β_2 -Adrenergic Receptors Are Expressed by Glia *in vivo* in the Normal and Injured Central Nervous System in the Rat, Rabbit, and Human

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Previous studies have demonstrated that glial cells in culture express several subtypes of functional adrenergic receptors. To determine if similar receptors are expressed by glia *in vivo*, we examined the expression of adrenergic receptors in the normal, crushed, and transected optic nerves of the rabbit and rat using quantitative receptor autoradiography. Additionally, we examined the expression of adrenergic receptors in the normal and damaged human optic nerve. High levels of α_1 -, α_2 -, β_1 -, and β_2 -adrenergic receptors were identified in the rabbit and rat forebrain. In the normal rabbit, rat, and human optic nerves, only α_1 and β_2 receptors were observed, and these were present in low to moderate densities. Combined immunohistochemistry and autoradiography suggests that the majority of β_2 -adrenergic receptors in the rabbit, rat, and human optic nerve are expressed by astrocytes.

After unilateral optic nerve crush or transection, only β_2 -adrenergic receptors were significantly increased. This increase in β_2 receptors was first detectable at days 7 and 28 post-transection in the rabbit and rat, respectively. The expression of β_2 receptors in the transected optic nerve continued to increase with time, so that by 90 d post-transection the density of β_2 receptors in both the rabbit and rat optic nerve was among the highest of any area in the forebrain. Taken together with previous studies, these results suggest that *in vivo*, β_2 -adrenergic receptors may provide a therapeutic target for regulation of astrocyte functions including glycogen metabolism, cytokine release, and the hypertrophy and proliferation that occurs in response to neuronal injury.

[Key words: neuroglia, adrenergic receptors, catecholamine, astrocytes, CNS, trauma]

There are three major classes of glia in the mammalian CNS: oligodendrocytes, astrocytes, and microglia (Miller et al., 1986, 1989). In the normal optic nerve oligodendrocytes provide the myelin sheaths that enhance the conduction efficiency of the axons they surround. In contrast, astrocytes are present between

the nerve fascicles, often with foot processes extending to an adjacent blood vessel, and appear to play a role in ionic buffering and maintenance of the blood–brain barrier (Tout et al., 1988; Hatten et al., 1991). The microglia are present in smaller numbers, in a distribution similar to the astrocytes, and are thought to play a macrophage-like role in the CNS (Miller et al., 1989; Mantyh, 1991).

The mammalian optic nerve is unique in that distal to the transection, there are no surviving axons, so that in time the injured nerve becomes a pure glial scar. After optic nerve transection oligodendrocytes atrophy and appear to degenerate, along with the transected retinal ganglion cell axons they ensheath (Villegas-Pérez et al., 1993). In contrast, the astrocytes and microglia proliferate, undergo substantial hypertrophy, and ultimately become the major cell types comprising the glial scar (Smith and Miller, 1991). Unlike fibroblasts, which form scars in non-neural tissue by secreting large amounts of collagenous extracellular matrix, astrocytes form scars by extending numerous processes that become packed with intracellular glial filaments (Maxwell and Kruger, 1965). Since the glial response to injury in part determines the success or failure of axonal regeneration (Savio and Schwab, 1989; Smith et al., 1990; Bray et al., 1991; McKeon et al., 1991), characterization of factors that regulate glial scar formation may allow the development of a pharmacological strategy for minimizing neuronal loss and dysfunction following CNS trauma.

In recent years substantial evidence has accumulated that glial cells *in vitro* express a diverse set of receptors, including (Wilkin et al., 1990) ciliary neurotrophic factor (Fulton et al., 1991), endothelin (Marsault et al., 1990; Höslí and Höslí, 1991), epidermal growth factor (Wilkin et al., 1990), fibroblast growth factor (Wilkin et al., 1990), tumor necrosis factor- β (Wilkin et al., 1990), platelet-derived growth factor (Richardson et al., 1988), interleukin-1 (Wilkin et al., 1990), interleukin-6 (Guillain and Lachman, 1985), α - and β -adrenergic (Salm and McCarthy, 1989, 1990, 1992; Dave et al., 1991), bradykinin (Cholewinski et al., 1991), substance P (Mantyh et al., 1989b; Torrens et al., 1989; Beaujouan et al., 1990, 1991; Delumeau et al., 1991), somatostatin (Wilkin et al., 1990), and vasoactive intestinal polypeptide (Cholewinski and Wilkin, 1988; Wilkin et al., 1991). Of these receptors expressed by astrocytes *in vitro*, the adrenergic receptors (ARs) have been the most extensively studied. Activation

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of ARs has been shown to produce alterations in the morphology (Hatton et al., 1991; Hansson and Rönnebeck, 1992), expression of early response genes (Arenander et al., 1989), membrane potential (Walz, 1989), and glycogen metabolism (Cummins et al., 1983; Rosenberg and Dichter, 1985; Hsu and Hsu, 1990; Subbarao and Hertz, 1990; Sorg and Magistretti, 1992) of astrocytes. Before any functional or therapeutic significance can be established for these *in vitro* observations, the presence of functional receptors on glial cells *in vivo* must be established.

The major thrust of the present report is to explore the glial expression of ARs *in vivo*. The models we use are the crushed or transected rat and rabbit optic nerves. Using these models we can examine, in an *in vivo* situation, the receptors that glia express after neuronal injury without surviving or intrinsic neurons complicating the analysis. Additionally, we have examined the ARs expressed in normal and damaged human optic nerves. In the present report, we address several questions, including the subtypes of ARs glia express in the normal and injured brain, whether the level of glial AR expression is altered in response to neuronal injury, the time course of any changes in AR expression by glia following neuronal injury, and whether there are significant species differences in the expression of ARs by glia.

Materials and Methods

Rabbit and rat tissue. Thirty-two adult male (10–12 weeks old, 4–5 lb) New Zealand White rabbits (Birchwood Valley Farms, Red Wing, MN) and 33 adult male (7–10 weeks old, 250 gm) Holtzman rats (Harlan Sprague Dawley, Madison, WI) were used. The rabbits were deeply anesthetized with intramuscular injections of 3 mg/kg xylazine, 50 mg/kg ketamine, and 50 mg/kg Nembutal, whereas rats were anesthetized with an intraperitoneal injection of 50 mg/kg Nembutal. A 1 cm incision was made away from the lateral canthus, the eyelid was gently retracted, and the eye pulled forward to expose the optic nerve. The optic nerve was then either unilaterally transected, approximately 1–2 mm from the retina, at the optic nerve head, or approximately 5 mm of the optic nerve was crushed using a forceps, and the skin was then sutured. For controls, we performed the same surgical procedure but did not transect or crush the optic nerve.

The animals that had undergone optic nerve transection were allowed to recover and survive for 0, 1, 2, 4, 7, 14, 28, or 90 d (a 60 d time point was included for rats), with three animals at each time point, except for 90 d, where there were six rabbits and five rats, whereas the animals that had undergone optic nerve crush were allowed to recover and survive for 7, 17, or 60 d for rabbits and 7, 14, or 120 d for rats.

To examine whether retinal ganglion cells synthesize and transport ARs to optic nerve axons and terminals, we examined unilaterally doubly ligated optic nerves to determine whether there was significant build-up of ARs proximal to the ligation. Double ligations were performed unilaterally on one optic nerve of two rabbits and one rat. The animals were anesthetized as described above, and the eye was gently pulled forward. Two silk sutures were then tied, until snug, around the optic nerve, with the proximal ligation being approximately 1–2 mm from the retina, and the distal ligation being 4–5 mm from the retina. Twenty-four hours after ligation the animals were killed and the optic nerves and retinas were removed and processed for AR receptor autoradiography as described below.

To obtain tissue from normal or experimental animals, the animals were first overdosed with Nembutal and perfused transcardially with phosphate-buffered saline (PBS) (pH 7.4, 4°C), and the whole optic nerve, forebrain, and, in some cases, retina were rapidly dissected out and blocked in the transverse plane, embedded in OCT Tissue Tek (Miles, Elkhart, IN) in a plastic mold, and frozen on dry ice. In the case of the retina, the retina was removed, filled with OCT Tissue Tek, and processed as described below.

Human tissue. Three human optic nerves were studied. At the time of enucleation for clinically indicated reasons (severe endophthalmitis and choroidal melanoma), any excess tissue not needed for pathological

examination was utilized. In all cases, appropriate informed written consent was secured. The first patient was a 89-year-old man with a long history of glaucoma who had endophthalmitis after cataract surgery 6 d previously. The second patient was a 69-year-old woman with choroidal melanoma of her macula, just inferior to her fovea, affecting only a small percentage of ganglion cell axons. The third patient was a 50-year-old man with a small peripheral choroidal melanoma and essentially no visual loss. Within 60 sec after the globe was enucleated, the distal 2–4 mm of nerve was placed in cold OCT Tissue Tek, frozen on dry ice, and processed for autoradiography as described below.

Tissue sectioning. The transected, crushed, and normal optic nerves were serially sectioned in the transverse plane at the distal end of the optic nerve, the double-ligated optic nerves were serially sectioned longitudinally, and the retinas were serially sectioned in the horizontal plane. In all cases where we used transected optic nerves, we used the optic nerve segment that was distal to the transection. All tissue was sectioned at $15 \pm 0.2 \mu\text{m}$ on a cryostat microtome (Hacker Instruments Inc., Fairfield, NJ) and thaw mounted onto gelatin-coated microscope slides. The tissue-mounted slides were stored for up to 2 months at -70°C in boxes containing desiccant.

Immunohistochemical and tissue staining. To determine the total number of cells in a $15 \mu\text{m}$ section of the rabbit and rat optic nerve at various time points after transection, the tissue sections were Nissl stained and cell nuclei counted. To determine the number of astrocytes in a section of optic nerve at various time points after transection, we used a rabbit antiserum raised against the astrocyte marker bovine glial fibrillary acidic protein (GFAP) (Dako, Carpinteria, CA). Immunohistochemistry was performed by immediately fixing the thaw-mounted sections in a PBS solution containing 4% paraformaldehyde (30 min at 4°C , pH 7.4) (Mantyh and Hunt, 1984; Kruger et al., 1988). The slides were then washed in PBS (pH 7.4) for 10 min at 22°C . The slide-mounted tissue sections were incubated at 4°C overnight by laying the slide flat and covering the tissue section with 1 ml of primary antibody solution (pH 7.4) containing 0.3% Triton X-100, 0.9% NaCl, 1.0% normal goat serum, and the anti-GFAP primary antibody (1:500).

Following the incubation with the primary antibody, the tissue sections were washed for 30 min at 22°C in PBS (pH 7.4) and then covered with the secondary antibody solution (pH 7.4) for 2 hr at 22°C . This secondary antibody solution was identical to the primary antibody solution except that fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG (Dako, Carpinteria, CA) was present (1:100) instead of the anti-GFAP antibody. Finally, the tissue sections were washed in PBS (pH 7.4) for 30 min at 22°C and coverslipped using PBS-glycerine containing *p*-phenylenediamine to reduce photobleaching of fluorescein (Johnson and Nogueira-Araujo, 1981).

To determine the number of microglia/macrophages within the optic nerve at various time points after optic nerve transection, the microglia/macrophage marker mouse anti-rat OX-42 (OX-42, which recognizes the rat C3b complement receptor; Harlan Bioproducts, Indianapolis, IN) was used with FITC-conjugated anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN). The same protocol described above was used for anti-OX-42 except that the slides were incubated overnight at 4°C with anti-OX-42 at a concentration of 1:10 in a solution of PBS + 0.5% bovine serum albumin (BSA) (pH 7.4). This was followed by a 10 min wash at 22°C in PBS (pH 7.4) and a 1 hr incubation at 22°C with the secondary antibody FITC-conjugated anti-mouse IgG, which was used at a concentration of 1:200 in PBS + 0.1% BSA.

Sections were examined using a Leitz Orthoplan II microscope equipped for fluorescence, bright-field, and dark-field microscopy. To determine the total number of cells in a $15 \mu\text{m}$ section of the normal and transected optic nerve, the Nissl-stained tissue was examined under bright field at $400\times$. Using a 1 cm^2 eyepiece grid that was divided into 100, 1 mm^2 units, the labeled cells in each of three pairs of transected and normal optic nerves were counted. This was performed by counting all the cells that were within the perimeter of the grid, in three different areas of each optic nerve. The area of the optic nerve was measured by tracing each nerve using the NIH IMAGE 1.51 image analysis program. The counts were averaged and multiplied by the total area of each optic nerve to obtain an estimate of the total number of cells in a $15 \mu\text{m}$ section of the optic nerve. The GFAP+ cells and OX-42+ cells were counted and examined in a similar manner under fluorescence using an I3 (450–490 nm) excitation filter (Wild Leitz, Rockleigh, NJ).

¹²⁵I-HEAT autoradiographic binding protocol (α_1 -ARs). Quantitative autoradiography was performed by first bringing the slide-mounted tissue sections to 22°C . The slides were then immersed for 10 min at 22°C

in a preincubation solution containing 170 mM Tris-HCl, 10 mM MgCl₂, and 0.01% ascorbic acid (pH 7.4). This was followed by a 2 hr incubation at 22°C in which the slides were laid flat and the tissue was covered with 1 ml of preincubation solution plus 80 pM of the α_1 -adrenergic-specific radioligand β -(¹²⁵I-iodo-4-hydroxyphenyl)-ethyl-aminomethyl-tetralone (¹²⁵I-HEAT; DuPont-New England Nuclear, Boston, MA) (Shao and Sutin, 1991, 1992). To determine the nonspecific binding, paired serial sections were incubated as described above with the exception that 1 μ M phentolamine (an α -adrenergic antagonist; Research Biochemicals Inc., Natick, MA) was added to the incubation solution. To determine whether the ¹²⁵I-HEAT was binding to α_1 -ARs, serial sections were incubated as described above except that varying concentrations (10⁻¹¹ to 10⁻⁶ M) of prazosin (an α_1 -adrenergic antagonist; Research Biochemicals Inc., Natick, MA) or yohimbine (an α_2 -adrenergic antagonist; Research Biochemicals Inc., Natick, MA) were added to the incubation solution. Immediately following the incubation, the slides were washed twice in 170 mM Tris-HCl (pH 7.4) for 12 min at 4°C, and then rinsed for 5 min in distilled water at 4°C.

¹²⁵I-PIC autoradiographic binding protocol (α_2 -ARs). After being brought to room temperature, the slides were immersed for 20 min at 22°C in a preincubation solution containing 170 mM Tris-HCl and 10 mM MgCl₂ (pH 7.4) (Alburges et al., 1993). This was followed by a 2 hr incubation at 22°C in which the slides were laid flat and the tissue was covered with 1 ml of preincubation solution plus 100 pM of the α_2 -adrenergic-specific radioligand 2-[(2,6-dichloro-4-[(¹²⁵I]iodophenyl)-iminol]imidazolidine (¹²⁵I-PIC; DuPont-New England Nuclear, Boston, MA). To determine the nonspecific binding, paired serial sections were incubated as described above except that 1 μ M phentolamine was added to the incubation solution. To determine whether the ¹²⁵I-PIC was binding to α_2 -ARs, serial sections were incubated as described above except that varying concentrations (10⁻¹¹ to 10⁻⁶ M) of yohimbine (an α_2 -adrenergic antagonist; Research Biochemicals Inc., Natick, MA) or prazosin (an α_1 -adrenergic antagonist; Research Biochemicals Inc., Natick, MA) were added to the incubation solution. Immediately following the incubation, the slides were washed twice in incubation solution (pH 7.4) for 5 min at 4°C, then dipped twice in distilled water at 4°C.

¹²⁵I-CYP autoradiographic binding protocol (β_1 - and β_2 -ARs). The same general protocol that we used in the ¹²⁵I-HEAT binding was used for the ¹²⁵I-CYP binding, with the exception that 60 pM ¹²⁵I-cyanopindolol (¹²⁵I-CYP; DuPont-New England Nuclear, Boston, MA) was added to the incubation solution in place of ¹²⁵I-HEAT (Shao and Sutin, 1991, 1992). Nonspecific binding was defined as that binding remaining in the presence of 1 μ M isoproterenol (a β -adrenergic agonist; Research Biochemicals Inc., Natick, MA). To determine the β -AR subtypes present, serial sections were incubated as described above except that 200 nM betaxolol (a β_1 -adrenergic antagonist; Alcon, Fort Worth, TX), 200 nM ICI 118,551 (a β_2 -adrenergic antagonist; Research Biochemicals Inc., Natick, MA), or 20 μ M 5-HT (Research Biochemicals Inc., Natick, MA) was added to the incubation solution. In addition, competitive inhibition curves were generated by adding varying concentrations (10⁻¹¹ to 10⁻⁶ M) of betaxolol, 118,551, or 5-HT to generate a Scatchard plot and to determine the K_D and B_{max} of the β_2 -ARs in the normal and transected optic nerves.

To determine the B_{max} of the ARs, it was necessary to determine the amount of protein in the normal and transected optic nerves. This was done by performing a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) on homogenates of previously weighed rabbit cortex, normal optic nerve, and transected optic nerve in which insoluble protein had been removed. The ratio of percentage of protein between a defined area of the temporal cortex, normal optic nerve, and transected optic nerve was then calculated. Using homogenate binding techniques we then determined the B_{max} for each of the four ARs in this piece of temporal cortex. Finally, we autoradiographically determined the density of the ¹²⁵I-CYP, ¹²⁵I-HEAT, and ¹²⁵I-PIC binding sites in the defined piece of temporal cortex and in the normal and transected optic nerves. We then used this data to calculate a B_{max} for each of ARs in the normal and transected optic nerves.

Analysis of autoradiograms. After the final rinse all the slide-mounted tissue sections were dried at 4°C, stored, and desiccated overnight. Quantitative autoradiographic analysis was performed by placing the slide-mounted tissue sections in apposition to β -max Hyperfilm (Amersham, Arlington Heights, IL) alongside ¹²⁵I-Microscales (Amersham, Arlington Heights, IL). After 5–7 d, the film was developed in Kodak D-19 developer, fixed, and washed. The autoradiograms were analyzed by quantitative densitometry using a computerized image analysis program (NIH

IMAGE 1.51). For sections where a greater histological resolution of the binding sites was required, the bound slides were processed for emulsion-dipped autoradiography (Mantyh et al., 1989a). Dark-field and bright-field photomicrographs were then taken of the silver grains and counterstained sections, respectively. Controls for chemographic artifacts were generated by performing the binding exactly as described except that the radioligand was omitted from the incubation medium. Statistical analysis was performed using a Student's *t* test on STATWORKS (Computer Associates, San Jose, CA).

Results

The rabbit and rat optic nerve after optic nerve transection

The normal optic tract is opaque in appearance and is composed of myelinated ganglion cell axons grouped in fascicles, surrounded by cells with darkly stained nuclei and a clear cytoplasm (Fig. 1*b*). In addition to the oligodendrocytes there is a small but significant population of GFAP+ astrocytes (Fig. 1*d*) and OX-42+ microglia/macrophage cells (Fig. 1*f*). The astrocytes and microglia are positioned at the outside of the nerve fascicles and surround both the nerve bundles and the blood vessels within the optic nerve.

After optic nerve transection the lesioned optic tract showed histological changes typical of Wallerian degeneration. Grossly, the lesioned nerve loses its opacity over time and becomes translucent in appearance and reduced in size due to the loss of myelinated optic nerve axons. In Nissl-stained sections, the transected optic nerve (Fig. 1*a*) becomes more darkly stained and is composed of greater numbers of cells with darkly stained nuclei, foamy cytoplasm, and extensive processes that lack the distinctive fasciculated organization characteristic of the normal optic nerve. Staining with antibodies raised against GFAP (Fig. 1*c*) and OX-42 (Fig. 1*e*) suggests that a substantial percentage of the increase in darkly stained cell bodies consists of astrocytes and microglia/macrophages (Fig. 2). Thus, in terms of the total number and percentage of all cells in the optic nerve, both GFAP+ and OX-42+ cells show a substantial increase after optic nerve transection (Figs. 1, 2).

Adrenergic receptors associated with retinal ganglion neurons or retinal ganglion axons

Using receptor autoradiography, we were unable to detect any α_1 -, α_2 -, β_1 -, or β_2 -ARs associated with retinal ganglion axons in sections of rabbit or rat retina. In the rabbit and rat, we were also unable to detect any "pile-up" of α_1 -, α_2 -, β_1 -, or β_2 -ARs proximal to the unilaterally ligated optic nerve, which one would expect to find if these receptors were being transported, via the optic nerve axons, to the terminal targets of the retinal ganglion cells. For these reasons, it appears *unlikely* that a significant percentage of α_1 -, α_2 -, β_1 -, or β_2 -ARs found in the rabbit or rat optic nerve arises from retinal ganglion cells.

Localization and pharmacological characterization of α -adrenergic receptors in the normal and transected rabbit and rat optic nerve

A moderate to high density of saturable binding sites for ¹²⁵I-HEAT (labeling α_1 -ARs) and ¹²⁵I-PIC (labeling α_2 -ARs) is observed in the rabbit and rat forebrain including the cerebral cortex, caudate nucleus, and claustrum (Fig. 3). The ¹²⁵I-HEAT binding sites in the rat and rabbit cortex and claustrum are displaced by 1 μ M phentolamine (Fig. 3*a,b*), as are the ¹²⁵I-PIC binding sites (Fig. 3*c,d*). To characterize the subtype of α -ARs,

Figure 1. The rat optic nerve after unilateral optic nerve transection. *a* and *b*, Bright-field photomicrographs of a Nissl-stained 15 μm coronal section showing the optic nerve 90 d post-transection (*a*) and the contralateral normal optic nerve (*b*). *c* and *d*, Dark-field photomicrographs showing the localization of GFAP+ astrocytes in the transected (*c*) and normal (*d*) optic nerve. *e* and *f*, Dark-field photomicrographs showing the localization of OX-42+ microglia/macrophages in the transected (*e*) and normal (*f*) optic nerve. Note the striking increase in the number of cells (*a*, *b*), astrocytes (*c*, *d*), and microglia/macrophages (*e*, *f*) in the optic nerve after transection. Scale bar, 70 μm .

Figure 2. Histograms showing the increase in total numbers of cells (Nissl-stained nuclei), astrocytes (GFAP+ cells), and microglia/macrophages (OX-42+) in the rabbit (*a*) and rat (*b*) optic nerve after transection. Note that in the rabbit there is a statistically significant increase in both astrocytes and microglia by day 14, whereas in the rat the increase is significant by day 7.

we used varying concentrations of prazosin (an α_1 -antagonist) or yohimbine (an α_2 -antagonist) to displace 125 I-HEAT or 125 I-PIC. Using 100 pM 125 I-PIC in the presence of 1 μ M of prazosin, a high density of α_2 -ARs is observed in all layers of the cerebral cortex, a low density in the caudate nucleus, and α_2 -ARs were undetectable in the normal optic nerve (Fig. 3c). Using 125 I-HEAT in the presence of 1 μ M yohimbine, a high density of α_1 -ARs was observed in the claustrum and laminae 5 and 6 of the cerebral cortex (Fig. 3a), whereas very low but detectable levels of α_1 -ARs were present in the normal rabbit or rat optic nerve.

After unilateral optic nerve transection or crush, there was no change, at any of the time points examined, in the density of α_1 -ARs or α_2 -ARs in the rabbit or rat forebrain or in the normal or transected optic nerve. Thus, using 125 I-HEAT in the normal rabbit optic nerve, α_1 -ARs have a K_D of 0.4 ± 0.3 nM and a B_{max} of 3.0 fmol/mg protein, whereas in the transected optic nerve the K_D is 0.8 ± 0.4 nM and the B_{max} is 1.1 fmol/mg protein. In the normal rat optic nerve the α_1 -ARs have a K_D of

0.3 ± 0.2 nM and the B_{max} is 2.3 fmol/mg protein, whereas in the transected optic nerve the K_D is 0.2 ± 0.1 nM and the B_{max} is 2.0 fmol/mg protein.

Localization and pharmacological characterization of β -adrenergic receptors in the normal and transected rabbit and rat optic nerve

A high density of specific binding sites for 125 I-CYP was present in the rabbit and rat forebrain including the cerebral cortex and the caudate nucleus (Fig. 3e,f). Additionally, a low to moderate density of 125 I-CYP binding sites was present in both the normal rabbit and rat normal optic nerve (Figs. 3e, 4a). In the normal rabbit and rat optic nerves, specific 125 I-CYP binding sites are associated with the GFAP+ astrocytes that surround the bundles of optic nerve axons. Thus, in serially adjacent sections, one stained immunohistochemically for GFAP+ and the other bound with 125 I-CYP, there is a clear correlation between areas containing the GFAP+ astrocytes and areas displaying high densities of 125 I-CYP binding sites.

In the rabbit and rat forebrain and normal optic nerve, 125 I-CYP was displaced by 1 μ M isoproterenol (Fig. 3f). To characterize further the subtype of β -AR expressed in optic nerves, varying concentrations of betaxolol (a β_1 -antagonist) or ICI 118,551 (a β_2 -antagonist) were used to displace 125 I-CYP (Figs. 4, 5). Using 60 pM 125 I-CYP in the presence of 200 nM ICI 118,551 to visualize the β_1 -ARs, a moderate density of β_1 -ARs was observed in the cerebral cortex and striatum, whereas β_1 -ARs were undetectable in the normal optic nerve (Fig. 4d). Using 125 I-CYP in the presence of 200 nM of betaxolol demonstrated that a moderate density of β_2 -ARs sites was present in the cerebral cortex, striatum, and normal optic nerve (Fig. 4c).

After unilateral optic nerve transection there is no change in the density of β_1 -ARs or β_2 -ARs in the rabbit or rat forebrain or in the contralateral normal optic nerve. In the transected or crushed optic nerve, however, there was a marked increase in the density of β_2 -ARs (Fig. 4a), whereas β_1 -ARs were undetectable in serially adjacent sections. In the rabbit a statistically significant increase in the density of β_2 -ARs was first observed at 7 d post-transection (Fig. 6a), whereas in the rat a significant increase was first observed at 28 d post-transection (Fig. 6b). In both the rabbit and rat this increase in the density of β_2 -ARs continued until 90 d post-transection, the last time point examined. By 90 d post-transection the density of β_2 -ARs in the transected optic nerve was 392% of the control optic nerve in the rabbit, whereas in the rat it was 207% of the control optic nerve. Pharmacological characterization of these changes in β_2 -ARs 90 d after transection suggests that the increase in density of β_2 -ARs is due to an increase in B_{max} rather than a change in the affinity of the receptor. Thus, using 125 I-CYP in the normal rabbit optic nerve, β_2 -ARs have a K_D of 3.5 ± 0.5 nM and a B_{max} of 456 fmol/mg protein, whereas in the transected optic nerve the K_D is 3.9 ± 0.4 nM and the B_{max} is 1792 fmol/mg protein (Fig. 6a). In the normal rat optic nerve the β_2 -ARs have a K_D of 2.9 ± 0.4 nM and the B_{max} is 924 fmol/mg protein, whereas in the transected optic nerve the K_D is 3.1 ± 0.4 nM and the B_{max} is 1914 fmol/mg protein (Fig. 6b).

Localization and pharmacological characterization of adrenergic receptors in human optic nerve

A low to moderate density of specific binding sites for 125 I-HEAT (α_1 -ARs) was present in the normal and pathological human

Figure 3. Autoradiographic localization of α_1 -, α_2 -, and β -adrenergic receptors in a coronal section of the rabbit brain 90 d after unilateral optic nerve transection. In these and the following dark-field photomicrographs the receptor binding sites appear white. *a*, *c*, and *e*, Dark-field photomicrographs showing the total α_1 -adrenergic binding (125 I-HEAT) (*a*), α_2 -adrenergic binding (125 I-PIC) (*c*), and β -adrenergic binding (125 I-CYP) (*e*). To obtain the specific binding, subtract the nonspecific binding in *b*, *d*, and *f* from the total binding in *a*, *c*, and *e*, respectively. Note that while there is a high concentration of α_1 -, α_2 -, and β -adrenergic receptor binding sites in the cerebral cortex, only β -adrenergic receptors are present in moderate concentrations in the normal optic nerve (*N*) and increase in the transected (*T*) optic nerve. Scale bar, 2.2 mm.

Figure 4. Characterization of β -adrenergic binding sites in the normal and transected rabbit optic nerve: a series of dark-field photomicrographs showing the displacement of ^{125}I -cyanopindolol (^{125}I -CYP) binding (*a*, *b*) by a β_1 -antagonist (betaxolol) (*c*), a β_2 -antagonist (ICI 118,551) (*d*), and serotonin (5-HT) (*e*) in serially adjacent sections of normal and transected (90 d) optic nerve. Note that whereas the β_1 -antagonist (*c*) and 5-HT (*e*) show relatively little displacement of ^{125}I -CYP, in either the normal or transected optic nerve, the β_2 -antagonist (*d*) shows almost complete displacement of ^{125}I -CYP from both the normal and transected optic nerve. Scale bars: *a*, 1.30 mm; *b*–*e*, 1.15 mm.

Figure 5. Histograms showing the density and pharmacological profile of adrenergic receptors in the normal and transected rabbit and rat optic nerve. These histograms were generated using quantitative autoradiographic analysis of ¹²⁵I-CYP binding to a normal and contralateral optic nerve that was 90 d post-transection. Note that while there is a moderate concentration of ¹²⁵I-CYP binding sites to the normal optic nerve, there is a substantial increase in ¹²⁵I-CYP binding after optic nerve transection. In both the rabbit (a) and rat (b) the majority of these ¹²⁵I-CYP binding sites appear to correspond to β₂-adrenergic receptors.

optic nerves (Fig. 7), and these binding sites were displaced by 1 μM of either phentolamine or prazosin but not yohimbine. In adjacent sections of either normal or pathological human optic nerve no specific ¹²⁵I-PIC binding sites (α₂-ARs) could be detected. In the normal human optic nerves, specific ¹²⁵I-HEAT binding sites are associated with the GFAP+ astrocytes that surround the bundles of optic nerve axons (data not shown). Thus, in serially adjacent sections, one stained immunohistochemically for GFAP+ and the other bound with ¹²⁵I-HEAT, there is a close register of the GFAP+ astrocytes and areas displaying high densities of ¹²⁵I-HEAT binding sites. These data suggest that α₁-ARs are expressed by astrocytes in human optic nerve in substantially higher concentrations than is observed in either the rabbit or rat.

A high density of specific binding sites for ¹²⁵I-CYP was present in the normal and pathological human optic nerves (Fig. 7). The ¹²⁵I-CYP was displaced by 1 μM isoproterenol (a β-agonist), 200 nM ICI 118,551 (a β₂-antagonist), but not 200 nM betaxolol (a β₁-antagonist). In the normal human optic nerves, specific

Figure 6. The density of β₂-adrenergic receptors in the rabbit (a) and the rat (b) optic nerve at various time points after transection. Note that whereas ¹²⁵I-CYP binding is significantly increased at 7 d post-transection in the rabbit, in the rat a significant increase in ¹²⁵I-CYP binding is not evident until 28 d post-transection. Also note in both the rabbit and rat ¹²⁵I-CYP binding sites continue to increase until 90 d post-transection, the last time point examined.

¹²⁵I-CYP binding sites were in register with the GFAP+ astrocytes that surround the fascicles of optic nerve axons (Fig. 8). Thus, in serially adjacent sections of human optic nerve, one stained immunohistochemically for GFAP+ and the other bound with ¹²⁵I-CYP, the densest concentrations of ¹²⁵I-CYP binding sites are directly over the GFAP+ astrocytes. These data suggest that β₂-ARs are expressed in moderate to high concentrations by astrocytes in both the normal and pathological human optic nerve.

Discussion

Adrenergic receptors expressed by glia in vivo

In the present report we have demonstrated that in both the normal and transected optic nerve, glial cells *in vivo* express β₂-ARs. After neuronal injury the density of β₂-ARs increases, and this increase appears to be due to both the hypertrophy and proliferation of glia that form the glial scar. An issue addressed but not definitively resolved in the present study is the exact glial cell type(s) expressing β₂-ARs. Although the location of β₂-ARs appears in close register with GFAP+ astrocytes in the

Figure 7. Autoradiographic localization of α_1 - and β_2 -adrenergic receptors in a coronal section of the human optic nerve. These sections are taken from tissue obtained from a 50-year-old man with a small peripheral choroidal melanoma and essentially no visual loss. *a* and *b*, Bright-field photomicrograph of a 15 μ m Nissl-stained coronal section (*a*) and a dark-field photomicrograph (*b*) showing distribution of cells (*a*) and the localization of GFAP+ astrocytes (*b*). *c* and *e*, Dark-field photomicrographs showing the total α_1 -adrenergic binding (125 I-HEAT) (*c*) and the total β -adrenergic binding (125 I-CYP) (*e*) in adjacent sections of the human optic nerve. To obtain the specific binding subtract the nonspecific binding in *d* and *f* from the total binding in *c* and *e*, respectively. Note that there are moderate concentrations of both α_1 - and β_2 -adrenergic receptor binding sites in the human optic nerve. Scale bar, 650 μ m.

rabbit and human optic nerve, the density and overlapping distribution of the glial cells within the optic nerve, combined with the diffusion of the bound ligand from the β_2 -AR, make the unequivocal single-cell resolution needed to answer this question difficult to obtain. However, this question has been addressed in a previous study that utilized immunohistochemistry to localize β_2 -AR-like immunoreactivity in the rat CNS (Aoki, 1992; Aoki and Pickel, 1992a,b). These immunohistochemical studies utilized antibodies raised against synthetic peptides corresponding to either the entire β_2 -AR or the carboxyl-terminal tail of the β_2 -AR. In these studies it was demonstrated that β_2 -AR-like immunoreactivity is present in both neurons and glia in the cerebral cortex, and the glia displaying the intense β_2 -

AR-like immunoreactivity have the morphological appearance of astrocytes (Aoki, 1992). In these studies it was also notable that, with the exception of astrocytes, no other glial cell types in any of the normal brain areas examined appeared to express β_2 -ARs, suggesting that the principal glial cell type expressing β_2 -ARs in the CNS is the astrocyte.

Previous *in vitro* studies have shown that there is a marked regional heterogeneity in the receptors expressed by astrocytes. For example, whereas astrocytes cultured from rat cerebral cortex express high levels of substance P receptors, similar astrocytes cultured from spinal cord express low to undetectable levels of substance P receptors (Aoki, 1992; Aoki and Pickel, 1992a,b). A critical question, from the standpoint of attempting

Figure 8. Localization of β_2 -adrenergic receptors in relation to the optic nerve axon bundles and GFAP+ cells in the normal human optic nerve. This tissue is the same as that used in Figure 7. Dark-field photomicrographs of adjacent sections of human optic nerve showing, in *a*, the fascicles of optic nerve axon bundles that appear as *white bundles*; *b*, the location of β_2 -ARs, which appear as *white silver grains*; and *c*, the location of GFAP+ astrocytes, which appear as *bright areas* surrounding the optic nerve axon bundles. Note that there is a clear correlation between the areas containing β_2 -ARs (*b*) and GFAP immunoreactivity (*c*), as both appear to surround the bundles of optic nerve axon bundles (1–5) (*a*). Scale bar, 100 μ m.

to manipulate astrocyte function pharmacologically, is to determine whether astrocytes from brain areas other than optic nerve also express β_2 -ARs in the normal and injured brain.

Two previous studies using different techniques have addressed this question. In the first group of studies, β_2 -AR-like immunoreactivity was localized within astrocytes in the rat cerebral cortex, striatum, area postrema, and nucleus of the solitary tract (Aoki, 1992; Aoki and Pickel, 1992a,b). While these studies demonstrated that the percentage of astrocytes that express β_2 -AR-like immunoreactivity depended on the brain region examined, a significant percentage of astrocytes expressed the β_2 -AR-like immunoreactivity in all brain regions examined.

In a second group of studies the cytotoxin ricin (from *Ricinus communis*) was injected into the trigeminal or sciatic nerve inducing motor neuron death in the trigeminal motor nucleus or ventral motor neurons, respectively (Sutin and Shao, 1991, 1992; Sutin and Griffith, 1993). Following motor neuron death the tissue was examined, using autoradiographic binding techniques, to determine whether significant changes had occurred in the density of α_1 - or β -ARs. In these studies, injury and death of the motor neurons resulted in a decrease in α_1 -ARs, while the β_2 -ARs increased after nerve injury, and this increase was correlated with an increase in astrocyte GFAP immunoreactivity (Sutin and Shao, 1991, 1992). From these studies the authors suggested that whereas the α_1 -ARs appeared to be associated primarily with the motor neurons, the more numerous β_2 -ARs were associated with the surrounding astrocytes.

These studies together with the present results suggest that β_2 -ARs are expressed in moderate to high levels by astrocytes in all CNS areas examined to date. These studies also suggest that the increase in astrocyte β_2 -AR expression that occurs after nearby neuronal injury is not confined to the optic nerve. Rather, the expression of β_2 -AR appears to be a general property of a population of astrocytes that is distributed throughout the mammalian CNS. Additionally, the expression of β -ARs by astrocytes does not appear to be limited to one species. Thus, we have shown that astrocytes from the rat, rabbit, and human appear to express β_2 -ARs. This widespread expression of β_2 -ARs by astrocytes suggests that manipulation of astrocytic function by β_2 -AR analogs may be therapeutically possible in most areas of the CNS and in most mammalian species including human.

Potential source of ligands that would interact with the β_2 -adrenergic receptors expressed by glia in vivo

A key question concerning the functional significance of β_2 -AR expression by glia is the source of endogenous noradrenaline that might occupy these astrocyte β_2 -ARs *in vivo*. There are at least two probable sources. The first source is the noradrenaline released from intrinsic noradrenaline-synthesizing neurons within the CNS. An example of such noradrenergic neurons are those located in the locus coeruleus. Noradrenergic locus coeruleus neurons are known to have widespread projections throughout the brain and noradrenaline released from nerve terminals could presumably occupy β_2 -ARs expressed by neighboring astrocytes (Beaudet and Descarries, 1978). Morphological support for this possibility comes from studies at the electron microscopic level (Aoki and Pickel, 1992a) that demonstrate that numerous synaptic contacts are made between astrocytes expressing β_2 -AR-like immunoreactivity and nerve terminals with catecholaminergic-like immunoreactivity. While catecholaminergic neurons in the CNS may be an important source

of noradrenaline in brain areas that receive a heavy noradrenergic innervation, it is clear that the presence of a direct noradrenergic innervation is not directly correlated with the level of β_2 -AR expression by astrocytes. For example, the normal optic nerve receives virtually no detectable noradrenergic input and yet astrocytes in the normal optic nerve express β_2 -ARs. This lack of correlation between neuronal noradrenergic input and the astrocytic expression of β_2 receptors is even more apparent after neuronal injury. After optic nerve transection there are no surviving neurons or axons in the optic nerve distal to the transection, yet astrocytes in the transected optic nerve express high levels of β_2 -ARs.

A second possible source is the catecholamine present in blood and plasma. In normal human plasma noradrenaline and adrenaline levels are generally relatively low, on the order of 1–2 nM and 0.1–0.2 nM, respectively (Cryer, 1980; Felig et al., 1987). The major sources of blood-borne catecholamine are the adrenals and sympathetic neurons, which contribute approximately 75% and 25%, respectively, to noradrenaline plasma levels (Cryer, 1980). In response to injury and trauma, however, the circulating levels of noradrenaline and adrenaline rise dramatically to the level of 10–20 nM and 5–10 nM, respectively. In the normal intact mammalian CNS, with the exception of brain areas outside the blood–brain barrier, it is unlikely that glial β_2 -ARs would be occupied by circulating catecholamine, which are unable to cross the blood–brain barrier to any appreciable extent (Felig et al., 1987). However, after neuronal injury there is frequently a breakdown of the blood–brain barrier (Cancilla et al., 1993) that would make circulating catecholamine accessible to astrocyte β_2 -ARs. This breakdown in the blood–brain barrier, accompanied by an increase in the circulating levels of noradrenaline, makes it likely that, at least in the areas near the neuronal injury, pharmacologically relevant levels of circulating noradrenaline could occupy β_2 -ARs expressed by astrocytes.

Glial functions that are modulated by β_2 -adrenergic receptors

Stimulation of β -ARs on astrocytes *in vitro* has been shown to influence several astrocyte functions including glycogen metabolism (Cummins et al., 1983; Rosenberg and Dichter, 1985; Hsu and Hsu, 1990; Subbarao and Hertz, 1990; Sorg and Magistretti, 1992), release of taurine (Hansson and Rönnbäck, 1991; Waniewski et al., 1991; Martin and Shain, 1993), synthesis and release of cytokines (Benveniste et al., 1990; Maimone et al., 1993), synthesis of neurotrophic factors (Schwartz and Mishler, 1990), amino acid uptake (Hansson and Rönnbäck, 1992), and the expression of interferon-induced major histocompatibility class II antigen (Frohman et al., 1988). In general, these effects appear to be mediated by β_2 -AR-evoked increases in cAMP (McCarthy and De Vellis, 1978), which in turn activates protein kinase A (Shao et al., 1993).

While these data clearly indicate that activation of β_2 -ARs on cultured astrocytes can modulate a wide range of astrocyte functions, demonstrating similar β_2 -AR regulation of astrocyte function *in vivo* has proven more difficult. Recently, however, one *in vivo* study suggested that β -AR antagonists can significantly block glial scar formation after neuronal injury (Sutin and Griffith, 1993). In this study, ricin was injected into the sciatic nerve to induce ventral motor neuron death (Aldskogius et al., 1988). Using this model it was shown that continuous subcutaneous infusion of the β -AR antagonist propranolol markedly reduced the astrocytic hypertrophy that normally accompanies the death

of the ventral motor neurons (Sutin and Griffith, 1993). These studies are provocative in that they suggest that circulating β -AR antagonists can have a substantial effect on astrocyte function after neuronal injury. Interestingly, it has been reported that elevated plasma noradrenaline levels after brain trauma can predict the outcome in patients with comparable neurological deficits, suggesting that circulating catecholamines may be used as markers that reflect the extent of brain injury (Hamill et al., 1987; Feeney and Sutton, 1988). While further studies are needed to define whether the effects of the β -AR antagonists on astrocytes were direct or indirect, these results suggest that activation or blockade of β -ARs expressed by astrocytes can produce profound effects on astrocyte morphology and function both *in vivo* and *in vitro*.

Summary

The present data demonstrate that glia *in vivo* express a relatively moderate to high level of β_2 -ARs in the normal and injured CNS of the rabbit, rat, and human. The major glial cell type expressing the β_2 -AR appears to be the astrocyte, and astrocytes in a wide variety of brain areas express β_2 -ARs. After injury there is an increase in the number of β_2 -ARs expressed by astrocytes, and this increase appears to be due to both hypertrophy and proliferation of astrocytes that express β_2 -ARs. This increase in β_2 -AR expression by astrocytes is first seen 7–28 d after injury and appears to depend on species, site, and type of injury. These data together with previous studies suggest that β_2 -ARs may provide a therapeutic target for regulating astrocyte function in both normal and injured human CNS.

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