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Annexin A1: A Central Player in the Anti-Inflammatory and Neuroprotective Role of Microglia

Simon McArthur,* Enrico Cristante,† Mario Paterno,* Helen Christian,‡ Federico Roncaroli,‡ Glenda E. Gillies,* and Egle Solito*

The brain microenvironment is continuously monitored by microglia with the detection of apoptotic cells or pathogens being rapidly followed by their phagocytosis to prevent inflammatory responses. The protein annexin A1 (ANXA1) is key to the phagocytosis of apoptotic leukocytes during peripheral inflammatory resolution, but the pathophysiological significance of its expression in the CNS that is restricted almost exclusively to microglia is unclear. In this study, we test the hypothesis that ANXA1 is important in the microglial clearance of apoptotic neurons in both noninflammatory and inflammatory conditions. We have identified ANXA1 to be sparingly expressed in microglia of normally aged human brains and to be more strongly expressed in Alzheimer’s disease. Using an in vitro model comprising microglial and neuronal cell lines, as well as primary microglia from wild-type and ANXA1 null mice, we have identified two distinct roles for microglial ANXA1: 1) controlling the noninflammatory phagocytosis of apoptotic neurons and 2) promoting resolution of inflammatory microglial activation. In particular, we showed that microglial-derived ANXA1 targets apoptotic neurons, serving as both an “eat me” signal and a bridge between phosphatidylserine on the dying cell and formyl peptide receptor 2 on the phagocytosing microglia. Moreover, inflammatory activation of microglia impairs their ability to discriminate between apoptotic and nonapoptotic cells, an ability restored by exogenous ANXA1. We thus show that ANXA1 is fundamental for brain homeostasis, and we suggest that ANXA1 and its peptidomimetics can be novel therapeutic targets in neuroinflammation. The Journal of Immunology, 2010, 185: 000–000.

Microglia are the resident immune cells of the CNS and function as critical regulators of the brain microenvironment (1). In normal brains, they show ramified, highly motile processes undergoing cycles of protrusion, extension, and withdrawal allowing them to monitor the local surroundings and detect any damage to brain tissue (2). In any disease affecting the CNS, such as neurodegenerative conditions, infections, traumatic brain injury, and stroke, the continued presence of “danger” signals prolongs microglial activation with subsequent production of proinflammatory molecules. Although the proinflammatory reaction helps to kill and remove dying neurons and cell debris, activated microglia may also damage and remove healthy neurons and thereby substantially contribute to the pathogenic process (3). A considerable research effort has focused on strategies to suppress microglial activation but with limited success (4, 5). In contrast, less attention has been given to promoting the protective role of microglia, which involves the detection and efficient removal of apoptotic cells (6–8). This disposal is of critical importance because apoptotic cells can enter secondary necrosis (9) and become potent triggers of inflammation (10, 11) that inevitably leads to further cell damage. An understanding of the factors dictating the switch from a protective to a damaging inflammatory response, and particularly of phagocytosis, will permit interventions aimed at limiting tissue damage.

In the peripheral immune system, the phospholipid-calcium-binding protein annexin A1 (ANXA1) plays a key role in the resolution of inflammation (12). In particular, it promotes macrophage phagocytic removal of apoptotic leukocytes without the production of inflammatory mediators (13) and therefore without escalation of the inflammatory cascade (14). In the CNS, there is little evidence to aid our understanding of the mechanisms for removal of apoptotic cells, but it is noteworthy that ANXA1 is present in microglia (15, 16) but absent from the majority of neurons (17). Moreover, enhanced expression of ANXA1 has been reported at sites of damage in the brains of patients suffering from multiple sclerosis (18) or Parkinson’s disease (19), although a role for ANXA1 in these conditions remains speculative.

In this study, we have tested the hypothesis that ANXA1 is a key regulator of microglial reactivity, promoting the phagocytic removal of apoptotic neurons while preventing the production of proinflammatory mediators, such as TNF, IL-6, and NO (20, 21). We have investigated microglial expression of ANXA1 in post-mortem brains with aging-related changes and brains with Alzheimer’s disease, and we have developed an in vitro system to model the interactions of microglia with apoptotic neurons. Specifically, we have used the neurotoxin 6-hydroxydopamine (6-OHDA) to induce apoptosis in neuron-like PC12 cells and characterized the involvement of ANXA1 in their interactions.
with murine BV2 microglial cells in both physiological, non-inflammatory conditions and pathological inflammatory conditions, modeled by in vitro stimulation with LPS, and amyloid β (Aβ) 1–42. In this paper, we provide the first evidence, to our knowledge, that ANXA1 plays a key role in ensuring the effective and selective removal of apoptotic neuron-like cells under non-inflammatory conditions. This protective role is overwhelmed under inflammatory conditions, indicating a mechanism by which central or peripheral inflammation could worsen cell death in neurodegenerative disease such as Alzheimer’s disease. Moreover, we provide evidence that pharmacological intervention with ANXA1 can reverse this effect, suggesting that ANXA1-dependent mechanisms contribute to microglial surveillance and maintenance of brain homeostasis and that ANXA1 and its peptidomimetics have the potential to suppress deregulated microglial phagocytosis, which typifies pathology in both chronic and acute brain disorders.

Materials and Methods

Immunohistochemistry and immunofluorescence in human postmortem brains

Expression of ANXA1 in microglial cells was analyzed by immunohistochemistry and immunofluorescence. Human postmortem brains with aging-related changes from three female and three male subjects aged 80 y and four age-matched brains of subjects with Braak’s stage VI Alzheimer’s disease (two males and two females) were retrieved from the Corssellis brain collection, held by the R&D Department of the West London Mental Health Trust, supported by the Starr Foundation (New York, NY) and the National Institute for Health Research (London, U.K.). Brains were selected using the following criteria: 1) availability of full clinical history; 2) death occurring within 1 h from a cardiovascular accident; 3) full post-mortem performed within 24 h from death; 4) no evidence of infections or cancer at postmortem; and 5) negligible arteriosclerosis of cerebral vasculature. Samples from the superior frontal gyri were examined in all subjects. The tissue was fixed in 10% buffered formalin and embedded in paraffin. From each paraffin block, 10-μm sections were cut and used for immunohistochemistry.

Immunostains were performed with Abs directed against Iba1 (1/750; Wako Chemicals, Neuss, Germany), MHC class II (MHC II) (CR3/43, 1/200; DakoCytomation, Cambridgeshire, U.K.), and ANXA1 (1/1000; Invitrogen, Paisley, U.K.). For immunohistochemistry, sections were deparaffinized in xylene and rehydrated in decreasing alcohols. Endogenous peroxidase was inhibited by incubation in 0.3% v/v H2O2 in 0.1 M PBS for 30 min at room temperature. Sections were rinsed twice in 0.05 M TBS and incubated for 1 h at room temperature with 10% BSA (Sigma-Aldrich, Dorset, U.K.) containing 0.05% v/v Triton X-100 to saturate nonspecific binding; sections were then incubated for 16 h at 4°C with the primary Ab, rinsed twice in TBS-containing 0.05% v/v Triton X-100, incubated in biotinylated goat-anti rabbit secondary Ab (Vector Laboratories, Peterborough, U.K.) at the dilution of 1/100 for 2 h at room temperature, rinsed twice with TBS, and incubated for 45 min in the avidin–biotin complex (ABC) conjugated with HRP in TBS (Vector Laboratories U.K.). Following two washings in TBS, the reactions were developed in 0.025% diaminobenzidine and 0.01% H2O2 (Sigma-Aldrich) in TBS for 5 min. Sections were rinsed twice in TBS, counterstained with hematoxylin (VWR International, Leicestershire, U.K.), dehydrated, and mounted under DPX mountant (VWR International) for bright-field microscopic analysis as described below.

To colocalize ANXA1 and MHC II in brains with Alzheimer’s disease, we performed a double immunohistochemical reaction. For ANXA1, sections were processed as above up to the step of incubation with diaminobenzidine. After this step, sections were incubated with an anti-MHC II Ab for 2 h at 37°C. After three washings in TBS, they were incubated for 1 h with a biotinylated secondary Ab (see above), washed three times in TBS, and then incubated for 45 min in ABC conjugated with alkaline phosphatase (Vector Laboratories U.K.). The reactions were developed using the Blue Vector Kit according to the manufacturer’s instructions. Sections were finally mounted in aqueous medium.

Amyloid and cells expressing ANXA1 were conversely colocalized using immunofluorescence. Sections were deparaffinized and brought to water as described above. Amyloid was first stained with Congo red (22).

Sections were deparaffinized and brought to water as described above. Amyloid was first stained with Congo red (22).

In the following day, sections were mounted with aqueous mounting medium with DAPI, which stains nuclei and amyloid following the reaction with Congo red (Vectashield Mounting Medium for fluorescence with DAPI; Vector Laboratories, Burlingame, CA). Slides were examined using a Nikon epifluorescence microscope (Nikon Eclipse 50i; Nikon, Tokyo, Japan) with appropriate filters, and images were captured with a digital camera (Digital Sight DS-2MBW; Nikon, Japan). Composite images were generated using Media Cybernetics Image Pro PLUS (version 5.0; Media Cybernetics, Wokingham, U.K.).

Sections of pharyngeal tonsil were used as positive control tissue for Iba1 and MHC II, and sections of normal pituitary gland were used as positive control tissue for ANXA1. These samples were retrieved from the files of the Department of Histopathology at Charing Cross Hospital in London, U.K. Immunoreactions with omission of the primary Abs were performed as negative controls.

Cell culture

Murine microglial BV2 cells were provided by Prof. R. Donato (University of Perugia, Perugia, Italy). These cells express typical microglial cell surface markers and exhibit the characteristic effector functions of native microglia (23) and therefore represent an excellent model for investigating microglial regulation and the underlying molecular mechanisms (24). BV2 cells were cultured in RPMI 1640 medium supplemented with 5% FCS, 100 μM nonessential amino acids, 2 mM L-alanyl-glutamine, and 50 μg/ml gentamycin (all Invitrogen U.K.) at 37°C in 5% CO2. The PC12 cells (American Type Culture Collection, Manassas, VA), derived from a rat pheochromocytoma, mediate entry into the cell of the neurotoxin 6-OHDA, an active dopamine transporter (25). Importantly, the dopamine transporter mediates entry into the cell of the neurotoxin 6-OHDA, allowing the induction of apoptosis via mechanisms that characterize neurodegenerative conditions, including oxidative stress and inhibition of the mitochondrial complex 1 (26, 27). PC12 cells were cultured in RPMI 1640 medium supplemented with 10% normal horse serum, 5% FCS, 100 μM nonessential amino acids, 2 mM L-alanyl-glutamine, and 50 μg/ml gentamycin (all Invitrogen U.K.) at 37°C in 5% CO2.

Phagocytosis assay

We developed a modified version of the model used to investigate the uptake of apoptotic leukocytes by peripheral macrophages (28, 29). Briefly, PC12 cells grown in suspension were fluorescently labeled with 5 μM 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen U.K.), according to the manufacturer’s instructions. The cells were then cultured in the presence of 37 μM 6-OHDA hydrobromide (Sigma-Aldrich) in Na2S2O3 vehicle or vehicle alone (final concentration of 40 μM Na2S2O3) for 16 h overtime. These conditions were optimized in preliminary time-course and dose-response studies (Results, Supplemental Fig. 1) in which the percentage of cells undergoing apoptosis was calculated using Hoechst H33342 (Sigma-Aldrich) nuclear staining, as described previously (30). Analysis also indicated that 4% of cells were necrotic for treatment conditions (including digitalis, toxocarcinobacarycin iodide binding and propidum iodide incorporation (data not shown)). BV2 cells were seeded at 3 × 105 cells/well in 12-well tissue culture plates for 24 h prior to the assay. PC12 cells (after prior overnight treatment with vehicle or 6-OHDA) were then added to the BV2 cells at a ratio of 3:1, chosen so that apoptotic targets were not a limiting factor, and cocultured for 2 h at 37°C. 5% CO2, in the BV2-conditioned medium unless otherwise stated. Cocultures were then rapidly washed four times with ice-cold PBS, fixed by incubation with 2% formaldehyde in PBS for 15 min at room temperature, washed once more with PBS, and mounted under glass coverslips using Moviol mounting agent. Cells were then examined at ×20 magnification with a Nikon TE2000U inverted microscope linked to a Hamamatsu C4742-95 charge-coupled device camera (Hamatsu Photonics, Welwyn Garden City, U.K.) and an Apple Macintosh G5 computer (Apple, Cupertino, CA) running OpenLab 5.5 software (ImproVision, Coventry, U.K.) under phase contrast (to visualize all cells) and fluorescent light (to visualize CMFDA-labeled PC12 cells). Images were captured from 25 random fields for each well assayed, and the proportion of phagocytosing BV2 cells was calculated for each well.

Primary microglial cultures

Primary murine microglial cultures were prepared from 12-wk-old female ANXA1 null and C57BL/6 wild-type mice, according to published protocols (31). Cells were plated at 1.5 × 105 cells/well in 12-well plates and cultured in DMEM supplemented with 10% FCS, 100 μM nonessential amino acids, 2 mM L-alanyl-glutamine, 50 μg/ml gentamycin (all Invitrogen
The resultant cDNA was used for PCR using published primer sequences for

**Western blotting**

Expression of total ANXA1 in BV2 and PC12 cells was analyzed by Western blot, essentially as described previously (33). Briefly, total protein was extracted from the cells by freeze/thawing (34), the protein content was estimated (35), and the sample was analyzed after storage at −80 °C. Samples (10 μg protein/well) were loaded onto 10% acrylamide gels and separated by SDS-PAGE in denaturing conditions at 50 mA for 90 min. The separated proteins were then transferred electrophoretically (100 mA blot, 70 min; 2117 Multiphor II; Pharmacia LKB, Bromma, Sweden) to nitrocellulose paper (Hybond-P; GE Healthcare, Buckinghamshire, U.K.) soaked in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol v/v; all from Sigma-Aldrich). Nonspecific binding was blocked by incubation in 5% milk powder (Marvel, Knighton Edbaston, Stafford, U.K.) in TBS-Tween [50 mM Tris, 150 mM NaCl, and 0.1% Tween v/v (all from Sigma-Aldrich)] for 60 min. After washing, blots were incubated overnight at 4 °C in a polyclonal Ab raised against ANXA1 (Invitrogen U.K.; diluted 1/1000 in 5% milk in TBS-Tween) and then for 1 h at room temperature with an HRP-conjugated sheep anti-rabbit polyclonal antiserum (Serotec, Oxford, U.K.; diluted 1/2000 in TBS-Tween). Immunoreactive protein bands were detected by chemiluminescence using ECL reagents and brief exposure to Hyperfilm (all from GE Healthcare). The blots were then scanned and analyzed with Adobe Photodeluxe Business Edition, version 1.1.

**RT-PCR**

RT-PCR analysis with gene-specific primers was used to detect mRNA for murine ANXA1, Fpr1, Fpr2, and Fpr3 and LPS signaling pathway molecules in BV2 or PC12 cells. Cells were collected by centrifugation at 800g for 5 min, the protein content of the pellets was estimated by a protein assay (MicroBeads; Pharmacia LKB, Piscataway, NJ), and 2 μg of total RNA were used for each reaction. The reverse transcriptase reaction was carried out in a final volume of 20 μl containing 2 μg total RNA, 50 μM of each 2′-deoxynucleoside 5′-triphosphate for 20 min at 42 °C. The resultant cDNA was used for PCR using published primer sequences for murine ANXA1, Fpr1, Fpr2, and Fpr3 and LPS signaling pathway molecules in BV2 or PC12 cells. Cells were prepared for electron microscopy as described previously (37). In brief, cells were fixed in 0.05% glutaraldehyde and 3% formaldehyde in 0.1 M PBS (pH 7.4) for 20 min at 4 °C, washed briefly in PBS, and fixed for 1 h at room temperature with 2% formaldehyde in PBS at 4 °C. The cryoprotected cells were then added to the plate in triplicates and incubated for 1 h at 37 °C in incubation buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1% FCS, and 0.05% Tween 20). Wells were washed, and incubated with a rabbit anti-ANXA1 polyclonal Ab, diluted 1/1000 in TBS-Tween, in incubation buffer for 1 h at 37 °C. Wells were washed and incubated with HRP-conjugated sheep anti-rabbit polyclonal antiserum (Serotec; diluted 1/500 in incubation buffer) for 1 h at 37 °C. Plates were washed and incubated with 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich) for 10 min at room temperature, with the reaction being stopped by addition of 0.5 M H2SO4. A spectrophotometer (Versamax Microplate Reader; MDS Analytical Technologies, Sunnyvale, CA) was then used to read absorbance at 450 nm, with plate correction being made at 570 nm.

**Flow cytometry analysis**

Aliquots of the BV2 or PC12 cells in suspension (106 cells in 200 μl, prepared by gentle scraping of cells in culture with a rubber policeman) were fixed by incubation for 10 min with 2% formaldehyde in PBS at room temperature, washed in PBS, and incubated for 30 min at room temperature in 1% NGS in PBS to block nonspecific secondary Ab binding. Cells were then incubated for 1 h at room temperature in the presence or absence (control) of primary Ab (50 ng/ml murine monoclonal anti-ANXA1 Ab, 5 μg/ml rabbit polyclonal anti-Fpr1, or 5 μg/ml rabbit polyclonal anti-Fpr2) in 0.1% NGS. The cells were then washed in PBS and incubated for 30 min with either 488-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rabbit IgG secondary Ab (diluted 1/100). Flow cytometry analysis was performed on a FACScan II flow cytometer (BD Biosciences, Cowley, U.K.) with an air-cooled 100-mW argon laser tuned to 488 nm, connected to Apple Macintosh G4 computer (Apple) running CellQuest II software (BD Biosciences). ANXA1 expression was recorded as units of fluorescence where the median fluorescence intensity for 10,000 cells was measured in the FL1 green channel (548 nm).

**Transfection of BV2 cells with sense or antisense cDNA for ANXA1**

BV2 cells were transiently transfected with pRc/CMV plasmids containing: 1) a 476-bp 5′ fragment from the full-length human ANXA1 cDNA sequence, termed ANXA1-sense; 2) an antisense sequence covering the same range of the ANXA1 cDNA, termed ANXA1-antisense (41); or 3) the full-length cDNA for ANXA1 (1040 bp). Briefly, cells were plated at 3 × 104 cells/well in 12-well tissue culture plates in RPMI 1640 medium containing 5% FCS for 24 h prior to transfection using Fugene-6 reagent (Promega), according to the manufacturer’s protocol. Cells were grown in the same medium for 72 h prior to their use in a phagocytosis assay as described above. The efficiency of the ANXA1-antisense construct was assessed by examining ANXA1 expression in BV2 cells transfected with the two constructs by flow cytometry: whereas ANXA1-sense-transfected cells showed similar expression of ANXA1 to untransfected controls, those cells transfected with ANXA1-antisense showed a significant reduction of 13.98 ± 0.86% (Supplemental Fig. 2A). Similarly, transient
transfection of BV2 cells with the full-length ANXA1 sequence resulted in a significant gain in immunoreactivity for ANXA1, compared with either native cells or cells transfected with an empty pRc/CMV vector (Supplemental Fig. 2B).

**Immunohistochemistry and immunofluorescence**

**Marine samples.** Male wild-type C57BL/6 mice were transcardially perfused with ice-cold 0.9% saline, followed by 4% formaldehyde in 0.1 M PBS; all animal housing and handling were in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986, under Project License 70/6483. Brains were rapidly removed and postfixed for 12 h in the same fixative, prior to cryoprotection in 30% sucrose in 0.1 M PBS, and storage at −80°C. Coronal sections (15 μm) were cut throughout the brain using a cryostat (Bright Instruments, Huntingdon, U.K.) maintained at −22°C and were stored in antifreeze (0.1 M NaH2PO4·H2O, 0.05 M Na2HPO4, 0.15 mM NaCl, 50% v/v ethanediol, 1% w/v polyvinylpyrrolidone, and 0.1% w/v Na3citrate) at −20°C until required. Free-floating sections were rinsed in 0.05 mM TBS, permeabilized by incubation for 5 min in 0.05%/v/v Triton X-100 in TBS, and incubated for 1 h at room temperature in 20% NGS in TBS (NGS; Serotec) to saturate nonspecific binding. Sections were then incubated for 16 h at 4°C in 1% NGS containing rabbit anti-mouse ANXA1 (1/2000; Invitrogen U.K.) and mouse antismouse CD11b (1/1000, Abcam, Cambridge, U.K.). Following three rinses in 1% NGS, sections were incubated for 2 h at room temperature in AF594-conjugated goat anti-rabbit (1/500; Invitrogen U.K.) and AF488-conjugated goat anti-mouse (1/500; Invitrogen U.K.) in 1% NGS. Sections were rinsed three times in TBS and mounted on gelatin-coated microscope slides, allowed to air-dry, and coverslipped using Vectashield (Vector Laboratories U.K.) mountant. Staining was then analyzed by confocal microscopy as described below.

**Cell lines.** CMFDA-labeled PC12 cells were plated in bovine type I collagen-coated microwell slides (Labtek II; Fisher Scientific, Loughborough, U.K.) at a density of 5 × 10⁶ cells/cm² for 24 h prior to treatment with 40 μM Na3AsO3 vehicle or 80 μM 6-OHDA hydrobromide in vehicle overnight. BV2 cells were plated at a density of 5 × 10⁴ cells/cm² for 24 h, prior to coculture with CMFDA-labeled PC12 cells, previously treated overnight with 80 μM 6-OHDA or vehicle, at a ratio of 3:1 for 2 h at 37°C and 5% CO₂ in the BV2-conditioned medium. Cells were washed four times with ice-cold PBS, fixed by incubation with 2% formaldehyde in PBS for 15 min at room temperature, and immunostained for the presence of cell surface ANXA1. Briefly, nonspecific binding was blocked by incubation for 30 min with 5% NGS, cells were then incubated for 2 h with a polyclonal rabbit anti-ANXA1 Ab (diluted 1/500 in 0.1% NGS; Invitrogen U.K.), rinsed three times with 0.1% NGS, and incubated for 1 h with an AF594-conjugated goat anti-rabbit secondary Ab (diluted 1/300 in 0.1% NGS; Invitrogen U.K.). Cells were rinsed three times in PBS and mounted under glass coverslips using Moviol mountant, prior to analysis by confocal microscopy as described below.

Confocal images were captured using a Leica TCS SP2 confocal laser scanning microscope system (Leica Microsystems, Heidelberg, Germany) fitted with argon/argon-krypton and helium-neon lasers, exciting at 488 and 561 nm, respectively, and attached to a Leica DM IRBE inverted microscope fitted with a ×100 objective lens. Images were captured with Leica confocal software version 2.6.1 and were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Bright-field images were captured using a Nikon Eclipse E800 microscope fitted with a CoolSNAP-ProCF charge-coupled device camera (Roper Scientific, Marlow, U.K.) using a ×40 objective lens. Images were captured using ImageProPlus, version 4.5.1 (Media Cybernetics, Finchampstead, U.K.).

**Statistical analysis**

All quantified data are derived from at least three independent experiments, performed in triplicate, and are expressed as the mean ± SEM. Data were analyzed by one- or two-way ANOVA as appropriate, with post hoc comparison using Bonferroni’s corrected Student t test. In all cases, a p ≤ 0.05 was taken as indicating statistical significance.

**Results**

**ANXA1 is expressed in human microglia and upregulated in Alzheimer’s disease**

Aging human brains revealed expression of ANXA1 in a few microglial cells identified with Iba1 and MHC II. ANXA1-positive microglia were present in the gray matter and exhibited amoeboid

![FIGURE 1. Analysis of ANXA1 expression in human microglia and characterization of the phagocytosis of PC12 cells by BV2 cells. Representative photomicrographs of serial sections from the gray matter of an aging human brain show Iba1-positive microglia (ABC/peroxidase, original magnification ×10; A) and a single ameboid microglial cell (asterisk and inset, original magnification ×10 and ×60, respectively; B). Coexpression of ANXA1 in activated microglia in a case of Alzheimer’s disease is seen in C using combined ABC/alkaline phosphatase (ANXA, blue color indicated by asterisk) (MHC II in brown, original magnification ×40). In the cortex of a male subject with Alzheimer’s disease (D), double immunofluorescence histochemistry shows ANXA1 immunoreactivity (red) associated with amyloid (Congo red, staining blue following exposure to DAPI) (×20). The graph (E) shows that in cocultures, the proportion of BV2 cells actively phagocytosing PC12 cells was significantly greater when PC12 cells had been treated overnight with the neurotoxin 6-OHDA (80 μM) compared with PC12 cells exposed only to vehicle (sodium metabisulphite). Data are mean ± SEM, n = 3. *p < 0.05.**
morphology (Fig. 1A, 1B). Brains of Alzheimer’s disease patients showed more robust expression of ANXA1 in activated microglia, which were mainly localized in the gray matter. ANXA1 was seen to colocalize with MHC II (Fig. 1C). Macrophages associated with amyloid plaques also expressed ANXA1 (Fig. 1D). Endothelial cells and mononuclear cells within vessels were positive in all samples and were considered an internal positive control.

**BV2 microglia nonphlogistically phagocytose apoptotic PC12 cells**

The expression of ANXA1 in human microglia, particularly in Alzheimer’s disease, led us to investigate whether and how ANXA1 plays a role in microglial phagocytosis of apoptotic neurons. Sixteen hours’ incubation of fluorescently labeled PC12 cells with 80 μM 6-OHDA routinely induced an apoptotic rate of between 30 and 40% of cells. These experiments also confirmed that under these conditions the effects of the neurotoxin were selective for PC12 cells, because apoptosis could not be induced in the neuroendocrine AtT20 cells or BV2 cells (Supplemental Fig. 1). Fig. 1E shows that the phagocytosis of PC12 cells by BV2 microglia was readily detectable after 2 h of contact time and that preincubation of PC12 cells with the neurotoxin increased this 4-fold. The index of phagocytosis, a measure of the number of PC12 cells phagocytosed per BV2 cell, similarly showed a significant effect of exposure to apoptotic PC12 cells, rising from 1.26 ± 0.08 for vehicle-treated PC12 cells to 2.36 ± 0.25 with apoptotic PC12 cells (p < 0.05). To investigate whether this response was associated with a microglial inflammatory reaction, we assessed the levels of the proinflammatory cytokines IL-6 and TNF-α and the anti-inflammatory cytokine IL-10 in the medium at the end of the 2-h incubation period (Table I). As expected, no detectable levels of cytokines were produced by PC12 cells, irrespective of their pretreatment with neurotoxin or vehicle control. The levels of IL-6 in the BV2 microglial incubation medium were undetectable under all conditions tested. In contrast, TNF-α and IL-10 were secreted constitutively at relatively low levels, and contact with toxin-treated PC12 cells (PC12t), but not vehicle-treated controls (PC12v), significantly reduced TNF-α and increased IL-10 levels, strongly indicating that the phagocytic reaction was noninflammatory.

**A role for ANXA1**

The current view of ANXA1-mediated phagocytosis, derived from macrophage engulfment of apoptotic neutrophils, suggests that the dying cells externalize and release the protein, which acts as an “eat me” signal for the phagocytes (42, 43). However, in our model, neither intact nor apoptotic PC12 cells release ANXA1 into the incubation medium (see Table I). Moreover, RT-PCR, Western blot, and flow cytometry analyses, as well as electron microscopy, established that ANXA1 mRNA and protein were not present in PC12 cells (Supplemental Fig. 3). In contrast, significant amounts of ANXA1 were released by BV2 cells (Table I), which also expressed ANXA1 mRNA and protein (Supplemental Fig. 3), leading us to hypothesize that ANXA1 released by the microglia plays a role in the phagocytosis of the apoptotic neuronal cells.

To examine whether the ANXA1 released by BV2 microglia was a limiting factor contributing to the phagocytosis of apoptotic neuronal cells, we first investigated the effects of depletion of ANXA1 in the incubation medium. Rather than adding PC12 cells to BV2 cells bathed by 24-h BV2-conditioned medium, the BV2-conditioned medium was replaced with the medium in which the PC12 cells had been cultured. Reduction in BV2-secreted ANXA1 in this way significantly attenuated PC12 cell engulfment (Fig. 2A), suggesting an important role for microglial-derived soluble factors, such as ANXA1, in regulating phagocytosis. In support of this, treatment of apoptotic but not control PC12 cells with exogenous ANXA1 (500 pM) for 30 min prior to coculture with the microglia markedly augmented phagocytosis of neurotoxin-treated PC12 cells (Table II).

To investigate the role of ANXA1 in promoting microglial phagocytosis, a previously characterized neutralizing anti-ANXA1 mAb (39, 44) was included in the phagocytosis assay incubation medium. This Ab, but not an IgG2A isotype control, was able to completely abolish phagocytosis of apoptotic cells by microglia (Fig. 2B).

Confirmation of a role for endogenously produced ANXA1 was obtained by transfection of BV2 cells with an antisense sequence to ANXA1 (41). The treatment with antisense significantly reduced phagocytosis of 6-OHDA–treated PC12 cells but not the control PC12 (Fig. 2C). Overexpression of full-length ANXA1 in BV2 cells caused a marked augmentation in phagocytosis (Fig. 2D), further confirming the importance of ANXA1 in the regulation of phagocytosis.

To confirm the central role for ANXA1 in microglial phagocytosis, we analyzed the phagocytic ability of primary microglia isolated ex vivo from adult wild-type and ANXA1 null mice. Although microglia from wild-type animals exhibited a strong upregulation in phagocytosis when cocultured for 2 h with apoptotic PC12 cells, microglia from ANXA1 null mice did not respond to the presence of apoptotic cells (Fig. 2E).

**ANXA1 bridges apoptotic neurons and microglia**

Measurement of ANXA1 in the medium following incubation of PC12 cells with the BV2 cells in 24 h BV2-conditioned medium revealed that coincubation of apoptotic, but not control, PC12 cells with the microglia led to a significant reduction in free ANXA1 (Table I). Although the finding that a reduction in ANXA1 levels in the incubation medium was coincident with an increase in phagocytosis may at first appear counterintuitive to our hypothesis, this observation is also compatible with the view that “free” ANXA1 in the medium is required to bind to apoptotic cells to allow phagocytosis to proceed effectively. The subcellular localization of ANXA1 was then investigated using confocal microscopy. ANXA1 was localized on the surface of the PC12 cells that

### Table 1. Release of inflammatory factors by BV2 cells and PC12 cells alone or in coculture

<table>
<thead>
<tr>
<th></th>
<th>BV2</th>
<th>PC12v or PC12t</th>
<th>BV2 + PC12v</th>
<th>BV2 + PC12t</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (nM)</td>
<td>1.86 ± 0.14</td>
<td>Undetectable</td>
<td>1.91 ± 0.16</td>
<td>1.41 ± 0.03*</td>
</tr>
<tr>
<td>IL-6 (nM)</td>
<td>Undetectable</td>
<td>Undetectable</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
<tr>
<td>IL-10 (nM)</td>
<td>0.96 ± 0.26</td>
<td>Undetectable</td>
<td>0.10 ± 0.06</td>
<td>1.31 ± 0.11*</td>
</tr>
<tr>
<td>ANXA1 (pM)</td>
<td>67.14 ± 6.32</td>
<td>Undetectable</td>
<td>57.71 ± 6.45</td>
<td>38.72 ± 4.28*</td>
</tr>
</tbody>
</table>

Soluble factors were measured by ELISA in the medium of cultures of BV2 cells (24 h of incubation), PC12 cells treated with either vehicle (PC12v) or 6-OHDA (PC12t, overnight incubation), or BV2-PC12 cocultures (24-h incubation of BV2 cells, followed by 2 h coincubation with PC12 cells). Data are mean ± SEM, n = 3.

*p < 0.05 versus BV2 cells alone and BV2 cells cocultured with PC12v.
were actively undergoing phagocytosis (Fig. 3A). Furthermore, flow cytometry analysis confirmed that ANXA1 was localized on the surface of 6-OHDA–treated, but not vehicle-treated, PC12 cells 2 h after incubation with 24-h BV2-conditioned medium (Fig. 3B). Taken together, these data provide strong evidence that microglial-derived ANXA1 binds to the surface of apoptotic but not intact neuronal cells.

A key feature of apoptotic cells is the surface expression of phosphatidylserine (PS), to which members of the annexin family (ANXA1 and ANXA5) are known to bind with high affinity in a calcium-dependent manner (45). As ANXA1 is expressed on the cell surface of BV2 microglial cells in addition to binding to the surface of apoptotic PC12 cells, it is possible that it may directly bridge the two cell types via the formation of a homodimer involving the two N-terminal sequences (46). To investigate this, we depleted ANXA1 from the surface of the BV2 cells by incubation with 10 mM EDTA for 10 min (47), followed by resuspension of cocultures in either BV2-conditioned or fresh medium (see Fig. 3C). As may be predicted from our data in Table I, in the absence of ANXA1-containing medium, phagocytosis

**FIGURE 2.** ANXA1 is critically required for efficient phagocytosis by BV2 cells of apoptotic PC12 cells. A, Coculture of BV2 cells and PC12 cells in PC12-conditioned medium significantly attenuates the clearance of apoptotic PC12 cells when compared with resuspension in BV2-conditioned medium (white column, PC12V; gray column, PC12T; data are mean ± SEM, n = 3). B, Inclusion of a neutralizing anti-ANXA1 mAb, but not an irrelevant IgG, significantly reduces phagocytosis of apoptotic PC12 cells by BV2 cells (white column, PC12V; gray column, PC12T; data are mean ± SEM, n = 3). C, Depletion of BV2 cell ANXA1 content by transient transfection with an antisense, but not a sense, sequence to ANXA1 significantly inhibits the phagocytosis of apoptotic PC12 cells (white column, PC12V; gray column, PC12T; data are mean ± SEM, n = 3). D, Overexpression of full-length ANXA1 in BV2 cells (pRCMV-ANXA1) enhances the phagocytosis of apoptotic PC12 cells relative to either untransfected BV2 cells or BV2 cells transfected with the empty pRc/CMV plasmid (white column, PC12V; gray column, PC12T; data are mean ± SEM, n = 3). E, Impaired phagocytosis in primary microglial cultures from wild-type and ANXA1 null mice (white column, PC12V; gray column, PC12T; data are mean ± SEM, n = 3).
Efferocytosis of apoptotic PC12 cells by BV2 cells is potentiated by 30-min treatment prior to coculture of PC12 cells with either 500 pM hrANXA1 or 500 pM of a chimeric protein composed of the N-terminal of ANXA1 and the C-terminal PS-binding domain of ANXA5 (ANXA1-5) and is inhibited by occlusion of PS binding sites with 1 μM recombinant full-length ANXA5 (hrANXA5). Data are mean ± SEM, n = 3.*p < 0.05 versus relative PC12v control; **p < 0.05 versus untreated PC12v group.

failed to occur, and removal of ANXA1 from the surface of BV2 cells failed to prevent phagocytosis. These data reinforce the concept that microglial-derived ANXA1 is required for phagocytosis of apoptotic neurones and indicate that ANXA1 binding to the surface of apoptotic PC12 cells is absolutely required for efficient phagocytosis, but its surface expression on BV2 cells is not necessary.

Annexin A5 also binds avidly to PS but lacks the N-terminal sequence of ANXA1, which bestows much of its biological activity (48). Table II shows that treatment of apoptotic PC12 cells with ANXA5 blocked their engulfment by the BV2 cells. In contrast, a chimeric protein containing the N-terminal, bioactive fragment of ANXA1 and the core, PS-binding domain of ANXA5 (44) significantly enhanced phagocytosis (Table II). These data point to the importance of both the binding of the core domain to PS on the surface of apoptotic neuronal cells, as well as N-terminal signaling to microglia for efficient phagocytosis.

Thus far, our experiments have demonstrated a role for ANXA1 in the removal of apoptotic neuronal cells by microglia under non-inflammatory conditions. However, microglia are highly reactive to inflammatory stimuli, and indeed, our studies of human brains with

FIGURE 3. Binding of ANXA1 to the surface of apoptotic PC12 cells is required for efficient phagocytosis. A, Representative confocal photomicrographs (original magnification ×800) of surface ANXA1 expression (red) in normal PC12 (labeled with CMFDA, green) and BV2 cells and in cells undergoing active phagocytosis. B, Flow cytometry analysis indicating that ANXA1 derived from BV2-conditioned medium binds to the cell surface of apoptotic (PC12v) but not vehicle treated (PC12v). C, Although the removal of surface ANXA1 from BV2 cells alone has no effect on phagocytosis, removal of both surface and soluble ANXA1 significantly attenuates phagocytic clearance of PC12v by microglia.

Influence of inflammatory stimulus on microglia phagocytosis

These data strongly suggest that ANXA1 does indeed signal to the BV2 microglia via this pathway, with the Fpr2 family member being the most important receptor involved.
Alzheimer’s disease, where Aβ is known to trigger an inflammatory response (51, 52), indicated expression of ANXA1 in microglia associated with amyloid plaques. Therefore, we also sought to investigate microglial phagocytic behavior under inflammatory stress, as modeled by their preincubation with bacterial LPS or fibrillar Aβ1–42 (Anaspec, San Jose, CA). Initial characterization of BV2 cells by RT-PCR confirmed the presence of the major signaling molecules required for the response to LPS, namely TLRs 2 and 4, CD14, and MD2 (Supplemental Fig. 4).

Activation of the microglial cells by LPS was assessed by measuring NO$_2^-$ production; after 0-, 2-, 6-, 18-, 48-, and 72-h incubation periods with LPS, NO$_2^-$ levels increased steadily, measuring 7.8 ± 1.1, 13.8 ± 1.1, 15.7 ± 3.7, 20.5 ± 2.7, 27.9 ± 0.5, and 50.1 ± 4.4 μM/mg cell protein, respectively. In addition, flow cytometric analysis of cell surface markers indicated that LPS induced a classically activated phenotype (CD40/CD206% positive cells: basal 1.51 ± 0.10%/57.0 ± 2.69% versus LPS 35.85 ± 0.67%/0.25 ± 0.01%). We next analyzed the impact that preincubation with LPS had on the phagocytic activity of the microglial cells during the 2-h period of coculture with the PC12 cells. Initial studies indicated that exposure of PC12 cells to LPS had no significant impact on their phagocytosis by BV2 cells (without LPS exposure: PC12v 5.92 ± 0.22%, PC12r 9.32 ± 0.38%, following 2-h exposure of PC12 cells to 50 ng/ml LPS: PC12v 5.32 ± 0.38%, PC12r 10.87 ± 0.36%). In contrast, prior exposure of BV2 cells to LPS (50 ng/ml) for 2, 18, 48, or 72 h significantly enhanced their ability to phagocytose apoptotic, PC12r at all time points tested (p < 0.05). The increments were, respectively, 9.9 ± 0.7, 20.7 ± 0.2, 14.9 ± 0.9, and 11.7 ± 0.7%, demonstrating a bell-shaped response curve with maximal effect at 18 h. Most noteworthy, however, LPS pretreatment markedly increased the phagocytosis of nonapoptotic, vehicle-treated, control PC12 cells with the same pattern of time dependency as the apoptotic cells (Fig. 5A). Similarly, pretreatment of BV2 cells with LPS significantly enhanced the phagocytic index in cocultures with both normal and apoptotic PC12 cells (vehicle-treated PC12 cells 2.46 ± 0.24, 6-OHDA–treated PC12 cells 3.33 ± 0.32; p < 0.05 for both compared with their relative controls). To confirm that this phenotypic change had relevance for different proinflammatory activators of microglia and was not specific to LPS, we used fibrillar Aβ1–42 known to induce microglia activation in vitro through TLR2 (53), revealing highly similar changes in phagocytosis (Supplemental Fig. 5). These results indicate that inflammatory activation alters the behavior of BV2 cells such that they inappropriately phagocytose “normal” cells. To investigate the underlying mechanisms, the release of the major proinflammatory cytokines TNF-α and IL-6, and the anti-inflammatory cytokine IL-10 during the 2-h coculture window was measured.

Preincubation with LPS caused a significant elevation in the amount of all three cytokines released during coculture compared with untreated conditions, but the time course for the proinflammatory and anti-inflammatory cytokines was markedly different. Fig. 5B shows the concentrations in the medium of BV2 cells cocultured with nonapoptotic cells, which were not significantly different from the concentrations achieved in the medium after coculture with apoptotic cells (data not shown). Release of both TNF-α and IL-6 was greatest after 18-h exposure of BV2 cells to LPS, with lower but still elevated levels after 48 and 72 h. The anti-inflammatory cytokine IL-10, however, showed a very different pattern, as shorter preincubations of BV2 cells caused very high levels of IL-10 secretion. Interestingly, treatment of BV2 cells with LPS for 18 h caused a dose-dependent increase in release of ANXA1 into the culture medium (0 ng/ml, 78.3 ± 1.3 pm; 50 ng/ml, 160.8 ± 0.7 pm; 100 ng/ml 175.4 ± 3.6 pm; p < 0.05 versus untreated cells), suggestive of an endogenous attempt at resolution, given the known role of ANXA1 in this process.

Having determined that priming of BV2 cells with LPS caused a significant change in their behavior, we investigated whether exogenous ANXA1 was able to reverse the effects of LPS. Inclusion of 500 nM ANXA1 in the cocultures significantly reduced...
the phagocytosis by LPS-stimulated BV2 cells of vehicle-treated, nonapoptotic PC12 cells (Fig. 5C). Measure of NO\textsubscript{2}- accumulation over the coculture period of the phagocytosis assay, as a measure of its inflammatory nature revealed that ANXA1 significantly limited NO production, strongly suggesting that the protein was able to reverse the LPS-induced activation of BV2 cells (Fig. 5D).

Together, the data from this paper on the role of ANXA1 in microglial phagocytosis are schematised in Fig. 6.

Discussion
Recent years have seen an explosion in interest in the role of microglia in the brain, both as part of normal development and aging and in the context of neurodegenerative diseases (54). A key reason for this interest lies in the high vulnerability of neurones to inflammatory damage and the central role of microglia as coordinators of inflammatory processes (55, 56). In particular, the efficient removal of apoptotic neurones is critical for the maintenance of homeostasis within the brain parenchyma, and microglia, as the primary phagocytic cells of the CNS, are intimately involved in this process (57). However, the mechanisms for removal of apoptotic cells in the CNS are unclear. In this study, we show that under noninflammatory conditions, endogenous microglial-derived ANXA1 plays a key role in ensuring the effective and selective removal of apoptotic, but not healthy, neuron-like cells, involving the formation of a bridge from the apoptotic cells to the microglia by secreted ANXA1 signaling through the Fpr2 receptor.

ANXA1 was initially identified as a potent anti-inflammatory protein in the periphery where it has pluripotent anti-inflammatory actions serving, for example, to both limit leukocyte entry into tissue (52) and to promote leukocyte apoptosis (30, 41) and phagocytosis (43), aiding resolution. To our knowledge, the current study is the first report of an anti-inflammatory role of ANXA1 within the CNS, further reinforcing the function of this protein in limiting the extent of inflammatory reactions and providing a justification for its hitherto unexplained presence in microglia (15–17).

Our data strongly suggest that ANXA1 could be a limiting factor in the switch from the protective face of microglial phagocytosis (removal only of potentially damaging material) to the situation in inflammatory conditions where microglial phagocytosis is considered an important contributor to tissue damage and loss of healthy neurones (Fig. 6). In particular, the discovery that BV2 microglia constitutively express and release ANXA1 and that the phagocytosis of apoptotic PC12 cells induces an anti-inflammatory state supports the hypothesis that ANXA1 is an important component of the proactive surveillance function of the microglia (2), involving the rapid identification and removal of apoptotic neurones.

In the normal brain, one would expect negligible stimuli for microglial apoptosis in keeping with the very low expression of ANXA1. However, during development and in select areas of the adult brain, such as the hippocampal dentate gyrus and the subventricular zone (58), neurogenesis is an active process that is also associated with significant “physiological” cell death (59). Clearly, it is important that any apoptotic cells generated in this process do not induce an inflammatory reaction, a consideration that may explain the particularly high density of microglia in these brain regions (60). As our work has demonstrated, a fundamental role for ANXA1 in the phagocytic clearance of apoptotic cells by microglia, a role for ANXA1 in brain regions associated with the
cycle of neurogenesis and apoptotic cell death thus seems highly likely.

Our studies also provide the first demonstration, to our knowledge, that ANXA1 is upregulated in human microglia in Alzheimer’s disease, supporting a possible role for the protein in regulating the microglial response to amyloid plaques and inflammatory response in neurodegeneration (51). This view is strongly supported by our findings that exogenous ANXA1 can suppress microglial activation by an inflammatory challenge.

The mechanism of action we have identified for ANXA1 in phagocytosis clearly reinforces the concept that ANXA1 acts as a ligand for two specific sites, binding through its core domain to PS externalized on the surface of the apoptotic cell and to a cell surface receptor via its N-terminal sequence (44, 49). Extensive evidence has been accrued suggesting that Fpr family members, and in particular Fpr2, serve as this recognition site (61, 62), and our data would further support this idea. Although Fpr family members have received much attention because of their role in microglial chemotaxis (63, 64), we present what we believe is novel evidence for a complementary function of Fpr2 in the clearance of apoptotic cells by microglia, suggesting that this receptor has a key role in the neuroinflammatory response. Interestingly, recent reports have suggested an interaction between Fpr2 and the scavenger receptor MARCO in astrocytes and microglia (65), which is thought to be involved in the phagocytosis of small molecules such as Aβ1-42 (66). It remains to be seen, however, whether an interaction between these two receptors plays any role in the more complex, anti-inflammatory removal of apoptotic cells investigated in the current work.

The identification of microglial Fpr2 as a receptor for ANXA1, together with our identification of strong expression of ANXA1 in neuritic plaque-associated microglia in Alzheimer’s disease, suggests a fascinating connection with the published data indicating a link between Aβ and Fpr2 (67). Microglia have clearly been shown to phagocytose Aβ through this receptor, but they appear unable to digest this protein, leading to persistent internalization of Aβ/Fpr2 complexes and culminating in intracellular fibril formation and apoptosis (64). The binding of ANXA1 to Fpr2 in microglia may thus be able to disrupt the interaction of the receptor with Aβ, potentially being of significant benefit in the treatment of Alzheimer’s disease.

Beyond a potential interaction among ANXA1, Aβ, and Fpr2, however, and based on our understanding of the role of ANXA1 in other pathologies (68, 69), as well as the known damaging effects of unresolved inflammation upon neurons, we would suggest that the robust expression of ANXA1 at lesion sites may represent an attempt by the innate brain immune system to limit the inflammatory response to neuronal death and thus restrict further cell loss (70–72). Our in vitro study of the role of ANXA1 in inflammatory phagocytosis mimics this phenomenon and moreover suggests that in contrast to the ability of “resting” BV2 cells to discriminate between apoptotic and nonapoptotic PC12 cells, inflammatory activation of the microglia leads to indiscriminate phagocytosis of nonapoptotic neurones, an event associated with the release of proinflammatory cytokines and NO. Importantly, however, we have shown that application of high concentrations of exogenous ANXA1 can suppress both inappropriate microglial phagocytosis of nonapoptotic cells and the production of inflammatory mediators during the phagocytic reaction. Further studies are needed to determine whether this action is recapitulated in the inflamed brain and to realize the potential significance of ANXA1 in the control of neuroinflammatory disease.

A notable feature of microglia associated with amyloid plaques, and indeed of BV2 cells treated with inflammatory stimuli such as LPS, was a marked upregulation in ANXA1 expression. This is in
clear contrast to ANXA1 expression in microglia in areas of the brain distal to inflammatory stimuli, where levels of the protein were limited. Given the powerful anti-inflammatory/proresolution role of ANXA1 we have identified, it is intriguing to speculate that the protein may thus serve as a marker of a beneficial microglial phenotype, an idea ripe for future investigation.

In summary, our data provide clear evidence of two complementary roles for microglial ANXA1: 1) controlling the noninflammatory phagocytosis of apoptotic neurons and 2) promoting resolution of inflammatory microglial activation. Clearly, it is of great importance to investigate the potential role of ANXA1 in conditions of neuroinflammation, and in particular in Alzheimer’s disease, where it may have great potential for therapeutic development.

Disclosures

The authors have no financial conflicts of interest.

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12. Perretti, M., and J. C. Buckingham. 2006. Annexin A1 and glucocorticoids as markers of a beneficial microglial role of ANXA1 we have identified, it is intriguing to speculate that the protein may thus serve as a marker of a beneficial microglial phenotype, an idea ripe for future investigation.

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References

12 ANNEXIN A1’S ROLE IN MICROGLIA SURVEILLANCE


Supplemental Figure 1: Characterisation of the effects of 6-hydroxydopamine of PC12 and BV2 cells. A) Treatment of PC12 cells for 16 hours with 6-hydroxydopamine causes a dose-dependent increase in apoptosis, as assessed by Hoechst staining. This toxic effect of 6-hydroxydopamine is selective for PC12 cells, as the neuroendocrine cell line AtT20 was unaffected by the toxin at the doses used (data are mean ± SEM, n=3, * p<0.05 vs. untreated PC12 cells). B) Exposure of PC12 cells to 80μM 6-hydroxydopamine causes a time-dependent increase in apoptosis, as assessed by Hoechst staining (data are mean ± SEM, n=3, * p<0.05 vs. untreated PC12 cells). C) BV2 cell survival is unaffected by incubation for 2 hours with increasing doses of 6-hydroxydopamine, whereas equivalent doses of hydrogen peroxide cause a significant and dose-dependent decline in cell survival, as assessed using the MTT assay (BV2 cells treated with 6-hydroxydopamine, BV2 cells treated with hydrogen peroxide, data are mean ± SEM, n=3, * p<0.05 vs. untreated BV2 cells).

Supplemental Figure 2: Characterisation of BV2 cells over- or under-expressing ANXA1. A) Typical histogram showing by flow cytometry analysis that transient transfection of BV2 cells with anti-sense ANXA1 causes a significant downregulation in ANXA1 expression, compared with BV2 cells transfected with an empty pRc/CMV plasmid. B) Typical histogram showing by flow cytometry analysis that transient transfection of BV2 cells with full-length ANXA1 causes a significant increase in ANXA1 expression, compared with both untransfected BV2 cells and BV2 cells transfected with an empty pRc/CMV plasmid.

Supplemental Figure 3: Analysis of ANXA1 expression in BV2 and PC12 cells. A) RT-PCR analysis of ANXA1 mRNA expression in BV2 and PC12 cells, with GAPDH mRNA expression as a positive control. B) Western blot analysis of ANXA1 protein expression in PC12 cells treated overnight with 80μM 6-hydroxydopamine or 40μM sodium metabisulfite vehicle, and in BV2 cells, with β-actin as a loading control. C) Flow cytometry analysis of total ANXA1 protein content in PC12 and BV2 cells, in comparison to curves showing non-specific secondary antibody binding. D) Representative electronmicrographs of a PC12 cell and a BV2 cell immunolabelled for ANXA1, arrows indicate immunogold particles identifying ANXA1, scale bar = 200nm. E) Representative confocal fluorescence micrograph indicating ANXA1 immunoreactivity (red) in a CD11b-identified microglia (green) in the cortex of a male C57Bl/6 mouse.

Supplemental Figure 4: LPS signalling molecule expression in BV2 cells. RT-PCR analysis of the presence of the LPS signalling pathway molecules CD14, MD2, Tlr2 and Tlr4 in BV2 cells, with GAPDH mRNA expression as a positive control.

Supplemental Figure 5: Effects of Aβ1-42 on the phagocytosis of PC12 cells by BV2 cells. Incubation of BV2 cells with fibrillar Aβ1-42 significantly enhances the phagocytosis of both PC12V and PC12T cells compared with control conditions; (PC12V, PC12T, data are mean ± SEM, n=3, *p<0.05, +p<0.05 vs. relative control condition).
Supplementary figure S1

A

Apoptotic cells (%)

0 1 3 6 16 24 48

0

10

20

30

40

50

Exposure to 80µM 6-hydroxydopamine (hours)

BA

0 µM 20 µM 40 µM 80 µM

6-Hydroxydopamine (16 hours)

C

Cell Survival (% of control)

0 20 40 60 80 100 120

0 20 40 60 80 100 120

0 20 40 80 160

Dose (µM)

0 20 40 80 160

0 20 40 60 80 100 120

0 20 40 60 80 100 120

0 20 40 80 160

C

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Supplemental figure S2

A

[Graph showing fluorescence intensity counts for different antibody conditions and cell transfections.]

B

[Graph showing fluorescence intensity counts for different ANXA1 expression conditions and cell transfections.]
Figure S3

A. Gel electrophoresis showing the expression of ANXA1 and GAPDH in Vehicle and 6-OHDA treatments.

B. Western blot analysis showing the expression of ANXA1 (37kDa) and β-Actin (42kDa) in PC12 and BV2 treatments.

C. Flow cytometry analysis showing the expression of ANXA1 in PC12 and BV2 cells.

D. Immunofluorescence images of PC12 and BV2 cells stained with ANXA1 antibody showing co-localization with CD11b.

E. Confocal microscopy images showing CD11b, ANXA1, and merged images.
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Supplementary figure S4
Supplementary figure S5

Phagocytosing BV2 cells (%)

0 5 10 15 20 25 30 35 40 45 50

Control  Aβ1-42

* +