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Estrogen (E2) and glucocorticoid (Gc) effects on microglia and Aβ clearance in vitro and in vivo

Marni E. Harris-White [a,b], Teresa Chu [a,b], Sheryl A. Miller [a,b], Mychica Simmons [a,b], Bruce Teter [a,b], Darrell Nash [a,b], Greg M. Cole [a,b,c], Sally A. Frautschy [a,b,c],*

[a] *Department of Medicine, UCLA, C-128 RNRC, Los Angeles, CA 90095-1769, USA*
[b] *Geriatric Research Education and Clinical Center, Greater Los Angeles Healthcare System, 16111 Plummer St., Sepulveda, CA 91343, USA*
[c] *Department of Neurology, UCLA, C-128 RNRC, Los Angeles, CA 90095-1769, USA*

Abstract

The accumulation of fibrillar aggregates of beta Amyloid (Aβ) in Alzheimer's Disease (AD) brain is associated with chronic brain inflammation. Although activated microglia (µglia) can potentially clear toxic amyloid, chronic activation may lead to excessive production of neurotoxins. Recent epidemiological and clinical data have raised questions about the use of anti-inflammatory steroids (glucocorticoids, Gcs) and estrogens for treatment or prevention of AD. Since very little is known about steroid effects on µglial interactions with amyloid, we investigated the effects of the synthetic Gc dexamethasone (DXM) and 17-β estradiol (E2) in vitro in a murine µglial-like N9 cell line on toxin production and intracellular Aβ accumulation. To determine whether the steroid alterations of Aβ uptake in vitro had relevance in vivo, we examined the effects of these steroids on Aβ accumulation and µglial responses to Aβ infused into rat brain. Our in vitro data demonstrate for the first time that Gc dose-dependently enhanced µglial Aβ accumulation and support previous work showing that E2 enhances Aβ uptake. Despite both steroids enhancing uptake, degradation was impeded, particularly with Gcs. Distinct differences between the two steroids were observed in their effect on toxin production and cell viability. Gc dose-dependently increased toxicity and potentiated Aβ induction of nitric oxide, while E2 promoted cell viability and inhibited Aβ induction of nitric oxide. The steroid enhancement of µglial uptake and impedence of degradation observed in vitro were consistent with observations from in vivo studies. In the brains of Aβ-infused rats, the µglial staining in entorhinal cortex layer 3, not associated with Aβ deposits was increased in response to Aβ infusion and this effect was blocked by feeding rats prednisolone. In contrast, E2 enhanced µglial staining in Aβ-infused rats. Aβ-immunoreactive (ir) deposits were quantitatively smaller, appeared denser, and were associated with robust µglial responses. Despite the fact that steroid produced a smaller more focal deposit, total extracted Aβ in cortical homogenate was elevated. Together, the in vivo and in vitro data support a role for steroids in plaque compaction. Our data are also consistent with the hypothesis that although E2 is less potent than Gc in impeding Aβ degradation, long term exposure to both steroids could reduce Aβ clearance and clinical utility. These data showing Gc potentiation of Aβ-induced µglial toxins may help explain the lack of epidemiological correlation for AD. The failure of both steroids to accelerate Aβ degradation may explain their lack of efficacy for treatment of AD. © 2001 Published by Elsevier Science Ltd.

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1. Introduction

Alzheimer's Disease (AD) is characterized by chronic inflammation indicated by elevation of inflammatory cytokines and other markers. Data suggest that chronic inflammation contributes to progression of AD in part by the associated increase in exposure to neurotoxic mediators of inflammation. Neurotoxic mediators of inflammation include activated complement that can lead to cell lysis and release of reactive oxygen species such as superoxide and nitric oxide-related radicals. Despite chronic µglial activation, peri-plaque µglia in the AD brain frequently appear only partially activated, manifesting ramified rather than the ameboid morphology characteristic of phagocytic cells (Frackowiak et al., 1992). This suggests that phagocytic function is impaired during chronic inflammation, leading

* Corresponding author. Present address: Sepulveda VAMC, GRECC 11E, 16111 Plummer St., Sepulveda, CA 91343, USA. Tel.: +1-818-895-5892; fax: +1-818-895-5835.
E-mail address: frautsch@ucla.edu (S.A. Frautschy).

to further accumulation of Aβ. The occasional observation of chlamydia accumulation in and around plaques (Balin et al., 1998) may also be symptomatic of impaired phagocytosis. Facilitating Aβ clearance may be clinically beneficial and might be accomplished by stimulation of various pathways. Pathways known to mediate Aβ uptake by μglia are the scavenger receptor A or B (Paresce et al., 1996) and Fc-receptor mediated phagocytosis. The role of the lipoprotein receptor-related protein (LRP) is less clear, since although it may mediate Aβ uptake in rodent μglia which do express LRP (Marzolo et al., 2000), it may not be relevant to human μglia which do not appear to express LRP (Rebeck et al., 1993). In AD, LRP appears important for Aβ uptake by neurons (Hyman et al., 2000).

Several epidemiological studies have suggested that prior clinical use of non-steroidal anti-inflammatory drugs (NSAIDs) may reduce risk for or prevent AD (Breitner et al., 1994, 1995; Stewart et al., 1997). Similarly strong epidemiological data suggest that estrogen replacement therapy, but not Gcs, also reduces risk for AD (Kawas et al., 1997; Akiyama et al., 2000). While mechanisms of NSAIDs related to AD are being explored because of potent effects on reducing Aβ accumulation, neuritic pathology and chronic inflammation (Jantzen et al., 2000; Lim et al., 2000), potential mechanisms of steroids on AD-related inflammatory responses in vivo are largely unexplored. Steroids and NSAIDs are anti-inflammatory but likely affect multiple pathways in AD pathogenesis including non-inflammatory pathways regulating neurotrophic and synaptogenic effects and pathways regulating APP cleavage-mediated Aβ production (Xu et al., 1998; Petanceska et al., 2000). Several effects of steroids on inflammatory pathways have been investigated. Both estrogens and Gcs can inhibit μglial proliferation (Ganter et al., 1992; Tanaka et al., 1997). Gcs can also reduce μglia number by causing their rapid degeneration (Kaur et al., 1994; Tanaka et al., 1997). Both steroids can also inhibit cytokine, nitric oxide (Drew and Chavis, 2000a) and superoxide production (Bruce-Keller et al., 2000; Drew and Chavis, 2000b) by μglia. Thus, μglia numbers and production of potential neurotoxins are readily influenced by the steroid milieu.

Less understood are how steroids might impact functions more closely related to the balance of Aβ production and removal. For example, estrogen has been shown to enhance μglial phagocytosis (Pow et al., 1989; Li et al., 2000) particularly at higher doses (Bruce-Keller et al., 2000). This may relate, in part, to estrogen's effect on the complement 3 (C3) promoter (Fan et al., 1996). Estrogen can also stimulate synthesis of substances that could impact amyloid deposition such as chemokines like monocyte chemotactic protein (MCP)-1 (Frazier-Jessen and Kovacs, 1995) and the expression of apolipoprotein E (ApoE) mRNA in μglia

(Stone et al., 1997). Because epidemiological data suggest that prior use of estrogen decreases the risk of AD, a effect which is not observed with prior use of Gcs, we sought to compare the impact of these steroids on Aβ accumulation, clearance and toxin production in vivo and in vitro. We chose to test E2, a physiologically active estrogen, and the synthetic Gcs, prednisolone (in vivo) and dexamethasone (in vitro).

1.1. Experimental procedures

1.1.1. In vitro studies

The N9 clone (Corradin et al., 1993), a murine cell line with μglial-like characteristics, a generous gift of P. Riccardo-Castiglione, was used in in vitro experiments. Cells were maintained in a 5% CO_2 atmosphere at 37 °C in DME (high glucose 4.5 g/l) containing 2 mM L-glutamine, 1 mM sodium pyruvate and 0.05 mg/ml gentamicin sulfate. For experiments, cells were seeded in 24-well culture plates at a density of 100,000 cells/well in 500 μl of the above described medium and allowed to adhere for 24 h prior to treatment. All treatments were carried out in N2 serum-free defined medium (Michler-Stuke and Bottenstein, 1982). Cells were incubated for 24 h with 115, 230, or 1150 μg/ml HDL (Sigma, St. Louis, MO) in triplicate wells. For steroid experiments, cells were simultaneously treated with the steroid, Aβ (1–42), murine interferon gamma (mIFN-gamma, Boerhinger Mannheim, Indianapolis, IN), and HDL. The Aβ peptide was obtained from U.S. Peptide (Fullerton, CA), solubilized in 20% DMSO and used at a concentration of 2 μg/ml (Final DMSO 0.04%). The mIFN-gamma was diluted with N2 medium and used at a final concentration of 0.01 U/ml. In our serum free media, this was the maximum dose that could be used without toxicity. Steroids were obtained from Sigma Chemicals St. Louis, MO. Stock solutions of 20 μg/ml of E2 (cat # E2257) and 1 mg/ml of DXM (Sigma, cat # D1756) were prepared by dissolving the powder in 1 ml absolute ethanol. Working solutions of 1:100 in media were used to obtain final concentrations of 10–250 pg/ml (2.7–68 pM) for E2 and 0.5–25 μg/ml (0.1–6.8 μM) for DXM.

Nitric oxide production was measured indirectly through the detection of nitrite, a stable end product of the nitric oxide radical, via the Griess reaction (Ding et al., 1988). Briefly, N9 conditioned medium was mixed with the Griess reagent (0.1% *N*-1-napthylethylenediamine dihydrochloride and 1% sulfanilamide in 2% phosphoric acid) and triplicate samples read on a Titertek Multiskan MCC/340 microplate spectrophotometer at a wavelength of 550 nm. Lactate dehydrogenase (LDH) in the culture medium was used as a measure of cell viability. The CytoTox 96™ Non-radioactive assay (Promega, Madison, WI) was used to assay samples in triplicate.

Aβ sandwich ELISA was performed as previously described to assess Aβ levels in the trypsinized μglial cell pellet and Aβ remaining in media (Chu et al., 1998). As in that study, trypsin was used to remove Aβ bound to cell membranes. The pellet-associated Aβ, measured by ELISA and confirmed by Western blot, was both chloroquine and leupeptin dependent (Chu et al., 1998) suggesting the Aβ pellet measurements are representative of intracellular Aβ uptake. Furthermore, immunogold electron microscopy also showed an ultra-structural lysosomal/endosomal Aβ accumulation upon chloroquine treatment (Ard et al., 1996).

1.1.2. In vivo studies

Two-hundred microliters of Aβ pump solution was bilaterally infused into the ventricles (coordinates to Bregma in mm were: lateral: ± 1.8, posterior: 1.3, and ventral 4.0.) of female Sprague–Dawley (SD) rats using mini-osmotic Alzet pumps ($\#5004$, Durect Corporation, Cupertino, CA) over a 1-month period. Per day delivery was 2.6 μg HDL (80 μg total), 0.6 μg of Aβ40 (20 μg total), and 0.2 μg Aβ42 (6 μg total) in 4 mM HEPES, pH 8.0. Double connector cannulas separated by 3 mm (center to center) and cannula bifurcation connectors ($\#21/22Y$) were custom ordered from Plastics One (Roanoke, VA, Cat 3280PD/SPC. 4 mm). Prior to pump implantation, 10 ng of TGFβ1 (R and D Systems, Minneapolis, MN) was injected bilaterally into the anterodorsal thalamus in vehicle and Aβ-infused rats. This has been shown to enhance Aβ deposition in vivo (Frautschy et al., 1996) and in organotypic slice cultures (Harris-White et al., 1998). HDL has been shown to carry Aβ in vivo and was used in the pump to reduce aggregation of Aβ42 and act as an Aβ chaperone. In the E2 experiment, female SD rats (22 months old) were ovariectomized (OVX) and implanted with 12 mm silastic capsules filled with E2 powder (Sigma Chemicals, St. Louis, MO) or unfilled (vehicle) as described previously to induce sustained release of physiological concentrations of E2 (Kelner et al., 1977). Ovariectomy and silastic capsule implantation surgery was 2 weeks prior to Alzet pump implantation. In the Gc experiment, the synthetic Gc prednisolone (Sigma Chemicals, St. Louis, MO) was mixed in chow at a dose of 50 ppm (about 4 mg/kg body weight) 2 months prior to and during the month following pump implantation. The rats (average 375 g) ingested approximately 30 g per day of rat chow (Purina Test Diets, $\#5753C$-I, Richmond, IN). This dose of prednisolone is the ED_{50} dose for reducing Gc receptors in thymus (Luzzani et al., 1983) and approximately the dose that reduces inflammation in a rat model of arthritis (Kim et al., 1999). Prednisolone is a Gc with seven times less potency than the Gc agonist DXM used in the in vitro experiments. Rats were anesthetized with pentobarbital and perfused with a non-fixative protease inhibitor buffer as previously described for transgenic mice (Lim et al., 2000). Half the brain was immersion fixed in 4% paraformaldehyde and paraffin embedded and the remaining half of the brain was dissected and snap frozen for later formic acid extraction and Aβ ELISA analysis as described (Lim et al., 2000).

1.1.3. Immunostaining and image analysis

Anti-Aβ staining (10G4) was performed as previously described (Frautschy et al., 1996) at an antibody dilution of 1:1200. Sections were pretreated with trichloroacetic acid. Anti-phosphotyrosine (anti-PT, Sigma Chemicals, St. Louis, MO) staining (1:2000) was used to specifically identify reactive μglia in fixed brain sections (Korematsu et al., 1994; Frautschy et al., 1998). The assay was performed as previously described (Frautschy et al., 1998) with the following modification: sections were steamed for 30 min in citrate buffer followed by a 3 day incubation with primary antibodies at 4 °C. Biotinylated anti-mouse antibodies (1:1300) were used with Vector Elite kits. Sections were incubated in secondary antibodies followed by the ABC reagent for 1 h at room temperature. Slides were developed using peroxidase/Diaminobenzidiene (DAB; Pierce, Rockford, IL). For ring analysis (double antigen staining), sections were first stained for PT and labeled with the DAB chromagen followed by treatment with trichloroacetic acid and staining for Aβ using 10G4. 10G4 immuno-reactivity was developed with the chromagen alkaline phosphatase/Vector blue (Vector Labs, Burlingame, CA). A total of 7–10 plaques per section and three consecutive sections were used for ring analysis of plaque-associated glia.

For image analysis of Aβ deposits and μglial parameters, sections were assessed 1.8–2 mm posterior to Bregma (0.5–0.7 mm posterior to cannula). Images were acquired from an Olympus Vanox-T (AHBT) microscope with an Optronix Engineering LX-450A CCD video system. The video signal was routed into a Macintosh via a Scion Corporation AG-5 averaging frame grabber, and these digitized images were analyzed with NIH-Image public domain software available on the Internet at http://rsb.info.nih.gov/nih-image/. Custom Pascal macro subroutines were written to calculate plaque areas and ring analysis parameters. Quantitative ring analysis was used to evaluate DAB-stained μglia within and surrounding Vector blue stained Aβ deposits as previously described (Frautschy et al., 1998) with the following modification: instead of a three ring analysis around the plaque, one ring adjacent to the plaque, with the width of 2 plaque radii was measured and analyzed at high magnification ($60 \times$) for precise resolution of μglial processes.

Statistical analyses were performed using StatView (Abacus Concepts, Berkeley, CA). Statistical differences

were analyzed by a factor analysis of variance, and specific differences between treatment groups were assessed with Fisher's Protected LSD. Some values had to be logarithmically transformed to establish homogeneity of variance. Error values are expressed as standard deviation (Figs. 1 and 2) or 95% confidence interval (Figs. 4, 5 and 7). Results were considered significant at a level of $P \leq 0.05$.

2. Results

2.1. In vitro

In serum free cultures, Aβ increased nitrite in the media approximately four-fold relative to vehicle or murine IFN (Fig. 1A). The dose-dependent effects of the two steroids were strikingly different. E2 dose-dependently increased nitrite release relative to vehicle or murine IFN (almost to the level achieved by Aβ with no steroid) while DXM caused a dose-dependent reduction in nitrite: relative to vehicle or IFN. DXM at low doses modestly increased nitrite production, but decreased it at high doses (Fig. 1A). Compared to Aβ with no steroid, both Aβ + E2 and Aβ + DXM showed diminished nitrite release, however, striking differences were once again observed between these steroids. While addition of Aβ to E2-treated cells generally diminished nitrite, addition of Aβ to DXM-treated cells generally increased nitrite. This result was most pronounced at the highest dose of DXM where nitrite was increased more than two-fold (Fig. 1A).

Toxicity was assessed by measuring LDH (Fig. 1B). Although, treatment of cultures with IFN or Aβ/IFN did not alter toxicity, high doses of E2 and all doses of DXM caused significant increases in toxicity (Fig. 1B). There were striking differences in the effects of E2 and DXM. E2-associated μglial toxicity was exacerbated by IFN in the absence of Aβ but addition of Aβ appeared to protect μglial viability relative to E2 or E2 + IFN alone (Fig. 1B). In contrast, Aβ increased the toxic effect of DXM (Fig. 1B).

Fig. 2 shows the Aβ ELISA results of the media and trypsinized pellet. Both steroids enhanced Aβ uptake from the culture media (Fig. 2B, light grey bars). DXM was slightly more effective than E2 (Fig. 2A, light grey bars). Both E2 and DXM appeared to increase Aβ in the cell pellet. The increase in cellular Aβ with E2 was approximately 50% at all doses (Fig. 2A, dark grey bars), while the increase in cellular Aβ with DXM was dose-dependent (Fig. 2B, dark grey bars) reaching 250% of the level achieved with Aβ alone.

2.2. In vivo

Micrographs of entorhinal cortex from rat brain sections show Aβ-immunoreactive (ir) deposits in representative sections from female SD rats infused with Aβ from sections 1.8–2 mm posterior to Bregma (0.5–0.7 mm posterior to cannula) (Fig. 3). Aβ-infused rats on both diets showed widely dispersed deposits (Fig. 3C). At higher magnification, deposits from rats on control diet were predominantly diffuse with minimal Aβ-ir cellular staining within deposits (Fig. 3A), while those
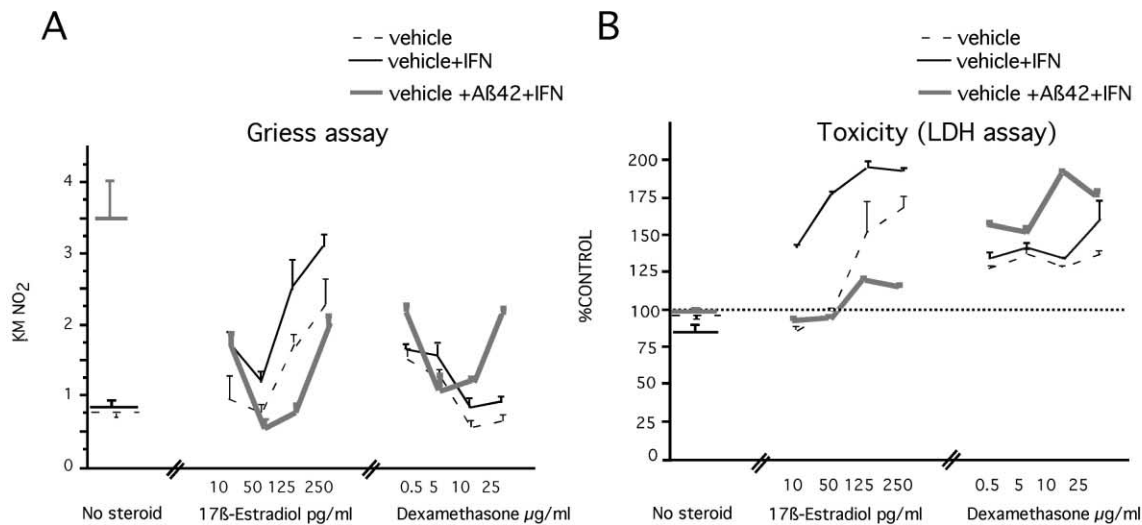

Fig. 1. Dose dependent effects of 17β-Estradiol (E2) and dexamethasone on Aβ and murine interferon gamma responses in (A) nitrite (Griess Assay) and (B) LDH. Error Bars signify standard deviations.
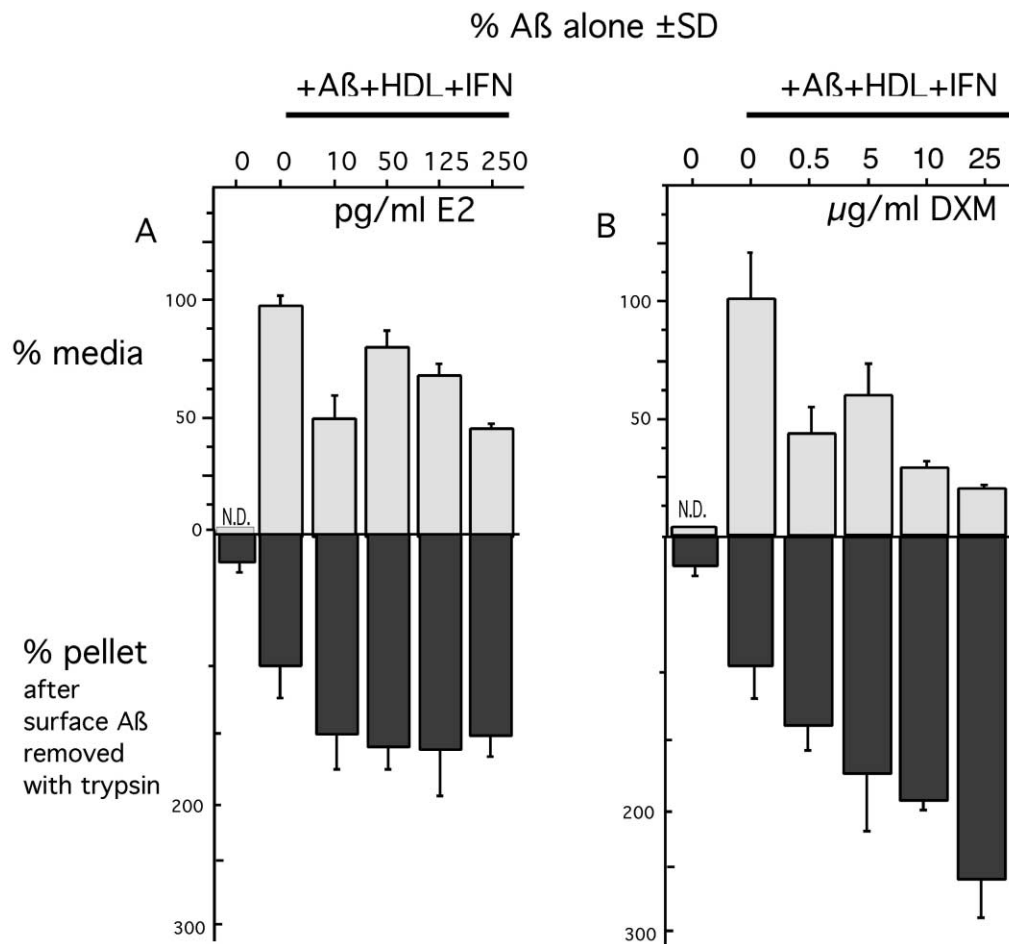
Fig. 2. Dose dependent effects of E2 (A) and dexamethasone (B) on Aβ remaining in media (light grey bars) and cell pellet (dark grey bars) following addition of 2 μg/ml Aβ42 to N9 μglial cells as measured by Aβ ELISA. Levels are normalized to percentage of Aβ alone. Error Bars signify standard deviations. N.D. = not detectable.

of Aβ-infused rats on prednisolone tended to be more compact with densely stained Aβ-ir processes in the center surrounded by lighter diffuse staining (Fig. 3B). Aβ-infused rats with subcutaneous (sc) silastic vehicle or E2-filled implants also showed widely dispersed deposits (not shown). At higher magnification, Aβ infused OVX rats with sc vehicle implants showed diffuse Aβ deposits with minimal cellular elements (not shown), while Aβ-infused rats with sc E2 implants showed diffuse Aβ deposits that had a darkly stained core (Fig. 3D). Double staining did not reveal that this core was of μglial origin (not shown).

Fig. 4 depicts the quantitative analysis of Aβ-ir deposits of hemi-brain sections from all rats 0.5–0.7 mm posterior to the cannula. In vivo, E2 and Gc had similar effects on Aβ deposition (Fig. 4A and B). E2 modestly increased plaque numbers. Without dramatically reducing the total area of diffuse Aβ deposition (total Aβ-ir area/section), both steroids appeared to shrink individual plaque size. Despite Aβ deposit shrinkage, Aβ ELISA revealed that total formic acid extractable Aβ levels in the cortex were increased with both steroids.

How these steroids altered overall μglial immunoreactivity was observed by quantifying the percentage area of PT-labeled cells in the entorhinal cortex layer 3 not associated with Aβ-ir deposits (Fig. 5) as well as examining μglia associated with Aβ-ir deposits (Fig. 7). Fig. 5 shows the results of analysis of μglia that are not necessarily related to Aβ-ir deposits but are more likely reflective of soluble Aβ. In non-Aβ infused, OVX rats, E2 tended to reduce total PT area but not affect PT-labeled particle size in most brain regions and in entorhinal cortex (Fig. 5A). However, when Aβ was infused, no effect of E2 was observed on total PT area, but E2 treatment was associated with an increase in particle size in most brain regions that was most altered in the entorhinal cortex layer 3 (Fig. 5B). Aβ treatment alone was associated with increased μglial staining. Unlike E2, dietary treatment with prednisolone prevented the Aβ induced increase in total PT area and particle size (Fig. 5C).

μglia not necessarily associated with Aβ deposits were analyzed in layer 3 of entorhinal cortex to evalu-

ate possible effects of soluble Aβ (Fig. 6A–C, Fig. 6F–G), while μglia associated with Aβ deposits were evaluated using double immunolabeling to assess inflammatory response of deposited Aβ (Fig. 6 D–E, Fig. 6H–I). The entorhinal cortex layer 3 of Aβ-infused rats revealed more μglial processes and larger μglia cell bodies (Fig. 6B) than the vehicle infused rats (Fig. 6A). The entorhinal cortex layer 3 of Aβ-infused rats fed prednisolone (Fig. 6C) was indistinguishable from vehicle infused rats. There was more μglial staining (shown in brown) associated with the Aβ-ir deposits (shown in blue) compared to outside deposits in both Aβ-infused rats (Fig. 6D), and Aβ-infused rat on prednisolone (Fig. 6E).

The entorhinal cortex layer 3 of Aβ-infused OVX rats implanted with E2 capsules revealed more and larger μglial cell bodies (Fig. 6G) compared to those without E2 (Fig. 6F). There was more μglial staining (shown in brown) associated with the Aβ deposits (shown in blue) compared to outside deposits in both Aβ-infused rats (Fig. 6H), and Aβ-infused rat implanted with E2 capsules (Fig. 6I). There appeared to be more and larger μglia associated with Aβ-ir deposits from E2 implanted rats (Fig. 6I) than in rats not on E2 (Fig. 6H).

The quantification of the inflammatory response to Aβ deposits from double stained sections (similar to those depicted in Fig. 6D–E, H–I) using the ring analysis is shown in Fig. 7. We calculated percentage μglial staining inside deposits (black circle, Fig. 7A) and in the surrounding ring (gray, Fig. 7A). In the absence of E2 or DXM, there was an increase in μglial area in the Aβ deposit compared to the surrounding ring (Fig. 7 B or C). Treatment with either E2 (Fig. 7B) or prednisolone (Fig. 7C) further increased the PT-labeled μglia within deposit relative to the outside ring ($P < 0.05$). The response to the deposit in steroid-treated rats was greater than the response in rats receiving no steroid ($P < 0.05$).
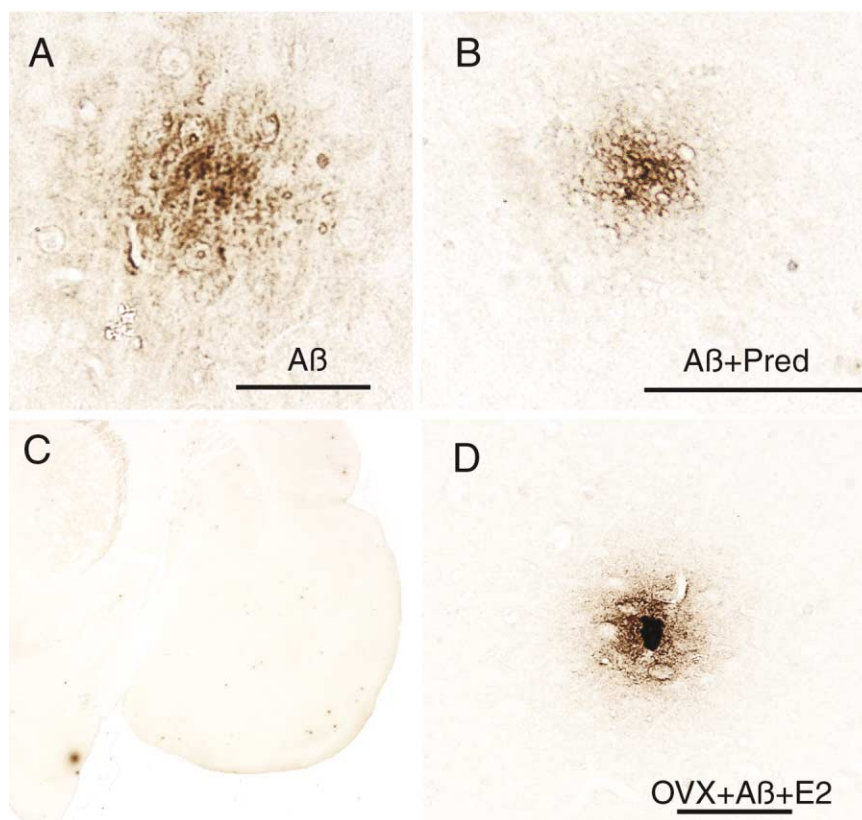

Fig. 3. Micrographs of rat brain sections show Aβ-immunoreactive (ir; 10G4 antibody) deposits in sections from female SD rats infused with Aβ. (A) At higher magnification (40 ×), deposits from rats on control diet were predominantly diffuse with minimal Aβ-ir cellular staining within the deposit (B), while those of Aβ-infused rats on prednisolone tended to be more compact with densely stained Aβ-ir μglial-like processes in the center surrounded by lighter diffuse staining. Within the deposit, unstained vacuoles were prominent. (C) Low magnification of Aβ-ir deposit distribution typical of Aβ infusion paradigms is shown (2 ×), from a OVX rat with sc E2 implants. OVX rats infused with Aβ and implanted with sc vehicle implants showed diffuse Aβ deposits with minimal cellular elements (not shown) (D), while Aβ-infused rats with sc E2 implants showed diffuse Aβ deposits containing a darker stained central core. Bar = 25 μm applies to panels A, B, D.

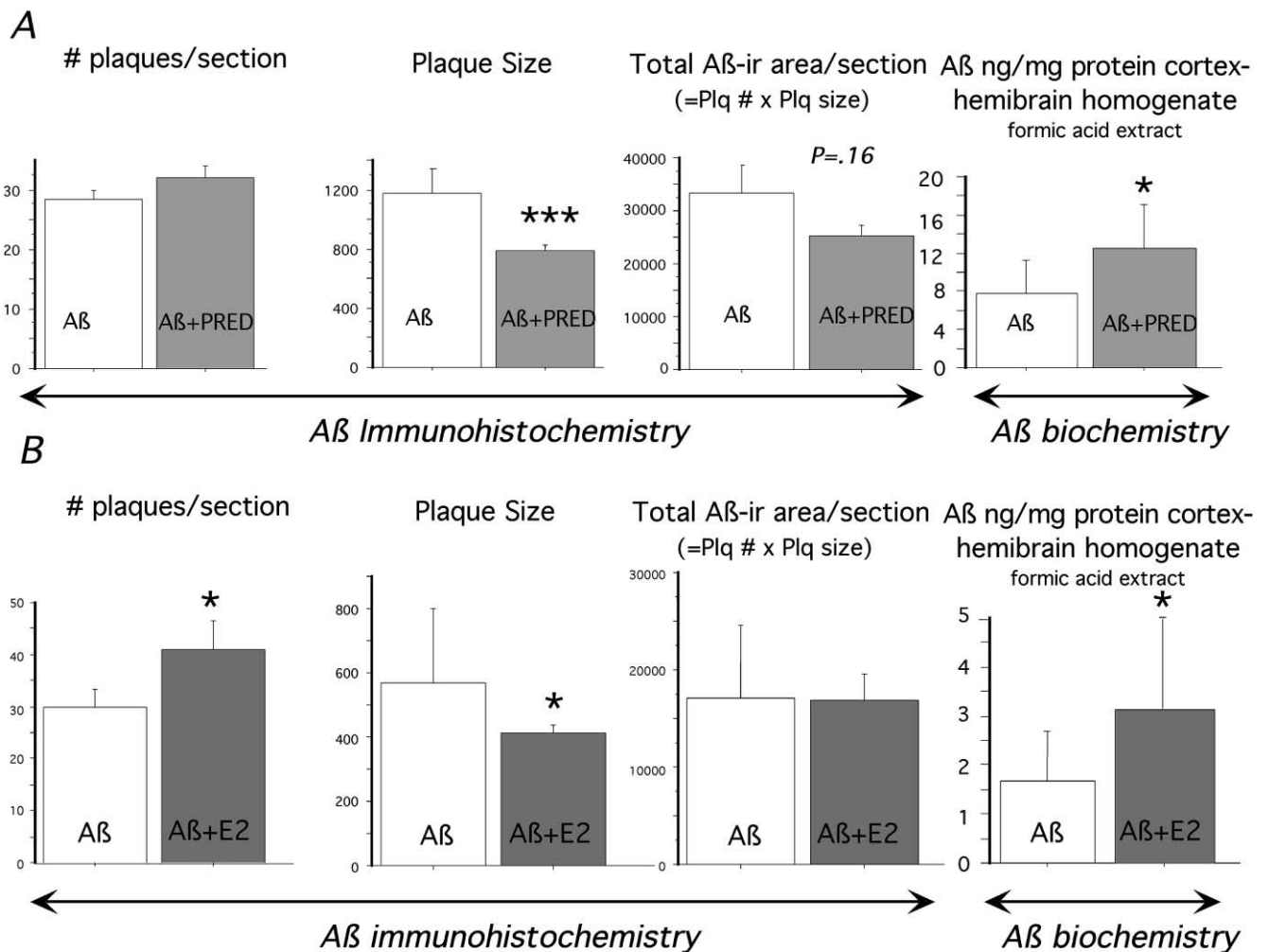Fig. 4. Aβ deposition in rat hemibrain and Aβ levels of formic acid-extracted cortex in Aβ-infused female SD rats. (A) Aβ-ir and quantitative Aβ ELISA results from brains of Aβ-infused rats treated with or without the synthetic Gc prednisolone in chow. (B) Aβ-ir and quantitative Aβ ELISA results in ovariectomized rats treated with subcutaneously implanted E2 or vehicle (corn oil)-containing silastic capsules followed by Aβ infusion. Error bars signify 95% confidence interval. Significance levels of treatment effects were analyzed by ANOVA factorial analysis (Plaque parameters × treatment). Asterisks signify: ***$P < 0.001$, *$P < 0.05$. Logarithmic transformation was needed for homogeneity of variance (Bartlett's Test) for analysis of E2 effects on plaque parameters. Depicted values are not log transformed.

3. Discussion

3.1. E2 alone and IFN effects on nitrite and viability

Our data demonstrate that physiological doses of E2 increase nitric oxide production, and this correlates with toxicity. Previously, it had been reported that supraphysiological doses of E2 reduced nitric oxide production (Bruce-Keller et al., 2000; Drew and Chavis, 2000a). In the periphery, E2 has potent effects on stimulation of inducible nitric oxide synthase (iNOS) in endothelial cells (Goetz et al., 1999), but it has been reported to have the opposite effects in macrophage cells (Hayashi et al., 1998). Our data and previously published work suggest that effects of E2 on μglial nitric oxide production are likely to be biphasic; stimulatory at low physiological doses and inhibitory at high doses. The presence of IFN did not alter nitrite on its own, but primed the nitrite production and toxicity response to E2, resulting in increased nitrite even with the two lowest doses of E2. These data further support the view that E2 potentiates the immune response and can have both anti-inflammatory and pro-inflammatory effects (Fox et al., 1991; Cutolo et al., 1995).

3.2. Aβ and E2: nitrite and viability

Despite the pro-inflammatory effects of E2, alone or in combination with IFN, on nitric oxide production, E2 dramatically reduced or abolished the Aβ induction of nitric oxide and associated toxicity. Aβ-stimulated nitric oxide does not occur without activated cells

(Meda et al., 1995). Although activation was not independently measured, our data are consistent with reduced activation with combined E2 and Aβ. The effect of E2 on reducing Aβ induced nitrite may be a mechanism in the possible role of E2 in AD prevention. Surprisingly, the addition of Aβ protected cells from the toxicity that occurred with E2 + IFN. That Aβ can

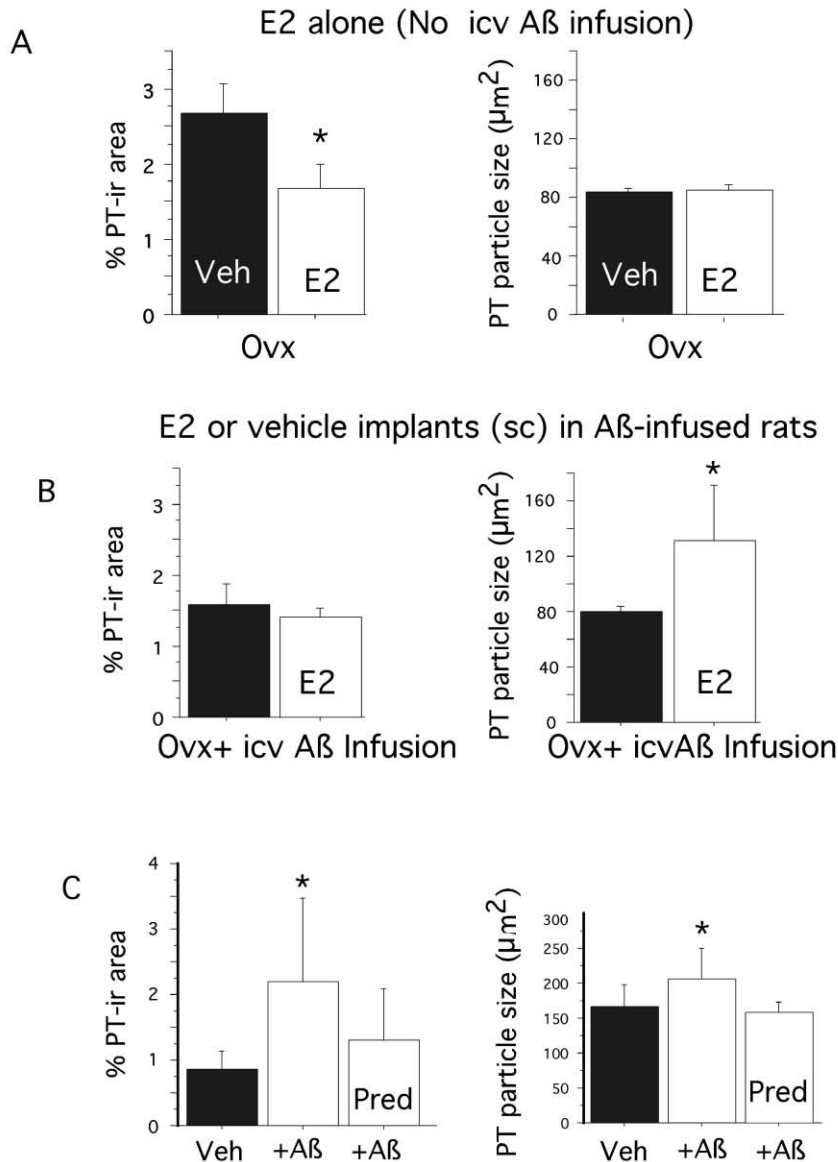Fig. 5. Effects of E2 (A and B) and prednisolone (C) on phosphotyrosine (PT)-labeled μglia parameters in layer 3 of the entorhinal cortex-% area stained (left) and particle area (μm², right). Panel A depicts E2 (sc) effects in non icv infused rats related to vehicle (sc). Panel B depicts E2 effects in Aβ-infused rats. Panel C demonstrates the effects of Aβ on PT and its reversal by the Gc prednisolone. Error bars signify 95% confidence interval. Asterisks signify: *$P < 0.05$ of ANOVA analysis. In panel C, the asterisk means significantly different from both other treatments. Because analysis of particle size for E2 and PT area for prednisolone did not reveal homogeneous variances, the analysis required logarithmic transformation. Real instead of log values are depicted.
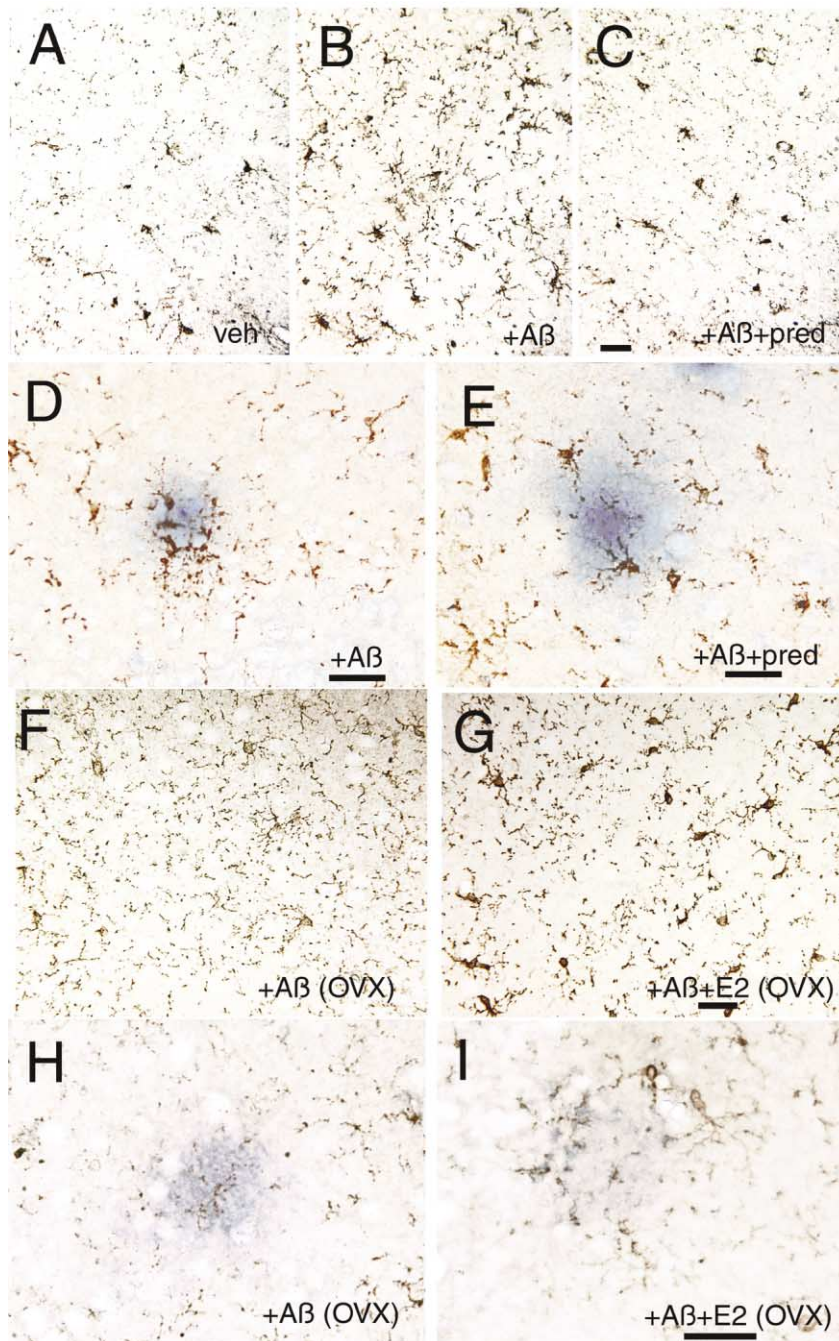
Fig. 6. (A–C, F–G) Phosphotyrosine-labeled μglia not necessarily associated with Aβ deposits were analyzed in layer 3 of entorhinal cortex to evaluate possible effects of soluble Aβ (D–E, H–I). Microglia associated with Aβ deposits were evaluated using double immunolabeling to assess inflammatory response (brown) of deposited Aβ (blue). (A) Vehicle infused rats, (B) Aβ-infused rats, (C) Aβ-infused rats fed prednisolone chow. (D–E) Aβ-ir deposits (blue) in Aβ-infused rats (D) or Aβ-infused rats on prednisolone (E) show more μglial cells and processes (brown) than compared to outside the deposit. The entorhinal cortex layer 3 of the Aβ-infused rats (F) show fewer and smaller μglial cell bodies than Aβ-infused rats implanted with E2 (G). (H–I) The μglial (brown) processes and bodies associated with the Aβ-ir deposits (blue) of Aβ-infused rats implanted with E2 (I) were larger and more frequent than deposits from Aβ-infused rats implanted with vehicle (H). Bars = 50 μm. Bars in G also pertain to F, and Bar in I also pertains to H.

have a differential effect on toxicity of μglia has previously been reported (Korotzer et al., 1993), but this is the first report that Aβ can actually protect the viability of μglia. Clearly, the environmental milieu effects the toxicity outcome. The clinical relevance of this observation is unknown. Although enhancing μglial viability could adversely prolong an inflammatory response normally limited by autotoxicity, our observed reduction in μglial neurotoxin release would suggest that E2 might have a net protective role.

3.3. DXM and IFN: nitrite and viability

DXM induced a biphasic effect on nitrite. At high doses we observed a reduction in nitrite similar to previously described Gc effects in µglial cells (Tanaka et al., 1997; Drew and Chavis, 2000b). However, at low doses, Gcs stimulated nitrite. A seemingly paradoxical proinflammatory response to Gcs is a phenomenon common at low doses. This is because while Gcs inhibit pro-inflammatory mediators such as interleukins and

IFN, they also stimulate other proinflammatory components including IFN and interleukin receptors (Strickland et al., 1986; Sadeghi et al., 1992; Calandra et al., 1995; Jüttner et al., 1998; Morand and Leech, 1999). Compared to DXM alone, the addition of IFN further increased the nitric oxide response and toxicity consistent with IFN priming. Toxicity of Gcs to µglia is consistent with the literature since Gc treatment has been reported to induce shrunken morphology in µglia and reduce ramification visible at low magnification
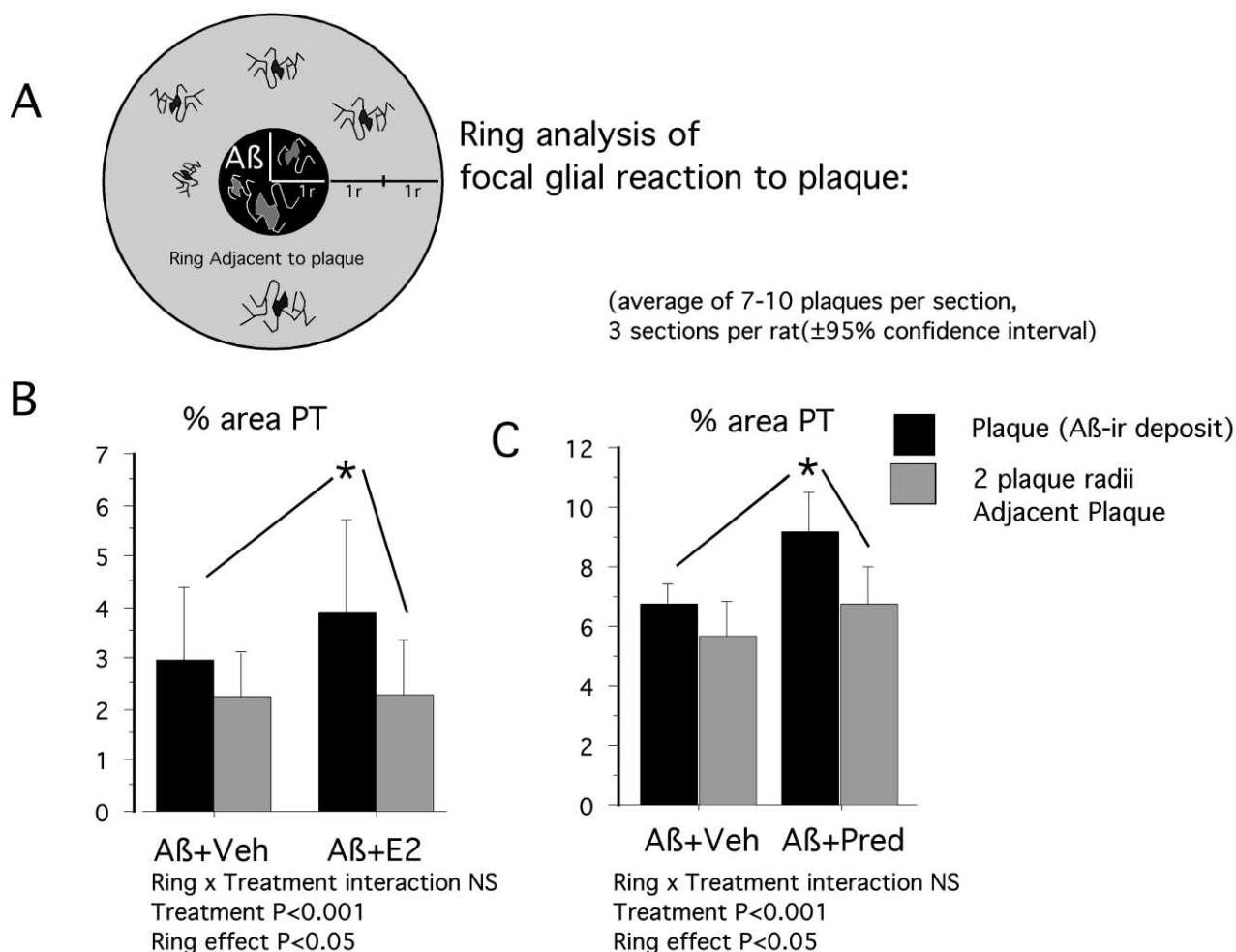

Fig. 7. Focal effects of Aβ deposits on percent phosphotyrosine (PT) ir area within Aβ-ir deposit and two plaque radii outside deposit using ring analysis. (A) Schematic diagram of ring analysis. Effects of E2 (B) and prednisolone (C) on PT response to deposit. Plaques were stained with 10G4 antibody using Vector Blue and µglia were stained with antibodies to PT and labeled with DAB (Brown). Ring analysis was performed at $60\times$ magnification for optimal resolution of µglial cells and processes. Graphs depict the averages of 7–10 plaques per section and three sections per rat $\pm 95\%$ confidence interval 2×2 ANOVA (treatment $\times$ ring) analysis showed that for both E2 and prednisolone analysis, treatment ($P < 0.001$) and ring ($P < 0.05$) effects were significant while the treatment ring interaction was not significant. This demonstrated that there was more% microglia immunoreactivity within deposit than in outside ring regardless of treatment. Asterisks denote significance of $P < 0.05$ of two planned comparisons indicated by lines (comparing different rings within same treatment or comparing same rings between different treatments.)

and to enhance the formation of lysosomal vacuolation ultrastructurally (Tanaka et al., 1997). Gcs also increased degeneration of ameboid μglia in vivo, leaving the remaining μglia ramified (Kaur et al., 1994).

3.4. Aβ and DXM: nitrite and viability

DXM reduced the nitrite levels induced by Aβ, but in direct contrast to effects with E2 and Aβ, this was associated with significant increases in toxicity at the two highest doses. The combined effects of Gc + Aβ may have been more toxic than E2 + Aβ because of protective antioxidant effects of E2 (Moosmann and Behl, 1999) or enhanced Aβ accumulation as suggested by Fig. 2. If Aβ accumulates in the endosome/lysosome, this could kill the cell by impairing endosomal/lysosomal permeability (Yang et al., 1998).

3.5. Steroids and Aβ accumulation

Our Fig. 2 data demonstrate that both steroids reduce Aβ in the media and enhance Aβ accumulation in the trypsinized μglial cell pellet. However, while increasing doses of E2 result in only slight increases in accumulation, increasing doses of Gc result in a dose-dependent increase in pellet Aβ.

3.6. E2 and Aβ uptake

Increased cell pellet Aβ may be due to increased uptake. Measurement of the trypsinized cell pellet by ELISA and confirmed by Western blot is chloroquine and leupeptin dependent (Chu et al., 1998) suggesting the Aβ pellet measurements are representative of intracellular Aβ uptake. That this represents intracellular uptake is further supported by electron microscopy showing that the same manipulations enhance ultrastructural lysosomal/endosomal Aβ accumulation by immunogold (Ard et al., 1996). E2 enhancement of Aβ uptake and phagocytosis in human μglia has been reported (Li et al., 2000). E2 also enhanced μglial endocytosis of neurons in the neurohypophysis (Pow et al., 1989) and macrophage phagocytosis (Chao et al., 1996). As with macrophages (Brown et al., 1990), E2 may stimulate μglial phagocytosis by its effect on complement protein C3 mRNA and the C3 promoter (Fan et al., 1996).

3.7. DXM and Aβ uptake

Our data demonstrate for the first time that Gcs can dose-dependently enhance cell pellet Aβ accumulation which could be due to increased Aβ uptake. Gcs can differentially alter phagocytosis dependent on milieu. In vivo, they do not impair phagocytosis associated with brain injury (Giulian et al., 1989). It is possible that

Gcs enhance Aβ uptake by induction of scavenger receptor mRNA and protein as demonstrated in monocytes (Ritter et al., 1999). Although Gc induction of LRP expression (Kancha et al., 1994) could conceivably be involved in the enhancement of Aβ uptake in rodent μglia, it is likely not relevant to human μglia which do not appear to express LRP (Rebeck et al. 1993). We did not test and cannot exclude a role for LRP in Gc enhancement of Aβ uptake and toxicity of neurons which could also explain the lack of efficacy of Gcs.

Because nitric oxide is an important inhibitor of μglial Aβ phagocytosis (Kopec and Carroll, 2000), steroid induced reductions in nitric oxide (NO) do not precisely parallel, but might contribute to, increased Aβ accumulation. For example, E2 dose-dependent increases in nitrite from 50–250 pg/ml might inhibit further Aβ accumulation. Thus, although E2 may not directly impair Aβ degradation, a compensatory NO response could explain why complete degradation does not occur.

3.8. Steroids and Aβ degradation

Although both steroids appeared to enhance intracellular Aβ accumulation, increased doses of Gcs led to larger increases in Aβ in the cell pellet coupled with larger decreases in the media, particularly at the high doses which lead to a 2.5-fold increase. This effect was different than E2 which only stimulated an approximate 50% increase in Aβ that plateaued at the lowest dose (10 pg/ml) while Aβ in the media continued to decline from 50 to 250 pg/ml. This Gc-dependent Aβ accumulation occurred despite considerable toxicity, suggesting that the Aβ accumulation of viable cells may have been even higher than 2.5-fold. In summary, together these data are consistent with E2 allowing partial degradation of phagocytosed Aβ and Gcs significantly impeding degradation.

3.9. Steroids in vivo

Both steroids appeared to shrink Aβ deposit size in vivo, yet Aβ assayed by ELISA in formic acid-extracted cortical homogenates was increased, suggesting the reduced size of Aβ deposits resulted from focal concentration of Aβ and not increased degradation. We interpret our data to mean that the steroids are impeding Aβ degradation in vivo, and this interpretation is further strengthened by the in vitro data on Aβ degradation. Nevertheless, an alternative explanation would be that the increase in formic acid extracted Aβ could occur if formic acid was extracting Aβ from a clearance compartment (for example a vascular compartment). This interpretation might be consistent with the recent report by Janus et al. (2000) who observed a trend for a 20% non-significant increase in Aβ42 by

ELISA despite dramatic reductions in plaques after vaccination. Our results do not support this interpretation because we perfused rats with buffer prior to sacrifice to reduce Aβ present in vasculature, and since changes in formic acid extracted Aβ usually parallel changes in deposition (Bard et al., 2000; Lim et al., 2000).

If steroids enhance intracellular Aβ accumulation, as suggested by in vivo and in vitro data, cells may form or phagocytose amyloid fibrils in the lysosome, rendering it indigestible (Burdick et al., 1997). Even though μglia can phagocytose and concentrate amyloid fibrils in vitro, degradation may be slow and incomplete so they eventually regurgitate it undigested (Chung et al., 1999, 2000). Our in vitro and in vivo data are consistent with a role for steroids in promoting increased μglial Aβ uptake without effective degradation leading to plaque compaction.

3.10. E2 and μglia

Microglia were analyzed in the entorhinal cortex layer 3 to assess effects of Aβ on μglia that were not necessarily plaque dependent but may also be soluble Aβ-dependent. In the absence of Aβ, E2 reduced μglia (not associated with plaques) in the entorhinal cortex neuronal layer 3. These data are consistent with an anti-inflammatory role in vivo of E2 that could be related to the toxicity that we observed in vitro in the absence of Aβ or due to an inhibitory effect on proliferation. In the presence of Aβ, E2 did not affect percentage μglial area stained (PT-ir is specific for μglia), demonstrating a very different effect of E2 in the presence of Aβ. This in vivo effect was consistent with our in vitro data demonstrating a protective effect of E2 in the presence of Aβ on μglial viability. Ring analysis was performed in Aβ-infused rats to evaluate μglia response to Aβ deposits and was found to be more robust in E2 treated than vehicle treated rats. The μglial stained particles inside the E2 deposits were larger, consistent with an ameboid shaped morphology. The common visualization of larger cells in E2 treated, Aβ-infused rats is consistent with E2 enhancing phagocytosis and consistent with the in vitro data. Phagocytic cells may undergo rapid degeneration (Gehrmann and Banati, 1995), which may explain the presence of the ameboid shaped core of Aβ that was most pronounced in E2 implanted rats and did not double label for phosphotyrosine. All these data are consistent with E2 stimulation of phagocytic morphology in Aβ-infused rats.

3.11. Prednisolone and μglia

In control diet fed rats, Aβ increased the percentage μglial area, consistent with a pro-inflammatory role of Aβ in vivo. However, in Gc-fed rats, Aβ did not increase μglial area. The effect of Gc on reducing non-plaque associated μglia in Aβ-infused rats is in contrast to the effect of E2 and consistent with a possible toxic effect of Gcs. Quantitative examination of the focal μglial response to Aβ deposits showed that compared to vehicle, both steroids quantitatively induced a more robust response within Aβ deposits. In summary these in vivo data show that both steroids show similar inflammatory responses to Aβ deposits, but divergent μglial responses not associated with deposits. This is somewhat parallel to in vitro data showing that both steroids enhance Aβ uptake but have divergent effects on Aβ-associated μglial toxicity.

These data raise the possibility that age-related changes in steroids modulate Aβ deposition. Increased secretion of adrenal steroids is positively correlated with AD severity in cross sectional studies (Weiner et al., 1993; Miller et al., 1998; Swanwick et al., 1998) and AD patients have been shown to have increased levels of cortisol secreted per burst (Hartmann et al., 1997; Swanwick et al., 1998).

In summary, these in vivo and in vitro data are consistent with the hypothesis that although E2 is not as deleterious as Gcs in impeding degradation, long term exposure to both steroids could impede Aβ clearance. In accordance with recently published work demonstrating that E2 reverses OVX-induced increases in brain Aβ levels (Petanceska et al., 2000), unpublished results from our laboratory show that E2 treatment results in a 25% decrease in endogenous Aβ by ELISA in OVX rats. However, it is currently unclear whether the alterations in Aβ levels reflect changes in production or clearance of Aβ. Our results suggest that E2 may decrease production of Aβ and may help to concentrate it into focal deposits, but is not effective in clearing Aβ. These data may explain the lack of correlation between anti-inflammatory Gcs and reduced AD risk in epidemiological studies and the lack of efficacy of both steroids for treatment of AD.

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