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Substance P receptor binding sites are expressed by glia *in vivo* after neuronal injury

(tachykinins/astrocytes/optic nerve)

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ABSTRACT **In vitro studies have demonstrated that glia can express functional receptors for a variety of neurotransmitters. To determine whether similar neurotransmitter receptors are also expressed by glia *in vivo*, we examined the glial scar in the transected optic nerve of the albino rabbit by quantitative receptor autoradiography. Receptor binding sites for radiolabeled calcitonin gene-related peptide, cholecystokinin, galanin, glutamate, somatostatin, substance P, and vasoactive intestinal peptide were examined. Specific receptor binding sites for each of these neurotransmitters were identified in the rabbit forebrain but were not detected in the normal optic nerve or tract. In the transected optic nerve and tract, only receptor binding sites for substance P were expressed at detectable levels. The density of substance P receptor binding sites observed in this glial scar is among the highest observed in the rabbit forebrain. Ligand displacement and saturation experiments indicate that the substance P receptor binding site expressed by the glial scar has pharmacological characteristics similar to those of substance P receptors in the rabbit striatum, rat brain, and rat and canine gut. The present study demonstrates that glial cells *in vivo* express high concentrations of substance P receptor binding sites after transection of retinal ganglion cell axons. Because substance P has been shown to regulate inflammatory and immune responses in peripheral tissues, substance P may also, by analogy, be involved in regulating the glial response to injury in the central nervous system.**

A major question in neurobiology is why damaged mammalian central nervous system (CNS) neurons do not regenerate *in vivo*. In recent years, the focus of attention has shifted from CNS neurons themselves, which appear to have the capacity to regenerate, to CNS glia, which apparently inhibit the regrowth of axons in the CNS. Thus, it has been demonstrated that, after injury, regenerating axons grow a short distance until they reach the glial scar, at which time they appear to stop growing and degenerate (1–6).

The major cellular constituent of a CNS glial scar is the reactive astrocyte (4). Unlike fibroblasts, which form scars in nonneural tissue by secreting large amounts of collagenous extracellular matrix, astrocytes form scars by extending numerous processes that become packed with intracellular glial filaments (7). Astrocytes proliferate in response to injury (8), and it appears that these "reactive astrocytes" are biochemically different from the major class of astrocytes present in the normal nonlesioned brain (4). Recently, several neuropeptides, including bombesin, substance K, and substance P, have been shown to be mitogenic (9, 10) for several cell types that may be involved in the inflammatory and wound-healing responses in peripheral tissues (11). *In vitro* studies suggest that glia are potential targets for a variety of neurotransmitters (12) including substance P (13–16), somatostatin (17, 18), and vasoactive intestinal peptide (15, 17, 18). By analogy with the inflammatory and immune responses to injury in peripheral tissues, these neurotransmitters may regulate glial mitogenesis and glial response to injury of the CNS. However, since glial cells exhibit different functional properties depending on their biochemical environment (12), it is imperative to demonstrate that these receptor binding sites, which have been shown to be expressed by glia *in vitro*, are also expressed by glia *in vivo*.

In the present report, we have used quantitative receptor autoradiography to define which neurotransmitter receptor binding sites are expressed *in vivo* by a pure glial scar. We have chosen the optic nerve as a model because of the ease of transection and because it is one of the few fiber tracts in the CNS in which all the axons arise from a single source. After a suitable time, the transected optic nerve is almost a pure glial scar composed almost exclusively of reactive astrocytes (4, 13, 19). The purpose of the present study is to use this glial scar as an *in situ* model to define which neurotransmitter receptors are expressed by glia in response to neuronal injury. The receptor binding sites examined include those for calcitonin gene-related peptide, cholecystokinin, galanin, glutamate, somatostatin, substance P, and vasoactive intestinal peptide.

EXPERIMENTAL PROCEDURES

Five male adult New Zealand White rabbits (Universal Animals, Inglewood, CA) were used. The animals were deeply anesthetized with a mixture of xylazine and ketamine and a 1-cm incision was made away from the lateral canthus. The eyelid was gently retracted, and the eye was pulled forward to expose the optic nerve. The optic nerve on one side was sectioned, the skin was sutured, and the animal was allowed to recover and survive for 37 (three animals: nos. 01, 02, and 03) or 99 (two animals: nos. 04 and 05) days. The animals were then deeply anesthetized with an overdose of sodium pentobarbital (Nembutal), perfused transcardially with isotonic phosphate-buffered saline (pH 7.4, 4°C), and the optic nerve and brain were rapidly dissected out, blocked in the transverse plane, placed on a brass microtome chuck, frozen on dry ice, and processed for quantitative autoradiography as described (20). The tissue was serially sectioned

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Abbreviation: CNS, central nervous system.

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in the coronal plane at 15 μm on a cryostat, thaw-mounted onto gelatin-coated microscope slides, and stored at −70°C in boxes containing desiccant for up to 3 months.

Radioligands. The radioligands used in the present study were ^{125}I-labeled radioligands purified by reverse-phase HPLC to essentially quantitative specific activity (≈2000 Ci/mmol; 1 Ci = 37 GBq).

Receptor Binding Protocols. Quantitative receptor autoradiography was performed by first bringing the slide-mounted tissue sections to room temperature. The slide-mounted tissue sections were then placed consecutively in a preincubation medium, an incubation medium, a wash solution, and a final dip in distilled water. The preincubations and washes were performed by immersing the entire slide in the appropriate solution, whereas the incubation with the radioligand was performed by placing the slides on a flat surface and covering the sections with 1.5 ml of the incubation medium. To estimate the nonspecific binding, paired serial sections were incubated as described above except that a 1 μM concentration of the appropriate nonradioactive peptide was included in the incubation solution. In the case of substance P, in which we undertook to characterize the pharmacological profile of the binding sites, we used the displacement and saturation protocol previously described (21). Optimal binding for each ligand required a unique set of conditions, which are given below.

Calcitonin Gene-Related Peptide. Slide-mounted tissue sections were preincubated in 50 mM Tris·HCl buffer (pH 7.4, 20°C) for 5 min followed by an incubation in a solution of 50 mM Tris·HCl buffer, pH 7.4/5 mM $MgCl_2$/2 mM EGTA/100 pM (2-[^{125}I]iodohistidyl^{10})calcitonin gene-related peptide type α (human) (Amersham) for 2 hr at 20°C. The slide-mounted tissue sections were then washed (four times, 3 min each, 4°C) in a solution of 50 mm Tris·HCl, pH 7.4/0.1% bovine serum albumin (22).

Cholecystokinin. Slide-mounted tissue sections were preincubated for 10 min in 50 mM Tris·HCl buffer (pH 7.7, 20°C) followed by an incubation in the same buffer with 5 mM $MgCl_2$/0.2% bovine serum albumin/0.02% bacitracin/1.0 mM dithiothreitol/100 pM CCK-8 (sulfated) labeled with (^{125}I-Bolton–Hunter reagent) (Amersham) for 60 min at 20°C. After this, the slide-mounted tissue sections were washed (four times, 3 min each, 4°C) in the same buffer with 0.1% bovine serum albumin (23).

Galanin. Slide-mounted tissue sections were preincubated for 10 min in 10 mM Hepes buffer (pH 7.4, 20°C) followed by an incubation in the same Hepes buffer with 100 pM ^{125}I-labeled galanin (monoiodinated porcine galanin labeled using chloramine T) added for 1 hr at 20°C. The slide-mounted tissue sections were then washed (four times, 3 min each, 40°C) in the same buffer (24).

Glutamate. Slide-mounted tissue sections were incubated for 45 min at 4°C in 200 nM L-[^{3}H]glutamate (New England Nuclear; diluted with unlabeled glutamate to a "working" specific activity of 4.5 Ci/mmol to label both the high- and low-affinity receptor binding sites; ref. 25) in 50 mM Tris·HCl (pH 7.4) containing 2.5 mM $CaCl_2$. Nonspecific binding was determined in the presence of 1 mM unlabeled glutamate. After the incubation, slide-mounted tissue sections were rinsed three times with buffer at 4°C, then rinsed twice with 2 ml of 2.5% glutaraldehyde in neat acetone at 4°C, to minimize dissociation during drying. The total rinse time was ≈10 sec (25).

Somatostatin. Slide-mounted tissue sections were preincubated in 170 mM Tris·HCl (pH 7.4, 20°C) for 5 min and then in an incubation solution of 170 mM Tris·HCl, pH 7.4/5 mM $MgCl_2$/1% bovine serum albumin/20 mg of bacitracin per liter/100 pM ^{125}I-labeled somatostatin-8[Tyr^3] cyclic analog (D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Cys-Thr-NH_2, which is similar to Sandostatin; ref. 26) for 2 hr at 20°C. The slides were then washed in 170 mM Tris·HCl buffer (pH 7.4, 4°C) and 0.01% bovine serum albumin (four times, 4 min each; ref. 26).

Substance P. Slide-mounted tissue sections were brought to room temperature and placed in a preincubation medium (20°C for 10 min) consisting of 50 mM Tris·HCl (pH 7.4) containing 0.005% polyethylenimine. The slide-mounted sections were then incubated at 20°C for 1 hr in a solution of 50 mM Tris·HCl (pH 7.4) containing 3 mM $MnCl_2$, 200 mg of bovine serum albumin per liter, 2 mg of chymostatin per liter, 4 mg of leupeptin per liter, 40 mg of bacitracin per liter, and 100 pM ^{125}I-Bolton–Hunter-labeled substance P. After this incubation, the slide-mounted tissue sections were rinsed with four washes of 50 mM Tris·HCl (pH 7.4)/0.2% BSA (4°C, 2 min each) and two washes of distilled H_2O (4°C, 5 sec each; refs. 11 and 20).

Vasoactive Intestinal Polypeptide. Slide-mounted tissue sections were preincubated in 10 mM Hepes buffer (pH 7.4, 20°C) for 5 min at 20°C followed by incubation in a solution of 10 mM Hepes buffer, pH 7.4/130 mM NaCl/4.7 mM KCl/5 mM $MgCl_2$/5 mM $MnCl_2$/1 mM EGTA/1% bovine serum albumin/1 mg of bacitracin per ml/100 pM (3-[^{125}I]iodotyrosyl^{10})vasoactive intestinal polypeptide (Amersham) for 2 hr at 20°C. After this, the slide-mounted tissue sections were washed (two times, 15 min each, 20°C) in the incubation solution minus the radioligand (27).

Analysis of Autoradiograms. After the final wash, all the slide-mounted tissue sections were dipped in distilled water, dried in a cold room (4°C), and stored overnight over desiccant. Quantitative autoradiographic analysis was performed by placing the dried labeled slide-mounted tissue sections in apposition

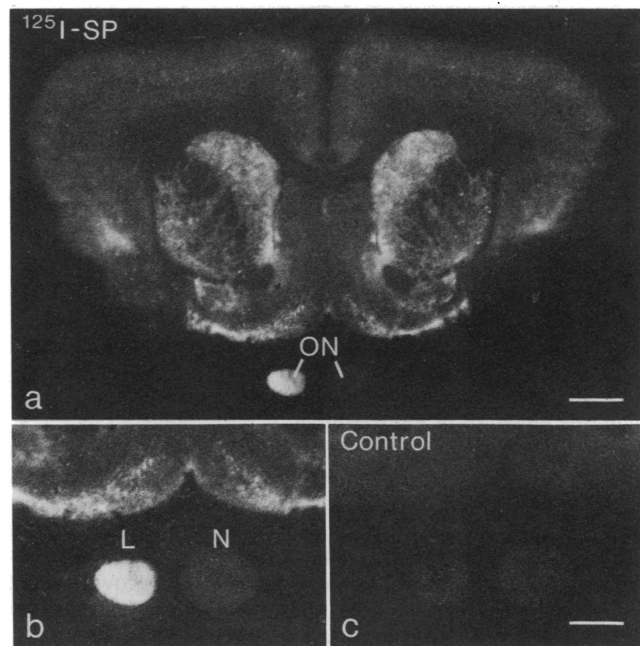

FIG. 1. A series of dark-field photomicrographs showing the autoradiographic localization of substance P (SP) receptor binding sites in a coronal section of the rabbit brain (animal no. 05) 99 days after unilateral transection of the optic nerve (ON). Autoradiograms in *a* and *b* show the total binding, whereas the nonspecific binding is shown in *c*. The control section (*c*) illustrating the nonspecific binding was treated identically to the adjacent section, which shows the total binding (*b*) except that nonradioactive substance P (1 μM) was added to the incubation medium. In all the dark-field autoradiograms, the highest density of white silver grains represents the highest concentration of binding sites. The specific binding is obtained by subtracting the binding in *c* from that in *b*. Note that whereas the lesioned (L) optic nerve is reduced in size, it expresses a high density of specific receptor binding sites relative to the larger normal (N) optic nerve, in which specific binding sites are not detectable. (*a*, bar = 1.9 mm; *b* and *c*, bar = 1.2 mm.)

to ^{3}H-sensitive film (Ultrofilm, LKB; Hyperfilm, Amersham) along with iodinated brain mash or commercially available standards (Amersham). After 1–4 weeks, the film was developed in Kodak D-19 developer, fixed, and washed. In sections in which a higher degree of histological resolution of the binding sites was sought, the tissue slices were overlaid with emulsion-coated coverslips or processed for standard emulsion-dipped autoradiography. After these autoradiograms were developed, the sections were placed in Carnoy's fixative for 3 hr, stained with cresyl violet (Nissl stain), and mounted with Histoclad. Dark-field or bright-field photomicrographs were then taken of the silver grains and counterstained sections, respectively. Using this approach, we generated three complementary images: the LKB or Ultrofilm autoradiograms, which were analyzed by quantitative densitometry; the autoradiograms of the emulsion-dipped slides, which provided detailed histological resolution of the binding sites; and the counterstained section, which allowed identification of the cell type expressing a specific binding site. Controls for chemographic artifacts were generated by performing the binding exactly as described except that the radioligand was omitted from the incubation medium. To quantitate the density of radiolabeled neurotransmitter binding sites, microdensitometry with ^{3}H-sensitive film was performed as described (21).

RESULTS AND DISCUSSION

Histology of the Rabbit Optic Nerve and Optic Tract After Unilateral Optic Nerve Transection. The normal optic tract is opaque in appearance and is composed of myelinated ganglion cell axons grouped in fascicles and surrounded by cells with darkly stained nuclei with a clear cytoplasm. Previous studies have shown that the supporting cells are primarily oligodendrocytes (4, 7, 28).

In all five rabbits, the lesioned optic tract showed the typical histological changes that occur after unilateral optic nerve transection (2, 5, 6, 19); here we describe specifically the changes in a 99-day animal (no. 05). Grossly, the lesioned optic nerve is translucent in appearance and reduced in size (Fig. 1) due to the loss of myelinated optic axons. In Nissl-stained sections, the lesioned optic nerve (Fig. 2) and tract (Fig. 3) were composed of numerous cells with darkly stained nuclei, foamy cytoplasm, and extensive processes, all of which lack the distinctive fasciculated organization characteristic of the normal optic nerve (Fig. 2). Cells with this morphology have previously been shown to correspond to reactive astrocytes (4, 7, 28).

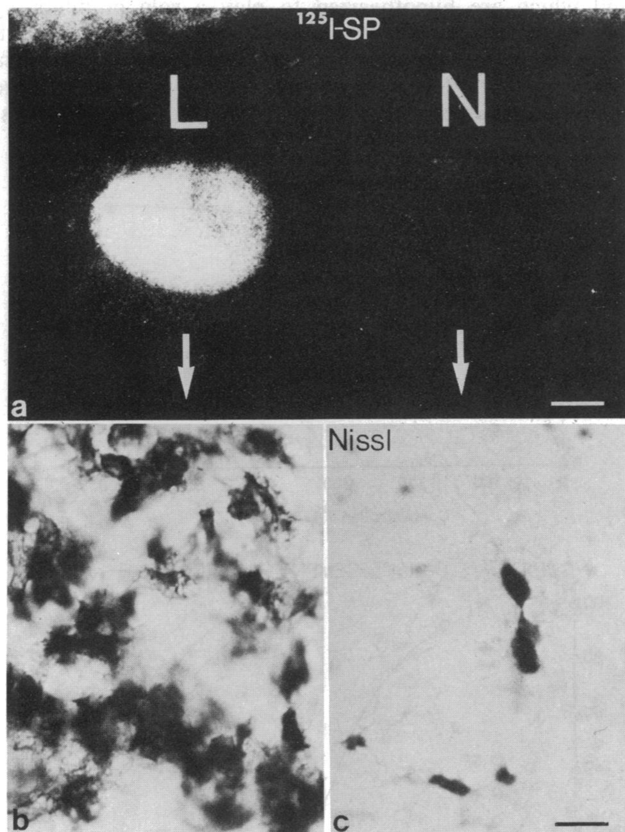

FIG. 2. Dark-field photomicrograph (*a*) showing the autoradiographic localization of substance P (SP) receptor binding sites in the lesioned (L) and normal (N) optic nerve. Bright-field photomicrographs obtained from the same Nissl-stained section showing the reactive astrocytes in the lesioned optic nerve (*b*) and in the normal unlesioned optic nerve (*c*). Note the numerous cells with darkly stained nuclei, foamy cytoplasm, and extensive processes, all of which are characteristic of the reactive astrocytes that are present in the lesioned (*b*) but not the normal (*c*) optic tract. (*a*, bar = 0.6 mm; *b* and *c*, bar = 0.01 mm.)

Receptor Binding Sites in Forebrain, Optic Nerve, and Optic Tract After Unilateral Optic Nerve Transection. Specific binding sites for calcitonin gene-related peptide, cholecystokinin, galanin, glutamate, somatostatin, substance P, and vasoactive intestinal peptide were observed in the rabbit forebrain. High concentrations of receptor binding sites were found in the striatum for calcitonin gene-related peptide; in laminae 1, 2, 3, 5, and 6 of the cerebral cortex, striatum, and nucleus accumbens for cholecystokinin; in the nucleus accumbens for galanin; in all laminae of the cerebral cortex, striatum, and nucleus accumbens for glutamate; in laminae 4 and 5 of the cerebral cortex for somatostatin; in the striatum and nucleus accumbens for substance P; and in laminae 1–3 of the cerebral cortex, striatum, and nucleus accumbens for vasoactive intestinal peptide.

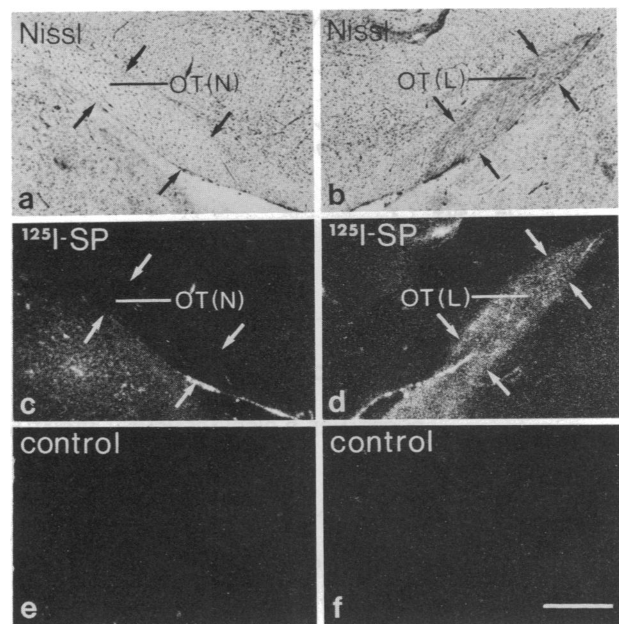

FIG. 3. Autoradiographic localization of substance P (SP) receptor binding sites in a coronal section of the rabbit brain (animal no. 01) 37 days after optic nerve transection. (*a* and *b*) Bright-field photomicrographs of Nissl-stained sections showing the normal (*a*) and lesioned (*b*) optic tract. (*c* and *d*) Dark-field autoradiograms showing the total ^{125}I-SP binding in the normal (*c*) and lesioned (*d*) optic tract. Arrows delineate the extent of the optic tract in these sections. (*e* and *f*) Nonspecific binding in the normal (*e*) and lesioned (*f*) optic tract. Note that there is a high concentration of substance P binding sites in the lesioned optic tract [OT(L)] in *d* but not in the normal optic tract [OT(N)] in *c*. Note also that the Nissl-stained lesioned optic tract in *b* stains much darker than the normal optic tract in *a*. (Bar = 1.1 mm.)

No specific binding sites for any of the ligands described above were observed in normal optic nerve or optic tract. In all transected optic nerves and tracts, the only specific binding sites that were detected were substance P binding sites. The specific/nonspecific binding ratios for substance P binding sites in the lesioned optic nerves were 90:10. All the lesioned animals showed a similar pattern after optic nerve transection, so we describe in detail the results from one animal 99 days after optic nerve transection. In this animal (rabbit no. 05), both the lesioned optic nerve (Figs. 1 and 2) and the optic tract (Fig. 3) expressed high concentrations of substance P receptor binding sites, although the optic nerve (Figs. 1 and 2) consistently expressed a higher density of specific binding sites than did the optic tract (Fig. 3). Specific substance P receptor binding sites were found throughout the lesioned optic nerve and throughout the optic tract to the level of the lateral geniculate nucleus where the specific binding sites declined to background levels.

Pharmacological Characteristics of Substance P Receptor Binding Sites in the Transected Optic Nerve. In previous papers we have defined the incubation conditions and pharmacological characteristics of substance P receptor binding sites (20, 21). To determine whether the receptor binding sites in the glial scar were pharmacologically similar to substance P receptor binding sites expressed in unlesioned areas of the rabbit brain, we generated saturation and competition curves for substance P receptor binding sites expressed by the transected optic nerve and the striatum. As shown in Fig. 4, the saturation curves for both regions were nearly identical. The concentration for half-saturating the substance P receptor binding sites was 69.5 pM for the lesioned optic nerve and 73.3 pM for the rabbit striatum (Fig. 4). The inhibition curve for ^{125}I-labeled substance P (Fig. 4) clearly shows that the binding sites expressed by the transected optic nerve is a substance P-preferring (21) receptor (sometimes called SP-P or NK-1). This receptor binding site has a similar specificity to that of the substance P receptor found in normal rabbit striatum (Fig. 4) and to substance P receptor binding sites found in rat brain and peripheral tissues (21). Thus the K_i for displacing ^{125}I-labeled substance P is 0.54 nM for substance P, 650 nM for substance K, and 720 nM for neuromedin K in the lesioned optic nerve and 0.71 nM for substance P and ≈1000 nM for both substance K and neuromedin K in the normal striatum (Fig. 4).

Substance P Receptor Binding Sites Expressed by Glia Appear To Be Functional Receptors. In the present report, we have demonstrated that after optic nerve transection, substance P binding sites are expressed in high concentrations by cells comprising the glial scar in the optic nerve and optic tract. These data are consistent with previous *in vitro* studies that demonstrated that substance P receptors are present in 2- to 3-week-old primary cultures of cortical astrocytes from newborn mice and that addition of substance P to these astrocyte cultures stimulates phosphatidylinositol turnover (16). Substance P has also been shown to stimulate the cyclooxygenase pathway of arachidonic acid metabolism in 2- to 3-week-old cultures of rat astrocytes and to evoke the formation of prostaglandin E and thromboxane B_2 in a dose-dependent manner (13). These studies together with the present results are consistent with the suggestion that "reactive astrocytes," which proliferate after neuronal injury, and which are hypothesized to play a role in inhibiting neuronal regeneration (4), express functional substance P receptors. These data suggest the hypothesis that when glia are separated from neurons either *in vitro* or *in vivo* after the transection and degeneration of retinal ganglion cell axons,

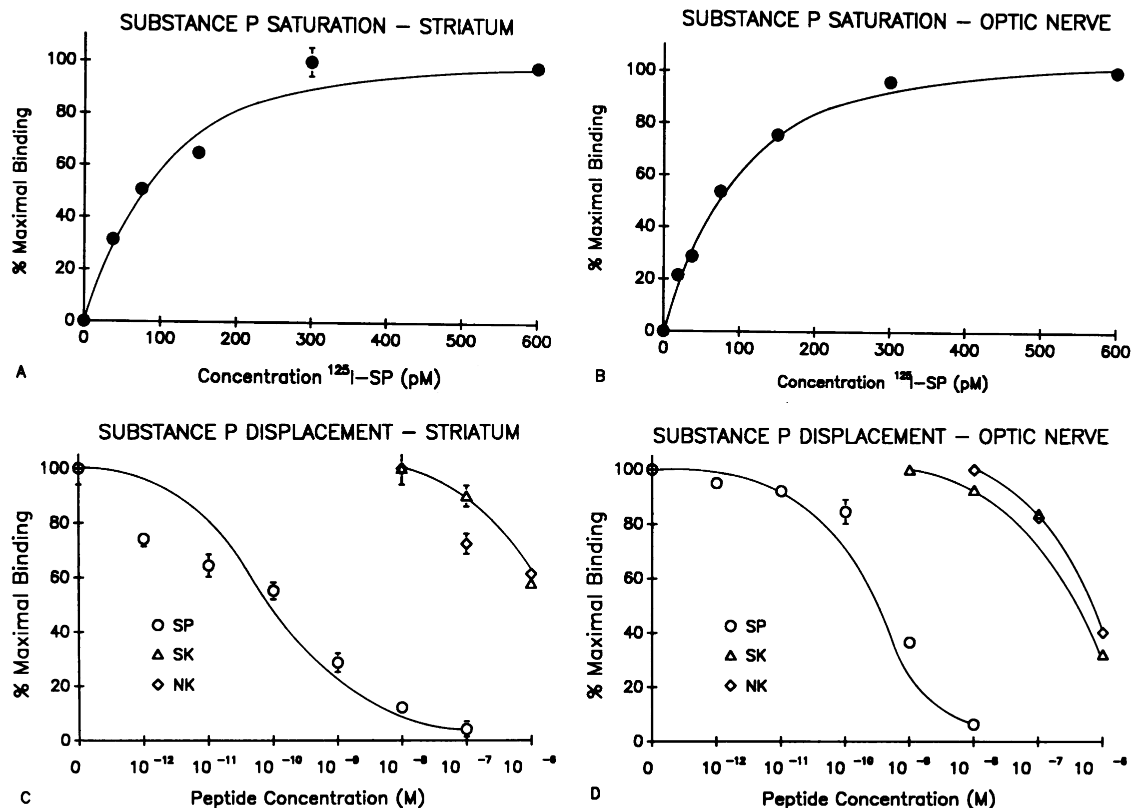

FIG. 4. Saturation (*a* and *b*) and displacement (*c* and *d*) curves for substance P receptor binding sites expressed by the striatum (*a* and *c*) and the lesioned optic nerve (*b* and *d*) 99 days after transection (rabbit no. 04). Note that both the saturation and displacement curves for substance P receptor binding sites in the normal striatum (*a* and *c*) and the transected optic tract (*b* and *d*) are similar, suggesting that the substance receptor binding site expressed by the lesioned optic nerve is similar to the substance P receptor binding site expressed by the normal striatum. Data are from a representative experiment. Each point represents the mean of triplicate determinations. SP, substance P; SK, substance K; NK, neuromedin K.

the glia that survive and proliferate express substance P receptors.

The present study has also demonstrated that, although receptors for glutamate and six neuropeptides were examined, the expression of binding sites by reactive astrocytes is unique for substance P binding sites in that none of the other seven radioligands examined showed detectable specific binding in the glial scar.

Proinflammatory Actions of Substance P. A proinflammatory function of substance P was not completely unexpected since a similar ectopic expression of substance P receptor binding sites was also observed recently in an inflammatory disease affecting human peripheral tissues (11). In these studies, quantitative receptor autoradiography was used to compare the expression of receptor binding sites for sensory neurotransmitters in histologically normal surgical specimens of colon vs. inflamed surgical specimens obtained from patients with ulcerative colitis and Crohn's disease. The sensory neurotransmitter receptors examined in that study included those for bombesin, calcitonin gene-related peptide, cholecystokinin, galanin, glutamate, somatostatin, substance K, substance P, and vasoactive intestinal polypeptide. The only tissues that exhibited a difference between the inflamed vs. the normal tissue were arterioles, venules, and lymph nodules, all of which expressed high concentrations of substance P receptor binding sites in the inflamed but not in the normal tissue. These studies suggest that substance P may be a multifunctional peptide that mediates the inflammatory, immune, and wound healing responses in peripheral tissues. Whether substance P is involved in mediating a similar response to injury in the CNS is still unknown, but the present results clearly demonstrate that under the appropriate conditions glia can express high levels of substance P receptor binding sites *in vivo*.

Together these findings suggest that substance P may have proinflammatory actions in both the CNS and in peripheral tissues. While the possibility that the same neuropeptide could have actions in both the brain and peripheral tissues is certainly not without precedent, there is a key difference in the source of the ligand in these tissues. Unlike peripheral tissues such as the gastrointestinal tract or skin, where there is a dense innervation by substance P-containing dorsal root ganglion neurons, the brain lacks such a sensory innervation. This important difference raises the question as to the possible origin of the substance P that could occupy the substance P receptors expressed by the CNS glia after neuronal injury. While the answer to this question is currently unknown, an important clue may be the findings that circulating leukocytes have been reported to synthesize neuropeptides such as corticotropin, opiates (29), and substance P (30).

The present study has provided an *in vivo* demonstration that glia can express neurotransmitter receptors. While a wealth of data has accumulated to document the fact that functional neurotransmitter receptors are expressed by glia *in vitro*, a key question remained as to whether glia in culture demonstrate such properties because of arrested development or isolation from other cell types. The present results suggest that glia can express *in vivo* a substance P receptor pharmacologically similar to that observed in the normal brain, but that the substance P receptor is expressed in detectable levels only after degeneration of the surrounding neurons. If the expression of substance P binding sites is a common feature of reactive gliosis in different regions of the CNS, it might offer an opportunity to define neuronal factors involved in regulating glial response to injury.

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