Supplementary information

Supplemental Experimental Procedures

Animals: Wild type (C57BL/6) or transgenic nestin-GFP (Mignone et al., 2004), nestin-CFPNuc (Encinas et al., 2006), fms-EGFP (Sasmono et al., 2003), POMC-EGFP (Overstreet et al., 2004) or hGFAP-GFP (Zhuo et al., 1997) mice were used in this study. Unless otherwise stated, animals were 1 month old. In BrdU pulse-and-chase experiments, mice were injected with BrdU (250mg/kg, i.p.) once (single BrdU paradigm) or every three hours for 24hr (cumulative BrdU paradigm), and sacrificed at different time points. In inflammation experiments, mice were injected with IdU (42.5mg/kg, Sigma) 6 or 20hr prior to receiving a single i.p. injection of phosphate-buffered saline (PBS; vehicle) or 1mg/kg LPS (*Salmonella typhimurium* lipopolysaccharides; Sigma, St. Louis, MO). Mice were then injected with CldU (57.5mg/kg, Sigma) and sacrificed 2hr later. IdU and CldU were injected in equimolar concentrations (Vega and Peterson, 2005). All mouse studies were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee and performed in accordance with institutional and federal guidelines.

Immunofluorescent labeling: Mice were transcardially perfused with saline and 50ml of 4% paraformaldehyde (PFA). The brains were dissected out, submerged into 4% PFA for 4h at room temperature (RT) and then transferred to a cryoprotectant solution (30% sucrose, 30% ethyleneglycol in PBS). Six series of 50μm lateral sections were collected with a vibratome. A full series (encompassing 8-9 sections) of free-floating sections were immunostained following conventional procedures (Sierra et al., 2007). All washes and incubations were done in PBS containing 0.5% bovine serum albumin (BSA; Sigma) and 0.3% triton X-100 (Sigma). An antigen retrieval step (2N HCl, 15min, 37°C) for BrdU, IdU or CldU detection was performed, followed by extensive washes with borate buffer (0.1M). Sections were pre-incubated in 5% normal goat serum (Vector Labs, Burlingame, CA) for 2 h at room temperature, followed by overnight incubation with primary antibodies (see below) at 4°C. After extensive washing, sections were incubated with the appropriate secondary antibody conjugated with Alexa 488 (Molecular Probes, Eugene, OR), RhodamineRed-X (Jackson Immunoresearch) and Cyanine 5 (Jackson Immunoresearch) together with DAPI (5μg/mL, Sigma) for 2 h at room temperature. They were then washed, and mounted on slides with Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA). The following primary antibodies were used: act-casp3 (1:200, BD); BrdU (1:400, Chemicon); CldU (anti-BrdU, 1:400, Accurate); CD11b (Serotec, 1:100); CD68 (Serotec, 1:100); DCX (Cell Signaling, 1:200); fractin (1:200, BD); GFAP (1:1,000, Sigma); Iba1 (1:1,000, Dako); IdU (anti-BrdU, 1:400, BD); MBP (1:200, Chemicon); NeuN (1:1,000, Chemicon); PECAM1 (BD, 1:200); Prox1 (1:1,000, Chemicon); and PSA-NCAM (1:400, Chemicon).

Confocal microscopy: Sections were imaged using a Zeiss confocal LSM or in a Leica confocal microscope. Two-three 20μm z-stacks (consisting of 30 optical slices of 0.8 μm thickness) were obtained from every section. The number of apoptotic cells and/or BrdU immunopositive cells per z-stack was estimated using the optical dissector method (Encinas and Enikolopov, 2008) The number of cells was evaluated as a function of the volume of the SGZ, defined as the SGZ length in the image multiplied by an optical thickness of 20 μm and a height of 20 μm (which we defined in these experiments as a layer of cells expanding 5μm into the hilus and 15μm into the granular layer), then extrapolated to the volume spanned by the SGZ in the hippocampus. Blind analysis was performed using AxioVision 4.5 (Zeiss) or LAS AF Lite (Leica). 3D rendering was performed using ImageSurfer (www.imagesurfer.org).

Electron microscopy: Animals were perfused as above and brains were post-fixed in 4%PFA for 4h at RT. They were then transferred to 30% sucrose in PBS and kept at 4^{o} C until samples sunk to the bottom of the vial. The brains were immersed in liquid nitrogen (N_2) for 5sec, and then put immediately in PBS at RT. 50μm lateral sections were collected with a vibratome and immunostaining was performed by pre-embedding (Encinas and Enikolopov, 2008). Slices were treated with 0.3% H_2O_2 in PBS for 20 min at RT to inhibit endogenous peroxidase activity and then washed thoroughly with PBS. Non-specific binding was blocked by 3% BSA in PBS for 30min and sections were incubated with anti-Iba1 (1:1,000, Wako) overnight at 4^{o}C. After rinsing, the slices were incubated with anti-rabbit antibody conjugated with biotin in PBS for 2h at RT, and then with Avidin-Biotin complex (1:150 in PBS, Pierce) for 1.5h at RT. After rinsing thoroughly, the slices were incubated with 6mg/ml 3'-3'-diaminobenzidine (DAB) in PBS for 10 min and the reaction was stopped by rinsing with PBS. The sections were then fixed with 5% glutaraldehyde in PBS for 30min, at RT, and then contrasted with 0.1% osmium tetraoxide, for 1hr at RT. Then, slices were dehydrated in 50% ethanol for 20min; 70% ethanol for 20min; 1% uranyl acetate in 70% ethanol for 30min; 100% ethanol for 20min each time (twice); propylene oxide for 10min; propylene oxide in Durcupan (1:1), for 10min. Slices were then covered completely in Durcupan and placed in a vacuum chamber overnight. Durcupan capsules were dried at 50^{0}C for 72hr, and ultra-thin sections of the SGZ were obtained using a Reichert-Jung Ultracult E ultra-microtome. Sections were imaged using a FEI BioIwinG2 Transmission Electron Microscope.

Supplemental Figures, Tables and legends

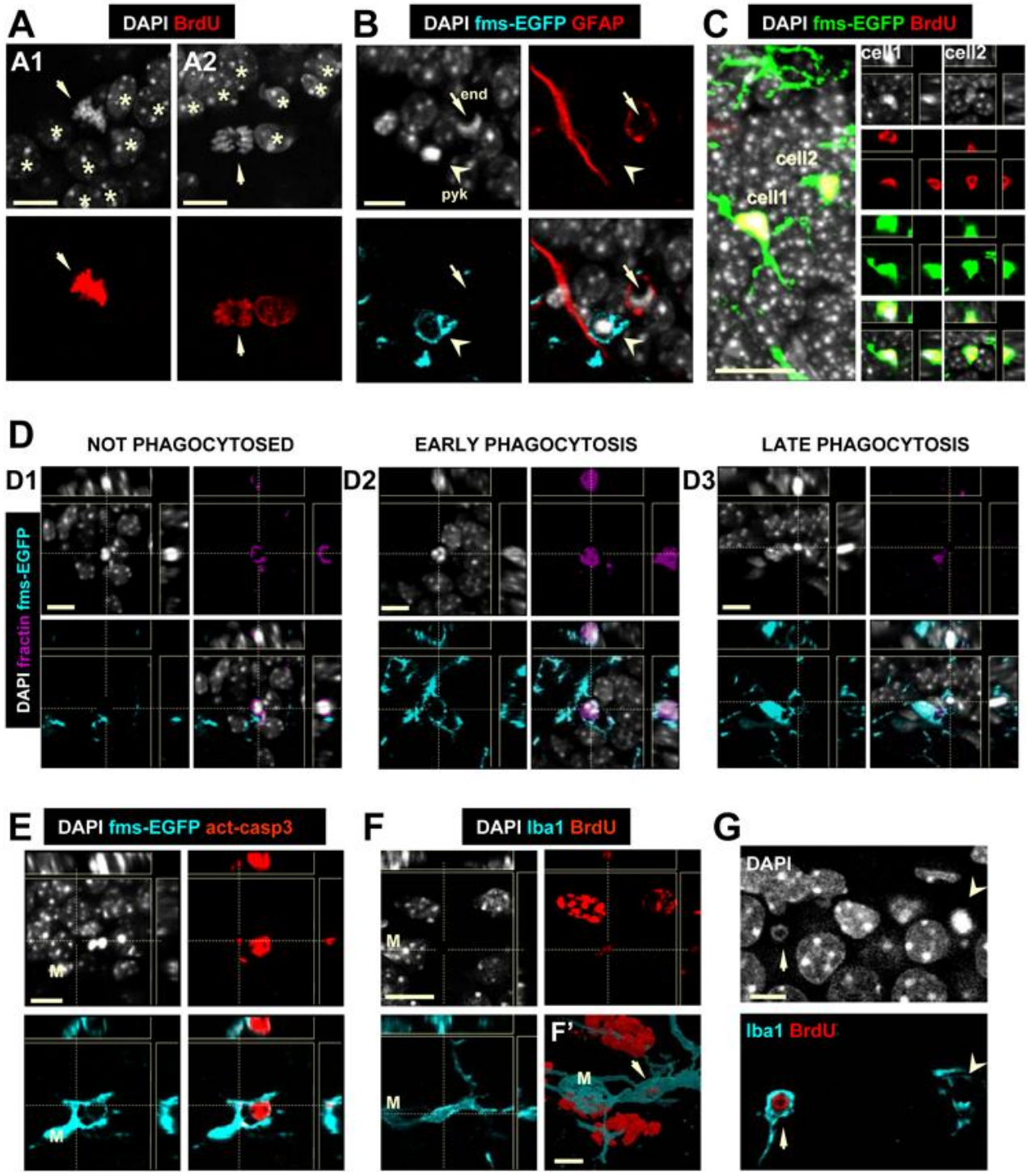

Figure S1, related to Figure 1. **Apoptotic cells have a unique nuclear morphology.**

(A) Cells in mitosis (arrows; B1, metaphase; B2, anaphase), labeled with BrdU, are morphologically different from apoptotic, pyknotic cells (B), and from healthy cells (asterisks).

(B) Endothelial cells (arrow) surrounded by GFAP+ astrocytic terminal feet, are morphologically distinct from apoptotic, pyknotic cells (arrowhead).

(C) Microglia labeled with BrdU are found occasionally. Small insets are orthogonal projections of confocal z-stacks, which show colocalization of the EGFP and BrdU.

(D) Different stages of phagocytosis in the SGZ. The microglial engulfment of the apoptotic cell (pyknotic, fractin positive) is assessed in orthogonal projections of confocal z-stacks. Apoptotic cells are either not phagocytosed (D1); undergoing early phagocytosis (loose microglial pouch, conspicuous act-casp3 immunolabeling; D2); or late phagocytosis (tight microglial pouch, dim act-casp3 immunolabeling; D3).

(E) Puncta of act-casp3 within fms-EGFP labeled microglial processes.

(F) Puncta of BrdU (arrow) within Iba1 labeled microglial process, next to the microglial cell body (M). B' shows a three dimensional rendering of the puncta of BrdU. (Same cell is shown in Fig. 4E).

(G) Terminal phagocytosis by Iba1 labeled microglia of a diminutive DAPI particle labeled with BrdU (arrow), close to another apoptotic cell undergoing early stage of phagocytosis (arrowhead). Scale bars; A, B, 10μm; C, 20μm (z=8.8μm); D, E, 10μm; E', 5μm; G, 5μm.

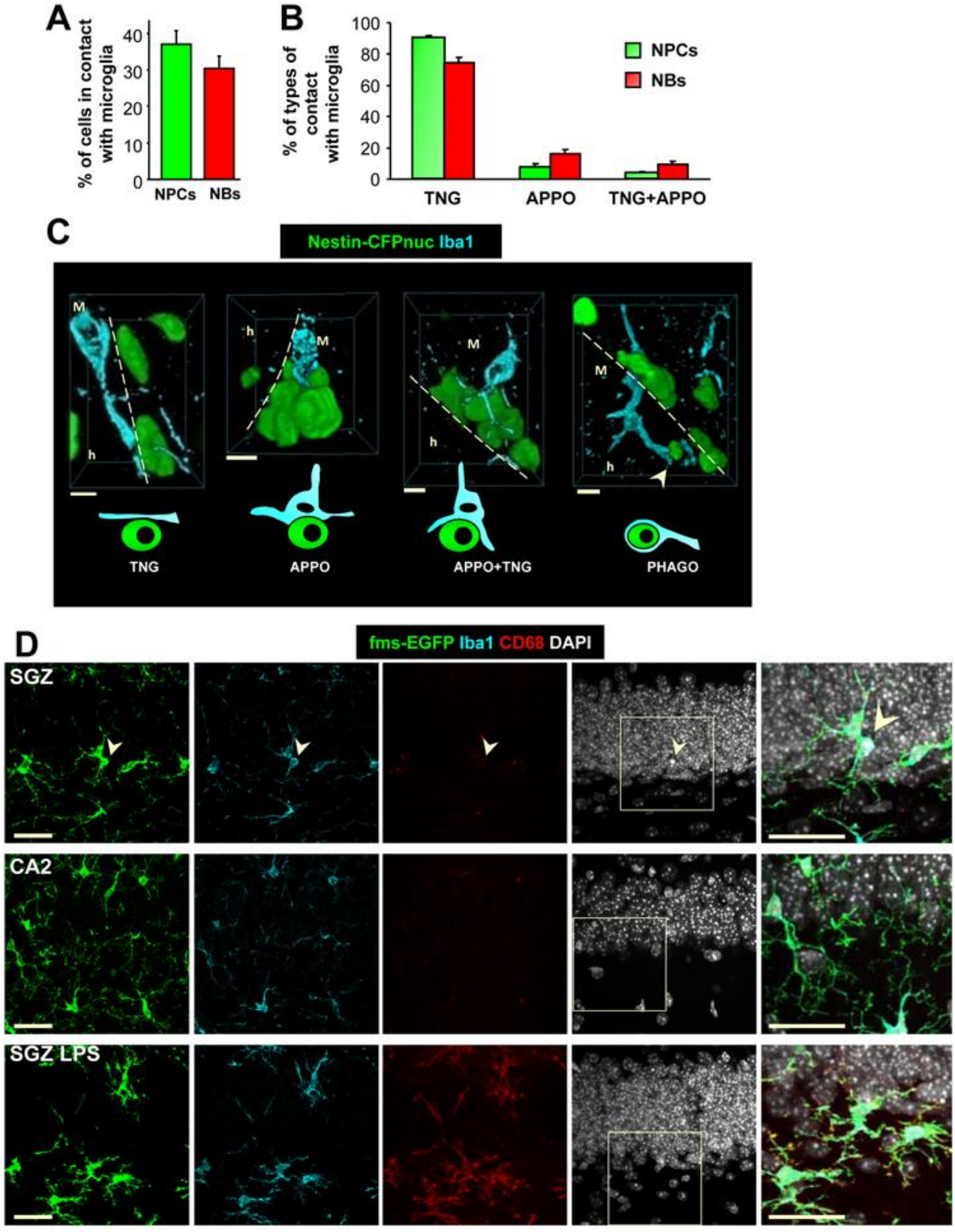

Figure S2, related to Figure 2. **Phagocytosis by unchallenged microglia**

(A) Sampling volume of microglia in the young adult SGZ niche, quantified as the percentage of NPCs or NBs contacted by microglial processes or somas. We estimate that approximately one-third (37 ± 4%) of the NPCs and 30 ± 3% of the NBs are in contact with microglial processes (n=4).

(B) The majority of contacts between microglia and NPCs or NBs are mediated through the processes (tangential, TNG) and only occasionally direct apposition (APPO) of a microglial cell body.

(C) Three-dimensional rendering examples of the different types of contacts between microglia and NPCs, visualized in nestin-CFPNuc mice, in which the cyan fluorescent protein is expressed under the control of the nestin promoter and regulatory elements and is directed by a nuclear localization signal (Encinas et al., 2006). Schematic representations outline the type of the contact (tangential, TNG; apposition (APPO; apposition and tangential, APPO+TNG; phagocytosis, PHAGO). Arrowhead points to the pyknotic nucleus of a nestin-CFPNuc+ NPC which is engulfed by the microglial process.

(D) Confocal photomicrographs of the expression of CD68 in phagocytic microglia. Phagocytic SGZ and non-phagocytic CA2 microglia express low levels of CD68, while LPS-challenged microglia express high levels of this marker. The expressions of fms-EGFP and Iba1 are similar in all the experimental groups studied. Identical confocal settings were used to collect images from all animals. Enlarged insets of the merged images are shown at the end of each row.

Scale bars: C, 5μm; D, 20μm (z=10μm); Arrowheads, phagocytic processes; M, microglia; h, hilus.

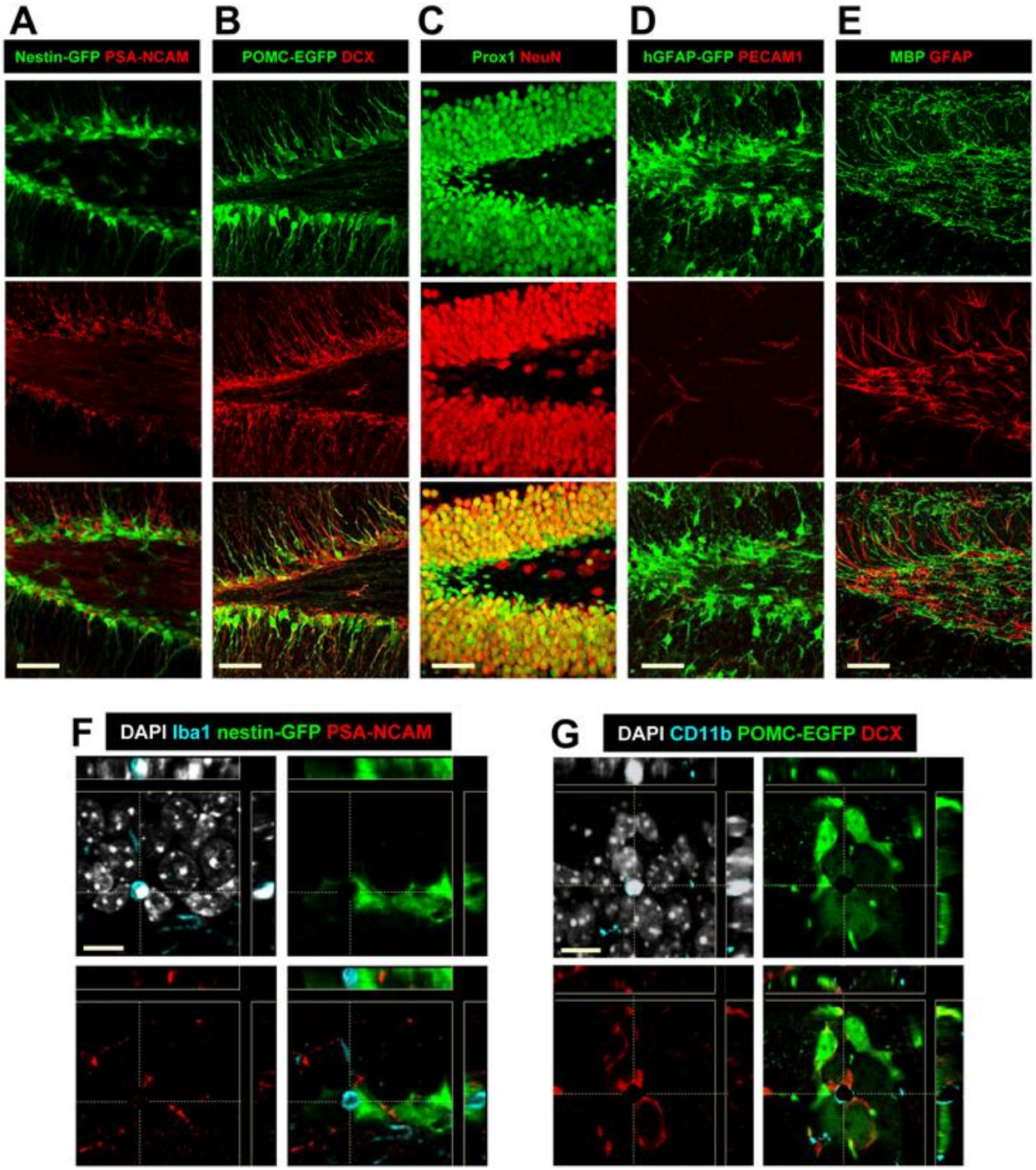

Figure S3, related to Figure 3. **Low magnification images of immunolabeling of the different cell-type specific biomarkers.**

(A) Nestin-GFP and PSA-NCAM

(B) POMC-EGFP and DCX

(C) Prox1 and NeuN

(D) GFAP-EGFP and PECAM1

(E) MBP and GFAP

(F) Phagocytosis by Iba1 labeled microglia of a non-identified apoptotic cell within the young adult SGZ neurogenic niche, surrounded by NPCs expressing nestin-GFP+.

(G) Phagocytosis by CD11b labeled microglia of a non-identified apoptotic cells within the young adult SGZ neurogenic niche, surrounded by NBs expressing POMC-EGFP and DCX.
Scale bars: A-E, 50μm (z=20μm); F, G, 10μm.

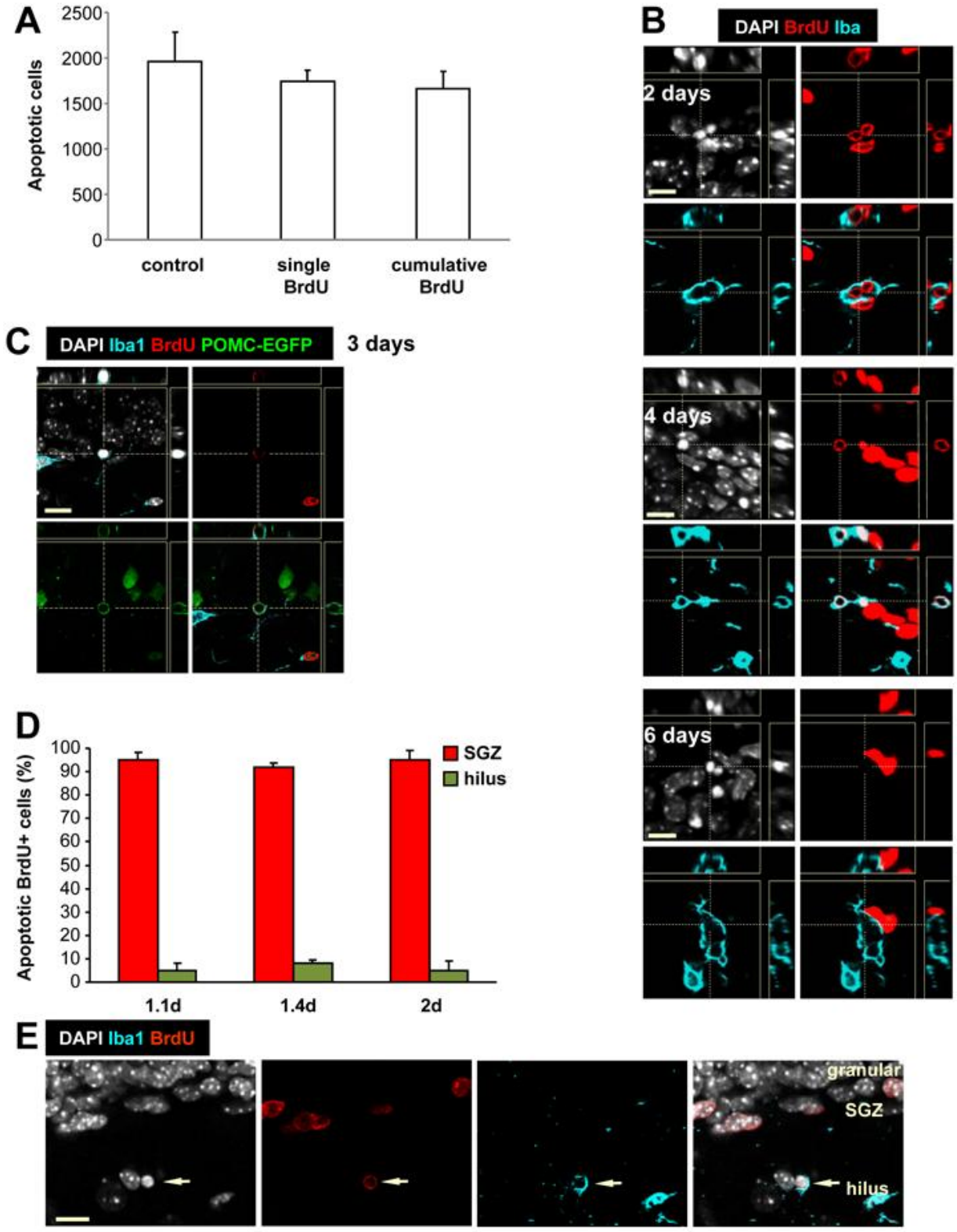

Figure S4, related to Figure 5. **SGZ apoptosis in the BrdU cumulative labeling paradigm.**
(A) BrdU labeling does not affect apoptosis in the young adult SGZ neurogenic zone. No significant differences are observed in the number of apoptotic cells per hippocampus when 1 m.o. animals were given single or cumulative BrdU injections, or vehicle (control). Quantification was done at 2 dpi. Bars represent mean ± SEM.

(B) Photomicrographs of apoptotic (pyknotic) cells, labeled with BrdU at 2, 4, and 6 dpi, phagocytosed by Iba1-labeled microglia, shown as orthogonal projections of confocal z-stacks.
(C) Photomicrographs of an apoptotic (pyknotic) cell, labeled with BrdU at 3 dpi, expressing POMC-EGFP and phagocytosed by Iba1-labeled microglia, shown in an orthogonal projection of a confocal z-stack.
(D) In the early time points after the cumulative BrdU labeling (1.1-2 dpi), the majority of apoptotic BrdU+ cells is located in the SGZ, and only a small percentage (5-8%) is located in the hilus.
(E) Photomicrograph of a hilar 1.4dpi BrdU+ apoptotic cell (arrow).
Scale bars: 10μm. Arrow, apoptotic cell.

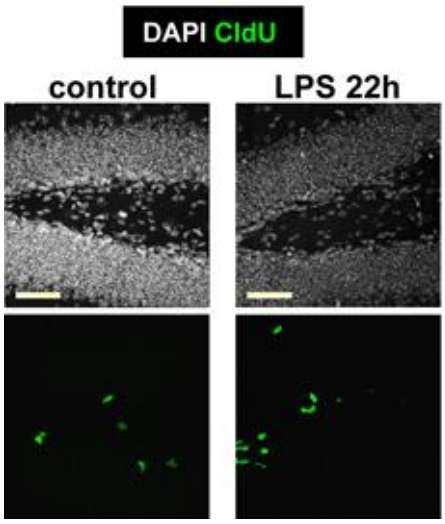

Figure S5, related to Figure 7. **Proliferation in the young adult SGZ 22h after LPS treatment.** Photomicrographs of the NPC proliferation (CldU+ cells) in control and LPS-treated animals 22 hr following LPS.
Scale bars: 50μm.

	pyknotic	pyk+act-casp3	pyk+fractin
Apoptotic total	287	181	151
Apoptotic phagocytosed	266	169	143
ratio	**0.927**	**0.934**	**0.947**
n	10	5	3

Table S1, related to Figure 1. **Phagocytic index quantified with different markers of apoptosis.** Total number of apoptotic cells (identified by pyknosis alone, pyknosis plus act-casp3, or pyknosis plus fractin immunolabeling, analyzed in three independent experiments), number of apoptotic cells phagocytosed by microglia, ratio, and the number of animals (n) analyzed.

	Nestin-GFP alone	PSA-NCAM alone	Nestin-GFP + PSA-NCAM	POMC-EGFP	DCX	Prox1	NeuN	PECAM1	GFAP-GFP	GFAP	MBP
Apoptotic total	71	71	71	393	382	104	104	146	146	132	132
Apoptotic biomarker	4	11	31	98	0	5	0	6	0	0	0
ratio	**0.056**	**0.155**	**0.437**	**0.249**	**0.000**	**0.048**	**0.000**	**0.041**	**0.000**	**0.000**	**0.000**
n	4	4	4	6	5	3	3	4	4	3	3

Table S2, related to Figure 3. **Apoptotic biomarker+ ratio.** Total number of apoptotic cells, number of apoptotic cells expressing each biomarker, ratio, and the number of animals (n) analyzed.

	2H	12H	1D	2D	3D	4D	8D	11D	15D	18D	22D	32D
Apoptotic total	101	153	201	171	165	165	166	49	78	48	84	53
Apoptotic BrdU+	0	0	4	12	6	2	1	0	3	0	0	1
ratio	**0.000**	**0.000**	**0.020**	**0.070**	**0.036**	**0.012**	**0.006**	**0.000**	**0.038**	**0.000**	**0.000**	**0.019**
n	3	3	4	4	4	3	4	3	4	3	4	3

Table S3, related to Figure 4. **Apoptotic BrdU+ ratio along the BrdU time course (single injection).** Total number of apoptotic cells, number of apoptotic BrdU+ cells, ratio, and the number of animals (n) analyzed per time point.

	1.1D	1.4D	2D	4D	6D	8D	11D	15D	18D	22D	32D
Apoptotic total	329	247	204	192	169	80	80	63	84	68	45
Apoptotic BrdU+	33	46	56	55	16	5	2	1	2	1	0
ratio	**0.100**	**0.186**	**0.275**	**0.286**	**0.095**	**0.063**	**0.025**	**0.016**	**0.024**	**0.015**	**0.000**
n	3	3	4	4	4	3	3	3	4	3	3

Table S4, related to Figure 5. **Apoptotic BrdU+ ratio along the BrdU time course (cumulative paradigm).** Total number of apoptotic cells, number of apoptotic BrdU+ cells, ratio, and the number of animals (n) analyzed per time point.

	1m.o.	2m.o.	5m.o.	12mo
Apoptotic total	71	26	8	6
Apoptotic phagocytosed	66	24	8	6
ratio	**0.930**	**0.923**	**1.000**	**1.000**
n	4	4	4	3

Table S5, related to Figure 6. **Phagocytosis throughout the adulthood.** Total number of apoptotic cells, number of apoptotic cells phagocytosed by microglia, ratio, and the number of animals (n) analyzed per age point.

	control 8h	LPS 8h	control 22h	LPS 22h
Apoptotic total	207	578	174	155
Apoptotic phagocytosed	199	538	161	141
ratio	**0.961**	**0.931**	**0.925**	**0.910**
n	4	5	4	5

Table S6, related to Figure 7. **Phagocytosis during acute neuroinflammation.** Total number of apoptotic cells, number of apoptotic cells phagocytosed by microglia, ratio, and the number of animals (n) analyzed per experimental group.