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RESEARCH ARTICLE

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Immunohistochemical visualization of newly formed quinolinate in the normal and excitotoxically lesioned rat striatum

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Abstract Intracerebral infusion of 3-hydroxyanthranilate (3HANA) rapidly increases the brain content of the endogenous excitotoxin quinolinate (QUIN). QUIN formation from 3HANA is readily prevented by coadministration of the specific 3-hydroxyanthranilate oxygenase inhibitor 4-chloro-3HANA (4-Cl-3HANA). This experimental paradigm was used to identify the cell populations which are responsible for the rapid de novo production of QUIN in the rat striatum in vivo. Rats received an intrastriatal infusion of 3HANA, alone or together with equimolar 4-Cl-3HANA, for 1 h. Striatal QUIN immunoreactivity (ir) was assessed immunohistochemically, using an antibody against protein-conjugated QUIN. This antibody displayed no significant crossreactivity with compounds structurally or functionally related to QUIN. QUIN-ir cells were detected after infusion with ≥300 μM 3HANA, but not in na striata or after coinfusion of 4-Cl-3HANA. Cellular staining was also abolished by preabsorption of the antibody with protein-conjugated QUIN. In the normal striatum, QUIN-ir was detected exclusively in cells of an apparent microglial morphology. When examined in the excitotoxically lesioned striatum, 3HANA-induced QUIN-ir localized exclusively to OX42-ir cells of an activated microglial/macrophage morphology. These data indicate that microglia and macrophages are the major source of QUIN in the rat striatum when hyperphysiological concentrations of 3HANA are used to drive QUIN synthesis. Comparison with earlier biochemical and immunohistochemical studies suggests that the enzyme responsible for microglial QUIN production is a distinct 3-hydroxyanthranilate oxygenase with high capacity and low affinity for 3HANA.

Keywords Astrocytes · Excitotoxicity · Kynurenines · Microglia · Quinolinic acid

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Introduction

The cellular localization of the endogenous excitotoxin and putative pathogen quinolinate (QUIN) in the mammalian brain has not been unequivocally resolved so far. Studies of various cell types in culture demonstrated that QUIN synthesis occurs primarily in cells of a monocytic lineage (Heyes et al. 1992, 1997; Alberati-Giani et al. 1996; Espey et al. 1997). In contrast, immunohistochemical studies, performed at both the light and electron microscopic level, indicated that QUIN metabolism in the rat brain is almost exclusively confined to astroglia. Thus, the use of antibodies directed against QUIN's biosynthetic enzyme 3-hydroxyanthranilate oxygenase (3HAO) resulted in the exclusive labeling of astrocytes (Köhler et al. 1988; Roberts et al. 1995), and antibodies that recognize the catabolic enzyme of QUIN, quinolinate phosphoribosyltransferase (QPRT), stained both astrocytes and a small neuronal population (Köhler et al. 1987). Notably, immunohistochemical studies with antibodies directed against QUIN itself, generated by using protein-conjugated QUIN as an antigen, revealed no QUIN-immunoreactive (ir) cells in tissue sections from normal rat brain (Moffett et al. 1993, 1994b, 1997; Sung et al. 1997; Barattè et al. 1998).

The present study was designed to revisit the issue using an antibody to protein-conjugated QUIN and taking advantage of the brain's ability to rapidly produce substantial amounts of QUIN following a focal intracerebral infusion of its immediate precursor 3-hydroxyanthranilate (3HANA; Speciale et al. 1989; Fig. 1). This approach also allowed us to perform control experiments by coadminis-

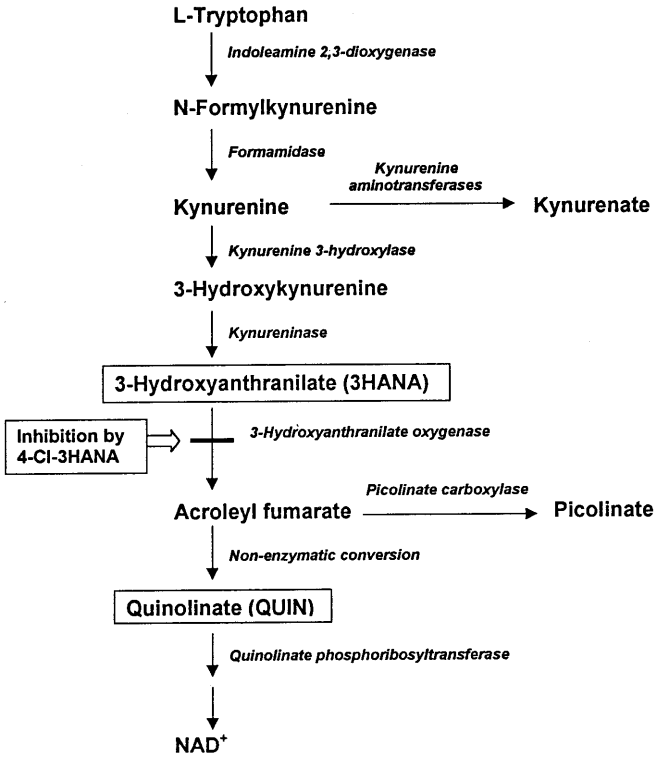

Fig. 1 Kynurenine pathway of tryptophan metabolism in mammalian cells. *4-Cl-3HANA* 4-Chloro-3-hydroxyanthranilate

tering 3HANA with 4-chloro-3HANA (4-Cl-3HANA), a specific, competitive 3HAO inhibitor, which potently interferes with 3HANA-induced QUIN production in vivo (Parli et al. 1980; Heyes et al. 1988; Saito et al. 1994; Walsh et al. 1994). QUIN-ir cells were characterized by morphological criteria, and the cellular localization of newly formed QUIN was examined in normal striatal tissue and in striata which had been depleted of neurons by a focal excitotoxin injection 1 week previously. Our results, which have been communicated in abstract form (Lehrmann et al. 1998), provide evidence that cells of a microglial/macrophage lineage, rather than astrocytes, are responsible for the rapid formation of QUIN when hyperphysiological concentrations of 3HANA are used in the normal and lesioned rat striatum.

Materials and methods

Materials

Unless specified otherwise, all chemicals were purchased from Sigma Chemical (St. Louis, Mo., USA).

Antibody production and in vitro characterization

QUIN is a hapten and thus not immunogenic per se, but can become antigenic when coupled to a polypeptide carrier such as keyhole limpet hemocyanin. This was achieved by reacting QUIN with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDAC), as previously described (Moffett et al. 1994b). Rabbits were injected with the purified reaction product, and antiserum was obtained for subsequent purification.

Solid-phase ELISA was used to evaluate the potency and specificity of the QUIN antibody in vitro (Barattè et al. 1998). Briefly, microtiter wells coated with bovine serum albumin (BSA) covalently coupled to QUIN by EDAC were incubated with a wide range of QUIN antibody dilutions (1:100 to $1:10^6$). A secondary peroxidase-labeled goat anti-rabbit IgG antibody was then added, and *o*-phenylenediamine (OPD) was used as the substrate of the chromogenic reaction. Chromogenic intensity was analyzed using an optical microplate reader. Control wells were coated either with BSA, BSA covalently linked to tryptophan, or serum from a normal, non-immunized rabbit.

Specificity of the QUIN antibody was assessed at a dilution which showed 50% of the maximal potency (1:6,400) by exposure to increasing concentrations of QUIN or compounds structurally, metabolically, or functionally related to QUIN (kynurenine, 3HANA, picolinate, phthalate, kynurenate, anthranilate, nicotinate, glutamate, and serotonin).

In vivo experiments

Solutions of 3HANA (300 µM or 15 mM) and 4-Cl-3HANA (15 mM; kindly provided by Dr. Mario Varasi, Pharmacia and Upjohn, Nerviano, Italy) were prepared immediately before use by dissolving the compounds in a small volume of 0.1 N HCl and adjusting the pH to 6.8 with 1 M NaOH and 0.1 M phosphate buffer (PB), pH 7.4. The solutions were shielded from light and kept on ice to minimize decomposition.

Animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Maryland. Male Sprague-Dawley rats (200–250 g; Charles River) were housed in an AAALAC-approved animal facility at a 12 h:12 h light:dark cycle with free access to food and water. On the day of the experiment, the rats were anesthetized with chloral hydrate (350 mg/kg, i.p.) and placed in a stereotaxic frame (Kopf, Tujunga, Calif., USA). Routinely, 8 µl of the drug(s) or vehicle solution were injected into the striatum (coordinates: +1.1 mm from bregma, 2.8 mm lateral from the midline, and 4.6 mm below the dura) over 1 h using an infusion pump (CMA100; Carnegie Medicin, Stockholm, Sweden). At the end of the infusion, animals were deeply anesthetized with chloral hydrate (500 mg/kg, i.p.) and perfused transcardially with an aqueous solution of 6% EDAC, 5% dimethylsulfoxide, and 1 mM *N*-hydroxysuccinimide, as detailed by Moffett et al. (1994a, b). The brain was removed and postfixed overnight in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 8.0. After sequential cryoprotection in 10% and 20% sucrose, the brain was rapidly frozen in CO_2 snow, and 40 µm coronal sections were cut on a cryostat.

Using the same stereotaxic procedure and coordinates, separate groups of animals received a unilateral intrastriatal injection of QUIN (120 nmol/1 µl per 10 min). These animals were either perfused after 1 h or given a focal 3HANA and/or 4-Cl-3HANA infusion 1 week later.

Immunohistochemistry

Immunohistochemistry was performed on free-floating tissue sections by a standard streptavidin-biotin labeling method, using anti-rabbit or anti-mouse IgG Elite Vectastain kits (Vector Laboratories, Burlingame, Calif., USA). Endogenous peroxidase activity was quenched with 1% H_2O_2 in equal volumes of methanol and distilled H_2O for 30 min at room temperature, followed by three rinses in 0.01 M PBS, pH 7.4 ("buffer"). Endogenous biotin was blocked using an avidin-biotin blocking kit (SP-2001; Vector), adding avidin during a 30-min preincubation with buffer containing 0.03% Triton X-100 ("T-buffer") and 2% normal serum. The following primary antibodies were used: (a) mouse monoclonal OX42 antibody (MCA275G; 1:500; Serotec, Raleigh, N.C., USA), recognizing the complement type 3 receptor present on cells of a microglial/macrophage lineage (Robinson et al. 1986), (b) mouse monoclonal glial fi-

brillary acidic protein (GFAP) antibody (814369; 1:1,500; Boehringer Mannheim, Indianapolis, Ind., USA), recognizing the astrocytic marker GFAP (Dahl et al. 1984), and (c) a non-commercial polyclonal rabbit antibody directed against protein-conjugated QUIN (1:1,000; see above). The tissue sections were incubated with gentle agitation for 48–72 h at 4°C using the primary antibodies in T-buffer with biotin. The sections were then warmed to room temperature, rinsed three times in T-buffer, and incubated with the secondary species-specific, biotinylated antigen in T-buffer for 1 h. After three rinses in buffer, the sections were incubated with the tertiary antibody (horseradish peroxidase-labeled streptavidin) for 1 h. They were then thoroughly rinsed in buffer and developed under the microscope, using diaminobenzidine hydrochloride (0.03%) or 3-amino-9-ethylcarbazole (AEC; Dako, Carpinteria, Calif., USA) as chromogenic substrates of H_2O_2. Sections were mounted onto gelatinized glass slides and coverslipped or dehydrated from Hemo-De (Fisher Scientific, Pittsburgh, Pa., USA) and coverslipped with Permount (Fisher).

Negative controls included the omission of either primary, secondary, or tertiary antibody, use of a non-crossreacting secondary antibody, or preabsorption of the QUIN antibody-containing solution. Preabsorption was accomplished by immobilizing BSA-coupled QUIN on a nitrocellulose strip (Moffett et al. 1994a), incubating the strip overnight with an aliquot of the QUIN-containing solution, removing the strip, and applying the solution to the cryostat sections. To test the specificity of the antibody further, aliquots of the QUIN-containing solution were also preabsorbed with phthalic acid–BSA or picolinic acid–BSA conjugates.

Results

Characterization of the QUIN antibody in vitro

The highest dilution of the antibody capable of producing an optical density reading that was significantly different from control (i.e., non-immunized rabbit serum) was 1:25,600 (Fig. 2a). Even at the highest concentration (1:100 dilution), the antibodies showed only very little or no reactivity in control wells coated with BSA or with BSA covalently coupled to tryptophan (data not shown).

Free QUIN dose-dependently inhibited the binding of the antibody to immobilized QUIN (Fig. 2b). Notably, the concentrations of free QUIN necessary for efficient displacement were beyond the physiological range of free QUIN concentrations in the brain (Morrison et al. 1999). Several test compounds with structural, metabolic, or potential functional relationships to QUIN, namely kynurenine, 3HANA, picolinate, phthalate (Fig. 2b), tryptophan, anthranilate, kynurenate, nicotinate, glutamate, and serotonin (data not shown), were tested but, with the exception of phthalate, did not show any significant crossreactivity at concentrations up to 500 µg/well, indicating considerable specificity of the QUIN antibody.

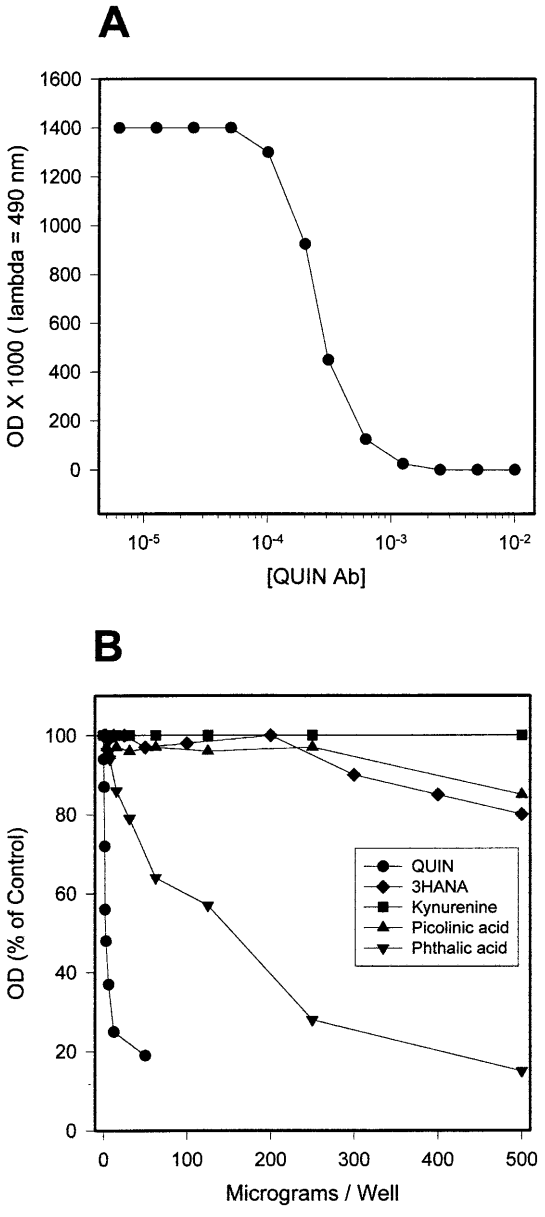

Fig. 2 a Titration curve demonstrating the potency of the quinolinate (QUIN) antibody (Ab) used in this study. Data are expressed as the optical density (OD) readings measured in comparison to normal, non-immunized rabbit serum. **b** Specificity of the QUIN antibody was tested in vitro by incubating the QUIN antibody (1:6,400 dilution) with immobilized bovine serum albumin–QUIN conjugate in the presence of free QUIN or structurally related compounds

Immunohistochemistry

Na striatum

No QUIN-ir was detected in the normal or vehicle-injected striatum. After a continuous infusion of 300 µM 3HANA up to 4 h, faint QUIN-ir was present in striatal cells of apparent microglial morphology (micrographs not shown). Next, the concentration of 3HANA was raised 50-fold (to 15 mM) in an attempt to increase staining intensity. Infusion of 15 mM 3HANA for 1 h resulted in the robust labeling of microglia-like cells, revealing fine crenellated processes and strongly labeled somata (Fig. 3a). Control experiments using either preabsorption of the antibody solution with immobilized BSA-QUIN (Fig. 3b) or co-infusion of 15 mM 3HANA with an equimolar amount of the 3HAO synthesis inhibitor 4-Cl-3HANA (Fig. 4d) abolished QUIN-ir, demonstrating the specificity of the cellular staining. In separate animals, an acute intrastriatal

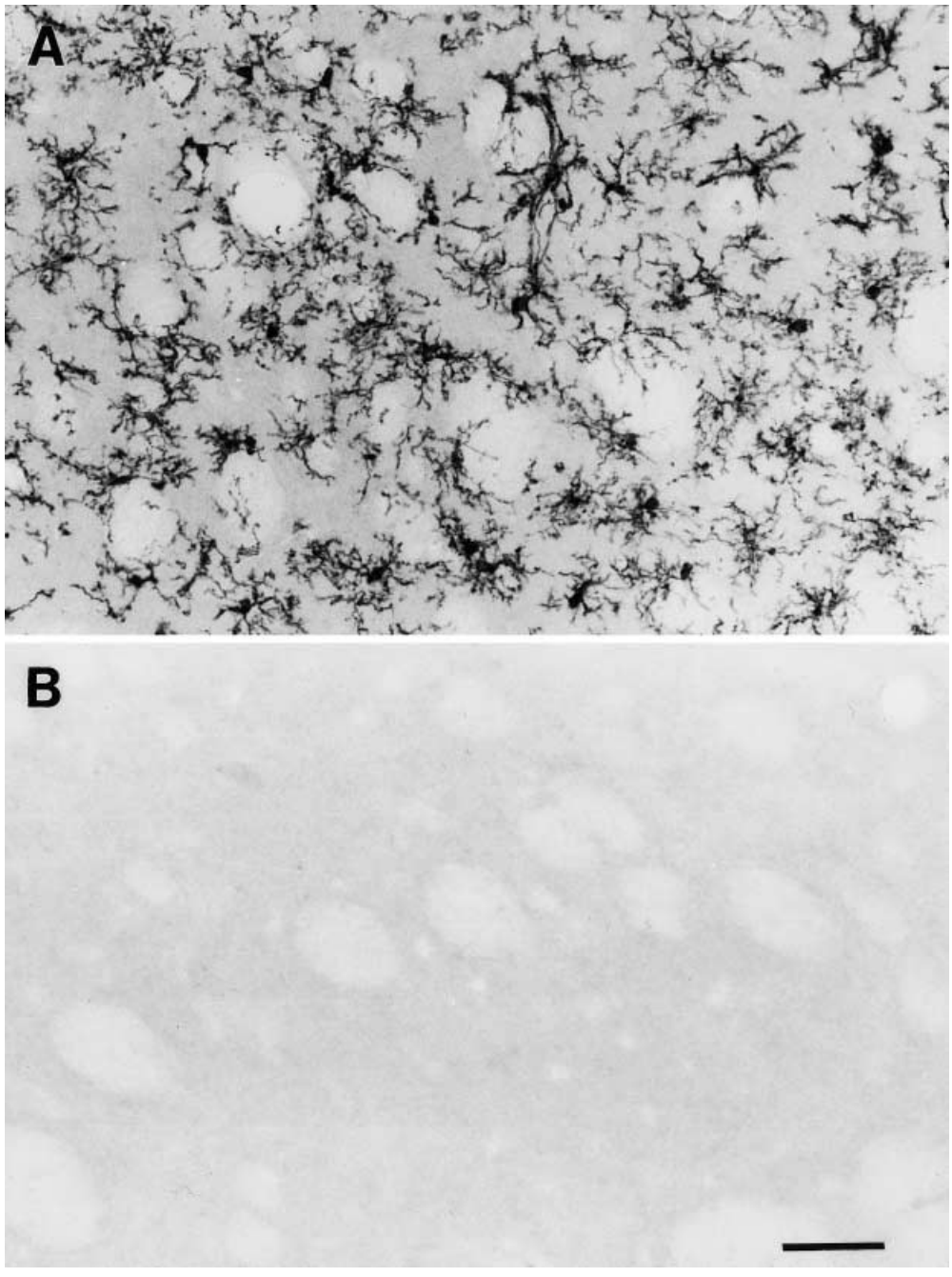

Fig. 3a, b QUIN immunoreactivity (ir) in the normal striatum after a continuous 1-h infusion of 15 mM 3HANA. QUIN-ir cells (**a**) display numerous different, delicate, microglial-like morphologies. Staining is abolished by preabsorption of the QUIN antibody with immobilized, protein-conjugated QUIN (**b**). *Bar* 50 µm

QUIN infusion (120 nmol/1 µl per 10 min) failed to elicit cellular QUIN-ir in animals killed 1 h later (n=2; micrograph not shown).

Parallel sections stained with QUIN, OX42, and GFAP antibodies were used to identify the cell types responsible for the rapid production of QUIN from 15 mM 3HANA in the striatum. The cytoarchitecture of all QUIN-ir cells was indistinguishable from that of OX42-ir microglial cells (Fig. 4a), but contrasted sharply with the morphology of GFAP-ir cells (Fig. 4e). Notably, QUIN-ir cells often had processes which appeared to form an extensive web linking several cytoarchitectonically similar cells. QUIN-ir cells in the periphery of the stained area were predominantly labeled in the soma, with a fainter staining of cellular processes. Co-infusion of 4-Cl-3HANA with 3HANA did not affect the appearance of OX42 or GFAP staining (Fig. 4b, f).

Lesioned striatum

No basal QUIN-ir was detected in striata receiving a focal injection of 120 nmol QUIN/1 µl 1 week earlier. QUIN-ir was only present after an infusion of 15 mM 3HANA (Fig. 5c) and was prevented by co-infusion of 15 mM 3HANA and 15 mM 4-Cl-3HANA (Fig. 5d).

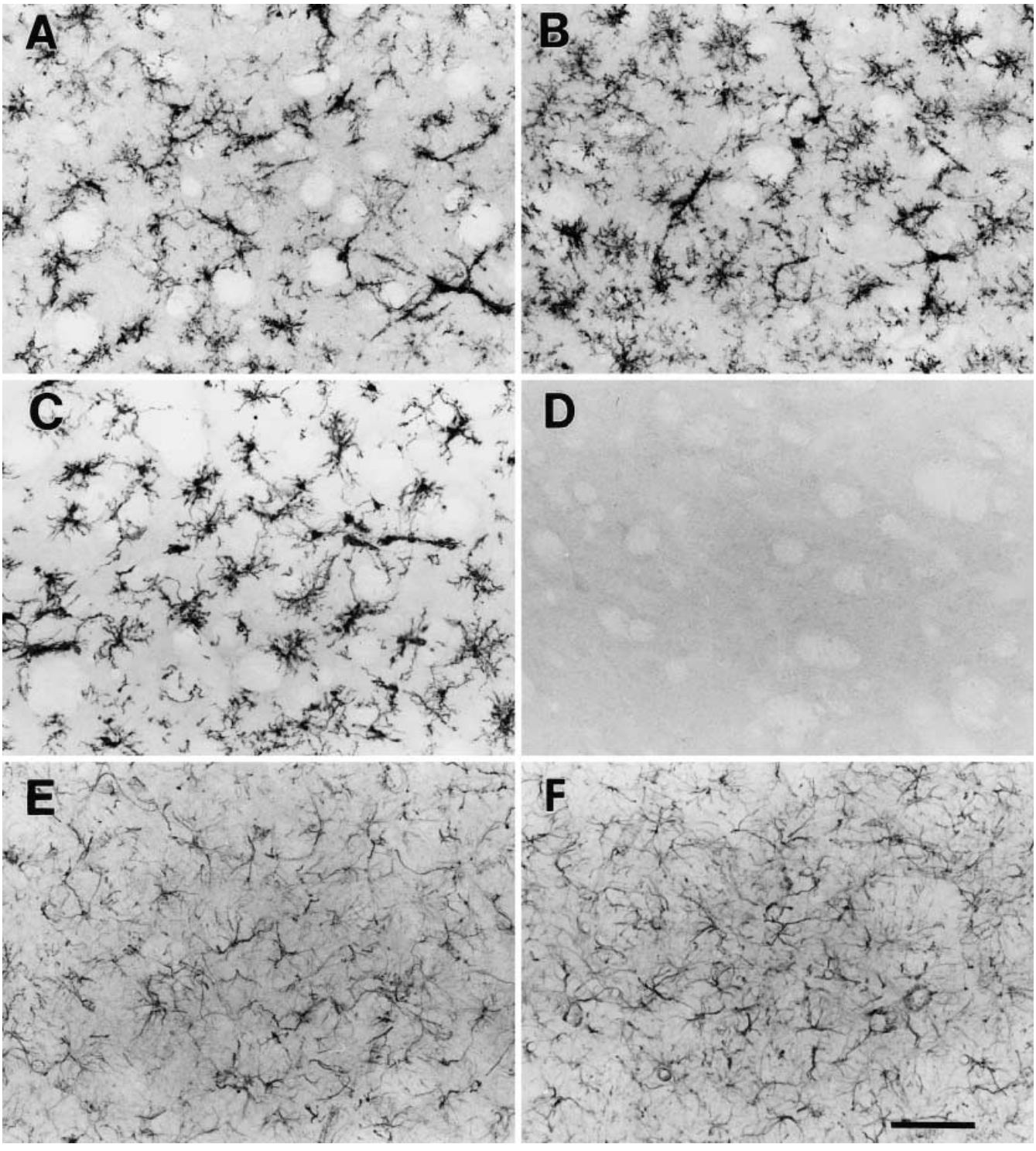

Fig. 4 Micrographs of the normal striatum after a continuous 1-h infusion of 15 mM 3HANA alone (**a**, **c**, **e**) or in combination with 15 mM 4-Cl-3HANA (**b**, **d**, **f**). Sections were stained with antibodies against complement type 3 receptor (OX42; **a**, **b**), QUIN (**c**, **d**) or glial fibrillary acidic protein (GFAP; **e**, **f**). QUIN-ir cells (**c**) display a microglial morphology and distribution, and staining is prevented by co-infusion with 4-Cl-3HANA (**d**). OX42-ir (**b**) and GFAP-ir (**f**) are not affected by 4-Cl-3HANA. *Bar* 50 µm

QUIN-ir cells had a round/oval shape and were almost devoid of processes. When detectable, immunostained processes were short and thick (Fig. 5c), similar to those seen in parallel OX42-immunostained sections (Fig. 5a). In the periphery of the lesion, penumbral zones of both OX42-ir and QUIN-ir revealed a transition from macrophage-like to microglia-like morphologies (micrographs not shown). Anti-GFAP antibodies showed the expected labeling of reactive, hypertrophic astrocytes, as de-

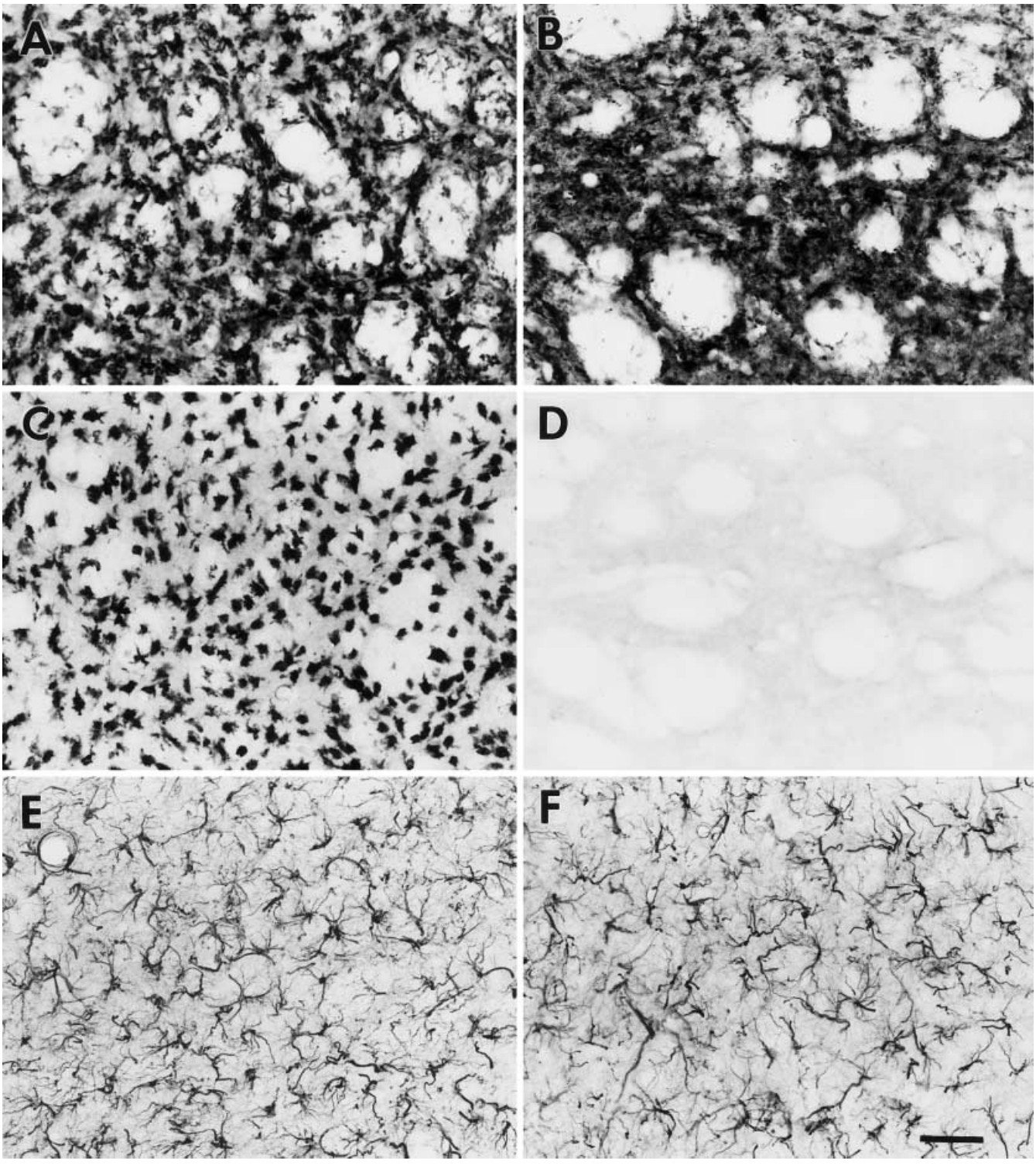

Fig. 5 Micrographs of the excitotoxically lesioned striatum after a continuous 1-h infusion of 15 mM 3HANA alone (**a**, **c**, **e**) or in combination with 15 mM 4-Cl-3HANA (**b**, **d**, **f**). Animals were used 1 week after a focal QUIN injection (120 nmol/1 µl). OX42-ir cells (**a**, **b**) are round, often confluent phagocytes. QUIN-ir cells (**c**, **d**) display a similar macrophage-like phenotype (**c**). No QUIN-ir cells are present after co-infusion of 4-Cl-3HANA and 3HANA (**d**). Anti-GFAP antibodies label hypertrophic astrocytes (**e**, **f**). Neither OX42-ir nor GFAP-ir are affected by 4-Cl-3HANA. *Bar* 50 µm

scribed previously (Björklund et al. 1986). The appearance of OX42-ir and GFAP-ir was not affected by coadministering 4-Cl-3HANA with 3HANA (Fig. 5b, f).

Discussion

The present study was designed to identify the cells that are responsible for the rapid de novo formation of

QUIN in the brain. To this end, rats were injected intrastriatally with QUIN's bioprecursor 3HANA, which is effectively converted to QUIN after intracerebral application (Speciale et al. 1989), and newly synthesized QUIN was visualized with antibodies directed against protein-linked QUIN (Moffett et al. 1993, 1994a, b; Barattè et al. 1998). Precursor administration became necessary because the low ambient concentrations of 3HANA (10–20 nM; Baran and Schwarcz 1990) and QUIN (<100 nM; Morrison et al. 1999) in brain parenchyma do not permit the immunohistochemical localization of QUIN in the normal rat brain (Moffett et al. 1998). Several control experiments validated our results. First, QUIN-ir was abolished by preabsorption of the QUIN antibody with immobilized BSA-conjugated QUIN; second, the antibody did not display crossreactivity with several structural, metabolic, or functional QUIN congeners, confirming the specificity of the antibody for protein-conjugated QUIN. Functional evidence was provided by co-infusion of 3HANA with the highly specific, potent 3HAO inhibitor 4-Cl-3HANA, which effectively blocks the de novo synthesis of QUIN from 3HANA in the rat brain (Heyes et al. 1988; Todd et al. 1989; Walsh et al. 1994).

One of the main findings of the present study was that the rapid production of QUIN in the na rat striatum (and hippocampus; unpublished observations) takes place preferentially in microglial cells when hyperphysiological concentrations of 3HANA are used to drive QUIN synthesis. This was demonstrated by the identical morphology and distribution of QUIN-ir and OX42-ir cells. Moreover, in the experimentally lesioned striatum the rapid conversion of 3HANA to QUIN was found to occur in intensely stained OX42-ir macrophages and activated microglia, both of which displayed strong QUIN-ir. The latter finding is in line with a report by Barattè et al. (1998), who demonstrated QUIN-ir cells with the morphology of activated microglia/infiltrating macrophages in the hippocampal CA1 subfield following experimentally induced cerebral ischemia. Taken together, these studies therefore suggested that microglial activation and/or the infiltration of blood-borne macrophages are responsible for enhanced cerebral QUIN formation following brain injury.

Early immunohistochemical studies, using antibodies directed against 3HAO or QPRT, had indicated that both QUIN formation and degradation in the brain take place in astrocytes. In particular, these light and electron microscopic analyses (Köhler et al. 1987; Okuno et al. 1987; Roberts et al. 1995), which included the assessment of lesioned striatal tissue (McCarthy et al. 1994), did not reveal any immunopositive cells of a microglial/macrophage morphology. It is noteworthy that the antibodies that were used for the visualization of QUIN's metabolic enzymes effectively neutralized the respective enzyme activities in immunotitration experiments (Okuno and Schwarcz 1985; Okuno et al. 1987), and that the 3HAO antibodies used for immunohistochemistry were also successfully employed to clone the 3HAO gene (Malherbe et al. 1994). These studies therefore strongly suggested that astrocytes are primarily responsible for QUIN synthesis and degradation in the mammalian brain (Schwarcz et al. 1996).

The formation and cellular origin of QUIN have also been extensively investigated using biochemical methods. Cell culture studies with monocytes, macrophages, and cells of related lineage (Heyes et al. 1992, 1997; Alberati-Giani et al. 1996; Espey et al. 1997) provided evidence that QUIN can be readily produced from upstream kynurenine pathway metabolites such as tryptophan, kynurenine, 3-hydroxykynurenine, and 3HANA. Moreover, QUIN formation in these peripheral cells is greatly stimulated when one or more enzymes in the biosynthetic cascade, especially indoleamine-2,3-dioxygenase, are activated under immunocompromised conditions (Werner-Felmayer et al. 1989). The situation is different in the brain, where the relatively low basal activity of indoleamine-2,3-dioxygenase (Fujigaki et al. 1998), and the efficient competing metabolism to serotonin, account for the poor incorporation of tryptophan into downstream kynurenine pathway metabolites (Speciale et al. 1989). Since the mRNAs for all three enzymes responsible for QUIN production from kynurenine are expressed in microglial cells (Guillemin et al. 1999), the enzymatic degradation of kynurenine, 3-hydroxykynurenine, or 3HANA to QUIN in the normal brain can clearly occur in these cells. In contrast, astrocytes express only the mRNAs for kynureninase and 3HAO, but not for kynurenine 3-hydroxylase, the first enzyme in the cascade (Guillemin et al. 1999; cf. Fig. 1). Thus, QUIN production from 3HANA can in principle take place in both microglia and astrocytes, whereas the conversion of kynurenine to QUIN may be largely limited to microglia. Notably, bioprecursor-induced QUIN synthesis has been demonstrated in cultured astrocytes, though the yield of QUIN was far inferior to that observed in monocytes, macrophages, and embryologically related cells (Heyes et al. 1992, 1997; Guillemin et al. 1999).

QUIN content and formation in the brain are elevated after various systemic or focal insults, including the administration of immunostimulants (Saito et al. 1993), cerebral ischemia (Saito et al. 1993), traumatic injury (Popovich et al. 1994; Blight et al. 1995), or exposure to viral agents (Eastman et al. 1994). Moreover, brain tissue slices from excitotoxically lesioned animals produce substantially higher extracellular QUIN levels after bath application of 3HANA than unlesioned control tissue (Speciale and Schwarcz 1993). Under many of these injurious conditions, all kynurenergic enzymes in the brain, including 3HAO, are stimulated (Schwarcz et al. 1989; Saito et al. 1993; Ceresoli-Borroni et al. 1999), and focal activation of brain cells is probably primarily responsible for the increase in QUIN production in most pathological situations (Heyes and Morrison 1997). Notably, high kynurenergic enzyme activities also occur in several human brain diseases such as Huntington's disease (Foster et al. 1985; Schwarcz et al. 1988), temporal

lobe epilepsy (Feldblum et al. 1988), and diseases caused by, or associated with, a compromised immune system (Heyes et al. 1993).

The currently available information, including the results of the study described here, is consistent with a scenario in which various non-neuronal brain cell types serve different roles in the formation and release of QUIN in the brain. Thus, the de novo formation of QUIN from millimolar concentrations of 3HANA clearly occurs preferentially in cells of a microglial lineage. Astrocytes, on the other hand, may be responsible for the synthesis of QUIN from (sub)micromolar 3HANA concentrations and for providing a steady, continuous flux of small quantities of QUIN for highly localized interactions with neuronal NMDA receptors (Roberts et al. 1995), so far the only unequivocally identified target of QUIN in the brain. Because of the low K_m value of astrocytic 3HAO (ca 3 μM; Foster et al. 1986) and the presence of QPRT in astrocytes (Köhler et al. 1987, 1988), even sudden surges in kynurenine pathway metabolism will not result in a proportional rapid increase in QUIN formation in these cells, avoiding a potentially detrimental QUIN-induced overactivation of NMDA receptors. The likely saturation of astrocytic 3HAO at low 3HANA concentrations also provides an explanation for the absence of QUIN-ir in astrocytes after acute tissue exposure to millimolar concentrations of 3HANA in the present study. By inference, it seems reasonable to postulate a 3HAO isoform with a high catalytic capacity and a relatively low affinity for 3HANA, which is preferentially localized in microglia/macrophages. The existence of such an isozyme would not only account for the lack of microglial/macrophage immunostaining with the currently available anti-3HAO antibodies (Roberts et al. 1995), but may also pertain to concepts of inflammatory and/or degenerative brain diseases. Thus, the activity of this hypothetical 3HAO isozyme may be controlled by distinct modulatory mechanisms, which would in turn influence the access of microglia/macrophage-derived QUIN to neuronal NMDA receptors (Chiarugi and Moroni 1999). If verified, this enzyme would therefore constitute an interesting new target for the development of drugs designed to modify NMDA receptor function.

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