in a Teflon/glass homogenizer (clearance 0.1–0.15 mm) by eight gentle up and down strokes at 800 rpm. The homogenate was spun at 1,000 *g* for 10 min to remove nuclei and cell debris. The resulting supernatant was centrifuged at 10,000 *g* for 20 min to obtain the crude synaptosomal pellet or P-2. The final pellet was washed with 2 ml of Krebs-Ringer phosphate buffer (KRP; 118 mM NaCl, 5 mM KCl, 4 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM KH_2PO_4, 16 mM sodium phosphate buffer [pH 7.4], and 10 mM glucose) and centrifuged (4 min, 2000 *g*, 4°C) before resuspension in KRP for incubations.

Dye Labeling

Calcein AM (final concentration 0.02 nM) was diluted in KRP buffer and added to 5–10 μl aliquots of P-2 (total volume 0.1 ml), incubated for 10 min at 4°C, then diluted in 0.5 ml PBS for immediate flow cytometry analysis.

Immunolabeling of P-2 Fraction

P-2 aliquots were immunolabeled for flow cytometry analysis using a previously published method for staining of intracellular antigens (10). Pellets were fixed in 0.25% buffered paraformaldehyde (1 h, 4°C) and permeabilized in 0.2% Tween20/PBS (15 min, 37°C). The monoclonal antibodies were diluted 1:1,000 in 2% FBS/PBS. Following a 30 min incubation with primary antibody at 4°C, pellets were washed two times with 1 ml 0.2% Tween20/PBS, then incubated (20 min, 4°C) with secondary antibody (FITC-conjugated anti-mouse IgG) followed by two additional washes before resuspension in KRP buffer for flow cytometry analysis.

Flow Cytometry

Samples of rat preparations were acquired on a FACScan flow cytometer, and samples of human preparations were acquired on a FACSCalibur (both from Becton-Dickinson, San Jose, CA); each type of preparation was always run on the same instrument to improve data consistency between experiments performed on different days. On both instruments forward angle light scatter (forward scatter, FSC), side scatter (SSC), and particle fluorescence were analyzed with logarithmic amplification; polystyrene beads (0.75, 1.4, and 4.5 μm) were run on the instrument to standardize forward scatter measurements. Green calcein AM fluorescence was collected through a 530 ± 30 nm filter. Sample flow rate was kept constant at approximately 3,000 events per second; 20,000–50,000 events were collected from each sample for analysis. A threshold was set on forward scatter (channel 42) to exclude debris. Analysis of the data was performed with FCS Express software (DeNovo Software, Ontario, Canada). Numeric results are expressed as mean ± SEM, and a Student's *t*-test for independent observations was used for all comparisons.

RESULTS

Size Distribution of Calcein-AM Positive Particles in P-2 Fractions from Rat and Human Brain

The synaptosomal population of interest consists of neuronal terminals that have resealed into a functional

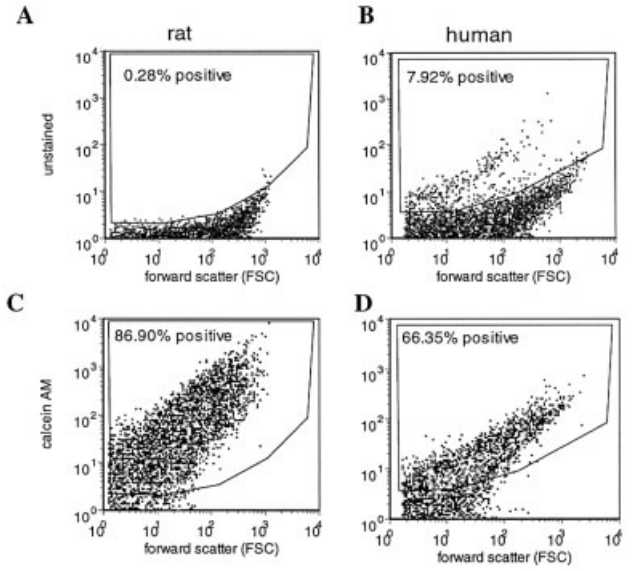

FIG. 1. Flow cytometric analysis of the viability dye calcein AM (0.02 nM). Representative plots are shown for rat (**A**,**C**) and human (**B**,**D**) P-2 fractions. The data analysis gate is drawn to include specific labeling above background; % positive indicates percent of total particles specifically labeled for calcein AM. See Results for data presented as mean ± SEM (n = 6).

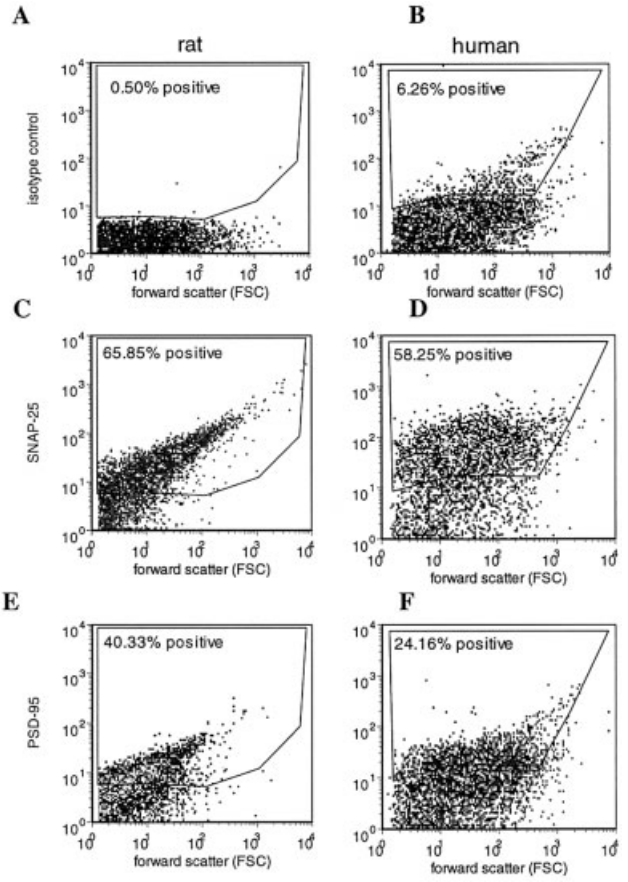

FIG. 2. Flow cytometric analysis of rat and human P-2 fractions immunolabeled for synaptic markers. The data analysis gate is drawn to include specific labeling above background with isotype control (**A**,**B**); % positive indicates the positive fraction for representative plots of the presynaptic marker SNAP-25 (**C**,**D**) and the postsynaptic marker PSD-95 (**E**,**F**). See Results for data presented as mean ± SEM (n = 6).

sphere. In order to distinguish synaptosomes from membrane fragments and myelin, the viability dye calcein AM was used to identify intact, esterase-positive particles in a P-2 prepared from rat (Fig. 1A, C). The analysis gate R1 contains P-2 particles that are positive for calcein AM based on background observed in an unstained aliquot (Fig. 1A). Consistent with our initial characterization (7), 88% ± 0.61 (n = 3) of the total elements are labeled with this viability marker (Fig. 1C).

For human synaptosomes (Fig. 1B, D), samples of A9 cortex were minced and cryopreserved at the time of autopsy. On the day of the experiment the frozen samples were rapidly thawed and P-2 fraction prepared as for rat brain. In contrast to the rat brain P-2, a small population with high autofluorescence (≈6–8%) was usually observed in the unstained sample in the analysis gate R1 (Fig. 1B); color dot plots show that much of this autofluorescence localizes to particles larger than 4 μm on light scatter analysis (data not shown). Compared to freshly prepared rat brain synaptosomes, fewer of total particles analyzed were calcein-AM positive in the human P-2, 88% ± 0.61, in rat compared to 59% ± 5.4 in human ($P < 0.01$; n = 4), a decrease in viability that was expected given the 3–10 h postmortem interval and freezing of the tissue. The forward angle light scatter distribution (FSC) indicates that for both rat and human synaptosomes, the largest particles are calcein AM–positive (Fig. 1C, D).

Size Distribution of Synaptic Markers in P-2 Fractions from Rat Brain

The calcein AM results described above indicate the integrity of the membrane-bound particles in the preparation, including free mitochondria, which are present at high levels in the P-2 fraction. Differentiation of synaptosomes from mitochondria requires presence of a synaptic marker. Therefore we next used monoclonal antibodies to label P-2 fractions for SNAP-25 following a fixation/permeabilization protocol for intracellular antigen labeling (10). SNAP-25 is a docking protein on the presynaptic membrane of synapses that is found only in neuronal terminals (11). We also measured levels of a postsynaptic marker, PSD-95, which is part of a scaffolding protein complex that anchors the N-methyl-D-aspartate (NMDA) receptor within synapses (12,13). Nonspecific fluorescence was measured in the presence of mouse $IgG_{1\alpha}$ (Fig. 2A, B); 65% ± 4.8 (n = 3) of the total P-2 particles were positive for SNAP-25 in the rat, and 61% ± 2.6 (n = 6) were SNAP-25 positive in the human (Fig. 2C, D). Because the unlabeled human synaptosomes show a small population that is highly autofluorescent, the gate for nonspecific labeling (Fig. 2B) in human synaptosomes is drawn based in part on the SNAP-25–positive population (Fig. 2D). The continuous nature of the size distribution is in agreement

with previous flow cytometry analysis of gradient purified synaptosomes (8), and is consistent with EM observations that the P-2 homogenate contains a heterogenous mixture of membrane fragments and vesicles in addition to synaptosomes (4). Also in agreement with EM observations, the P-2 contains fewer postsynaptic than presynaptic elements: 38% ± 4.6 (n = 3) and 22% ± 2.6 (n = 6) of the total P-2 was positive for PSD-95 in the rat and human, respectively (Fig. 2E, F). Because postsynaptic elements are not abundant in the P-2 and relatively low fluorescence is measured, a clear PSD-95–positive population is not visualized. Therefore the gate delineating percent above background for PSD-95 represents an estimate; however, measuring the percentage of particles positive for PSD-95 is a useful measure, particularly for the present purpose of assay development. Immunolabeling above background was not observed for other postsynaptic markers, including drebrin and the NMDA receptor subunit NR2B (data not shown).

The forward angle light scatter parameter demonstrates that in both rat and human synaptosomes, presynaptic and postsynaptic marker fluorescence is increased in the largest P-2 particles. Taken together with the calcein AM data reported above (Fig. 1C, D), these results suggest that the synaptosomal population of interest can be identified on the basis of relative size as indicated by forward light scatter.

Effect of Size-Gating Analysis on the Fraction of Synaptosomal Markers

To quantify the enrichment of synaptosomes within the larger P-2 population, we drew analysis gates identifying small and large P-2 particles on forward light scatter. Based on size standards, particles less than 1 μm were identified as small, and those between 1.4 and 4 μm were classified as large (Fig. 3A, B). Histograms from each population were used to quantify the fraction of positive particles in small versus large synaptosomes; representative histograms from rat and human preparations illustrate the resulting increases in the viability marker calcein AM (Fig. 3C, D), the presynaptic marker SNAP-25 (Fig. 3E, F), and the postsynaptic marker PSD-95 (Fig. 3G, H). These data were quantified using channel by channel histogram subtraction and are summarized in Figure 4; the enrichment obtained by gating on forward scatter is highly significant for all three markers (large versus small comparison $P < 0.001$ level for each marker in both human and rat, with the exception of PSD-95 in human, where $P < 0.01$, Student's *t*-test; n = 6). The large particles were 98% ± 0.82 (n = 3) and 75% ± 9.8 (n = 6) calcein AM–positive in rat and human samples, respectively. This result is not unexpected given that the rat tissue is homogenized immediately following animal euthanasia, while human tissue is cryopreserved (10% DMSO 0.32 M sucrose) following a postmortem interval of 3–10 h. Parallel single-labeled samples reveal that in this forward scatter gate the SNAP-25 positive fraction is 90% ± 3 (n = 5) for rat and 82% ± 3 (n = 6) for human.

Comparison of the gating analysis for SNAP-25 and PSD-95 in rat versus human in Figure 4 reveals, as was observed with calcein AM, that enrichment of synaptic markers is greater when synaptosomes are freshly prepared. The PSD-95–positive fraction of large particles in fresh rat P-2 was significantly greater than in the human preparation (76% ± 2.9 versus 36% ± 3.0; $P < 0.001$; n = 6). This relative loss of postsynaptic elements in human tissue likely results from alteration of the axon cleavage plane by the extended postmortem interval and cryopreservation before homogenization. SNAP-25 enrichment was also greater in fresh synaptosomes (90% ± 2.7 versus 82% ± 2.6; $P < 0.05$; n = 6). The error bars in Figure 4 reveal a relatively small interexperiment variability, indicating that, in our hands, the size gate boundaries are consistent from one preparation to the next. Because the population of interest consists of functional synaptic terminals with intact membranes, the calcein AM–positive and SNAP-25–positive fractions in both rat and human preparations indicates that gating analysis results in synaptosome purity equal or superior to density gradient purification (8).

DISCUSSION

The use of synaptosomes for the study of synaptic chemistry and physiology is routine, but analysis is limited by a number of factors, particularly the heterogeneity of the preparation. The present results demonstrate that the synaptosomal population of interest in the crude synaptosome preparation (P-2) can be identified based on size, and most nonsynaptosomal elements can be excluded by gating analysis. Enhancement of SNAP-25 fluorescence in large P-2 particles was noted in our initial characterization; the present results extend the original observation by measuring the degree of enrichment for calcein AM labeling and for both presynaptic and postsynaptic proteins. The experiments described also compared the rat and human preparations.

Synapse loss is considered a primary correlate of the memory loss in Alzheimer's disease (14), but with conventional methods, detailed study of synaptic regions in adult brain is problematic. The resolution of light microscopic immunolabeling is not sufficient for precise synapse identification, and biochemical measures such as Western blots do not yield information about cellular location or size and inevitably include contaminants. EM is semiquantitative and requires harsh fixation that may introduce artifacts. Primary culture systems for the study of synapses share similar problems, including contaminants, culture artifacts, and quantification. The key advantages of flow cytometry analysis for this purpose are: 1) the definitive localization of damage markers (e.g., proapoptotic proteins or amyloid-β) to the synaptic compartment within the complex structure of adult brain, and 2) the identification of synaptosome subpopulations of interest because multiple parameters are quantified for each sample. Additional advantages include the potential to sort damaged terminals for further biochemical and proteomic studies. The 3–10-h postmortem interval for human tissue clearly

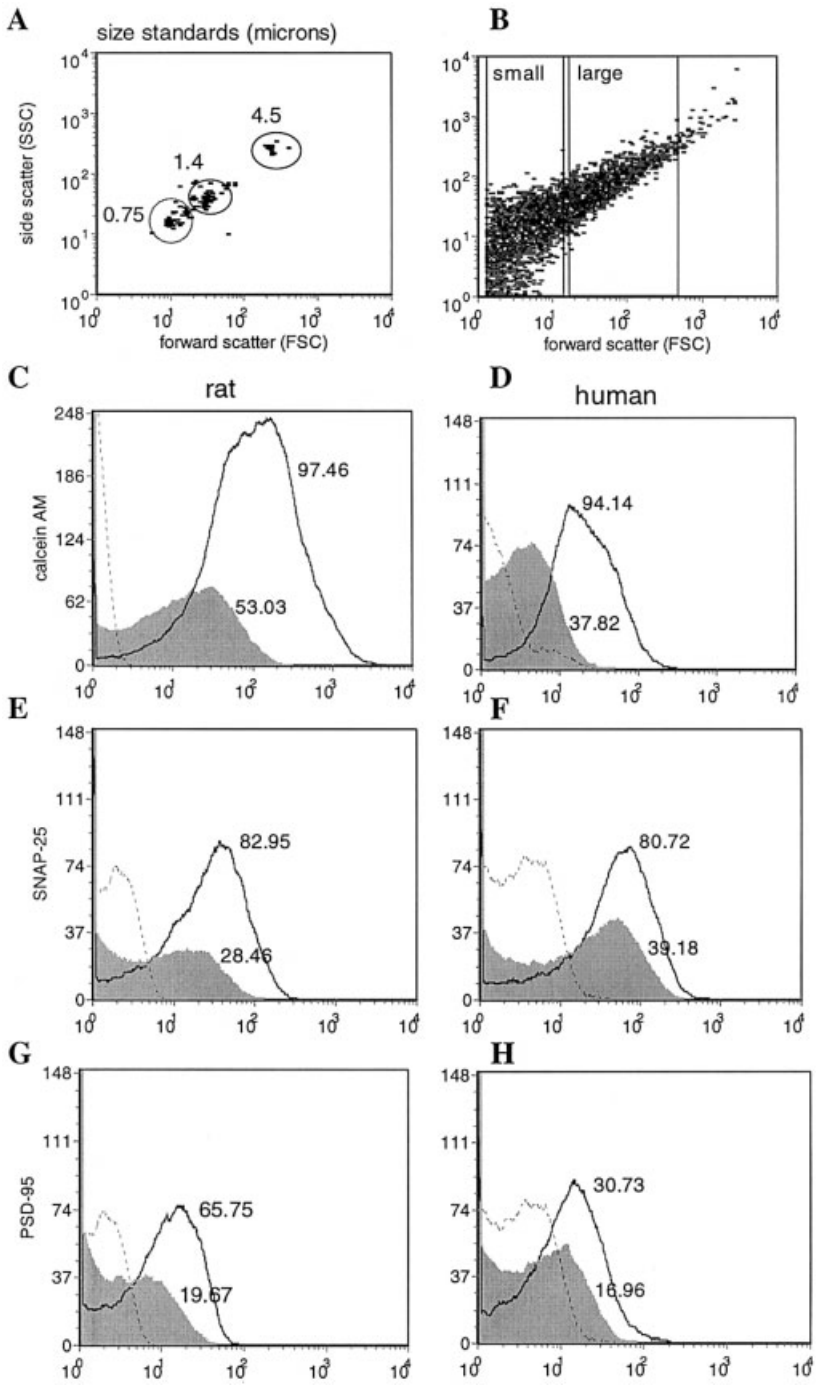

FIG. 3. Gating analysis of rat and human P-2 fractions. **A:** Size standards acquired with identical instrument settings. **B:** Size-based (FSC) analysis gates shown on a representative fresh rat P-2 sample; small particles are <1.4 μm; large particles are 1.4–4.5 μm. Histograms illustrate the positive fraction in small (filled curves) and large particles (open curves) from rat and human P-2 fractions for calcein AM (**C**,**D**), SNAP-25 (**E**,**F**), and PSD-95 (**G**,**H**). Background labeling with isotype control is indicated by dashed histogram at left for each plot.

limits use for examination of many dynamic cellular processes such as phosphorylation and membrane potential. However, the ability to directly localize and quantify stable disease-related molecules, particularly proteins and lipids, in human synapses is critical for understanding pathology and avoids the relevance issues associated with animal and in vitro models.

Although gating on populations of interest based on FSC is often useful (15,16), deduction of particle size in the low micrometer range based on FSC can be problematic. This is in part because FSC is a complex function of particle size and the refractive index of the particle and the suspending media (17). When isolated brain mitochondria are examined using flow cytometry, for example, swelling is associated with decreased SSC rather than increased FSC (18). Therefore, it is important to note that, in this range, FSC information about particle size is an index of approximate or relative size rather than an abso-

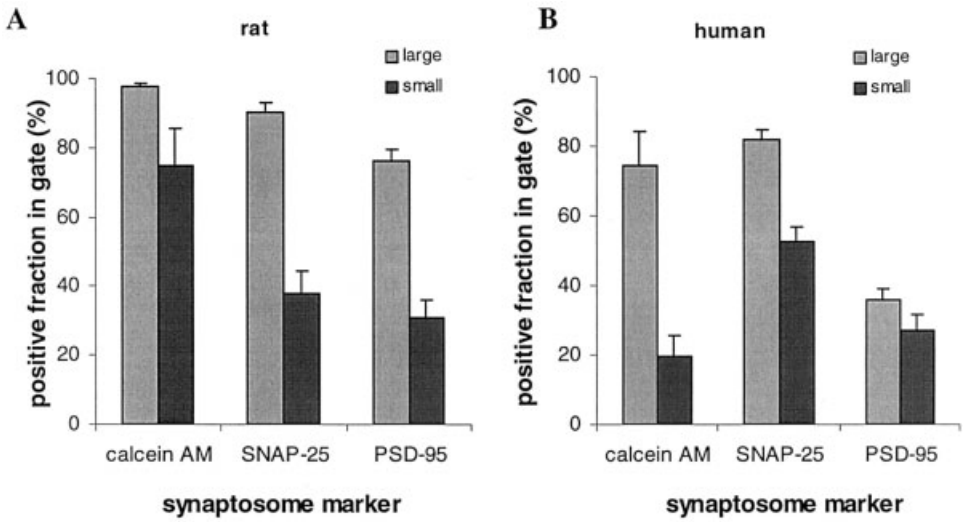

FIG. 4. Comparison of enrichment obtained by gating analysis in rat (**A**) and human (**B**) P-2 fractions. The difference between large and small particles was significant for all comparisons ($P < 0.0001$ except for PSD-95 in human, where the significance level was 0.05). Data are from four to six independent experiments.

lute measure. In the analysis described here, gates were drawn based on size standards, but enrichment of synaptic markers does not require careful placement of the analysis gate as long as it includes particles in a sample with higher FSC (data not shown). Application of size-based gating requires the additional consideration that fluorescence changes associated with markers of interest in a synaptosome preparation may be related to particle size as well as to the variable of interest (amount of antigen, membrane potential, etc). Therefore, if comparisons are made between populations of different sizes, additional analysis should be performed in order to remove size as a confound.

Flow cytometry analysis can not distinguish between a "snowman-shaped" synaptosome with an intact membrane-bound postsynaptic element (19) and those with an adherent fragment of postsynaptic membrane. However, the crude synaptosomes (P-2) that we use might be expected to contain more synaptosomes with a membrane-bound postsynaptic element, because less purification time and less manipulation is required. An alternative in vitro preparation, termed "synaptoneurosomes," uses a filtration step rather than the second centrifugation, which yields the P-2 (5,19). Synaptoneurosomes have been thought to contain more membrane-bound postsynaptic elements than a conventional P-2 fraction, but a comparison in our lab (data not shown) demonstrated more debris and less PSD-95 labeling in the synaptoneurosome preparation compared to the P-2 fraction.

Synaptosomes have historically been considered a primarily presynaptic preparation (20) and have been most widely used for the study of neurotransmitter synthesis and release. The finding that a majority (76%) of the large particles in fresh synaptosomes are PSD-95–positive was somewhat unexpected. This figure represents, to our knowledge, the first quantification of the postsynaptic component in synaptosomes. It is important to note, however, that synaptosomes and synaptic membrane preparations have been used as tissue sources for studies of proteins in the postsynaptic complex (21,22), and the postsynaptic fraction we observe is consistent with the relatively abundant Western blot signal observed in synaptosomes for the postsynaptic NMDA receptor and the dendritic spine protein RC3/neurogranin (5). Taken together, all of these results indicate that examination of changes in synaptic proteins need not be limited to presynaptic elements, an important consideration in light of recent evidence that postsynaptic loss may precede presynaptic loss in Alzheimer's disease (23). Compared to fresh synaptosomes, large synaptosomes from cryopreserved human tissue are less "viable" (calcein AM–positive), and contain fewer postsynaptic elements. However, the significant fraction (75–80%) of intact synaptosomes that we have identified within the large population in human tissue makes this preparation a unique model system in which to explore pathways leading to synaptic changes.

Recent work suggests that flow cytometry analysis of synaptosomes may prove a valuable tool for studies of presynaptic vesicle traffic and release (24,25). In addition, because lysis or damage to synaptosomes results in a loss of particles within the large gate (7), size gating can also be used as a direct toxicity indicator in synaptosome studies. We have previously shown, for example, that staurosporine-induced loss of large synaptosomes can be blocked by caspase-3 and calpain inhibitors (26). We have also measured amyloid-β uptake into synaptosomes using the present method (27), and are currently optimizing protocols for dual labeling of synaptosomes. Colocalization of amyloid-β protein with other damage and neurochemical markers will greatly enhance our ability to identify vulnerable synaptic subpopulations and clarify the sequence of events that leads to loss of synapses. Size-based gating analysis provides a simple and cost-effective way to identify the primary population of interest within

the P-2 and to monitor fluorescence changes in synapse regions from animal and human brain samples.

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