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Colchicine Affects Cortical and Amygdalar Neurochemical Changes Differentially After Middle Cerebral Artery Occlusion in Rats

R.T.F. CHEUNG AND D.F. CECHETTO*
John P. Robarts Research Institute and Departments of Physiology and Clinical Neurological Sciences, University of Western Ontario, London, Ontario N6A 5K8, Canada

ABSTRACT

Recently, we have shown increases in the immunoreactivity for neuropeptide Y and tyrosine hydroxylase in the insular cortex surrounding the focal infarction after middle cerebral artery occlusion. In addition, the immunoreactivity for neuropeptide Y, leucine-enkephalin, dynorphin, and neurotensin increased ipsilaterally in the amygdala. Increases in immunoreactivity were observed in nerve terminals and fibers; changes in the neuropeptides were maximal 3 days after stroke. Local excitotoxic injury of the insular cortex also elicited similar neuropeptide changes unilaterally in the same regions. In this study, immunohistochemistry was used following intracerebroventricular injection of colchicine and stroke to determine whether blockade of axonal transport would prevent these neurochemical changes. These experiments would also locate the putative cellular origins of the neurochemicals involved. Control rats received either colchicine injection or middle cerebral artery occlusion alone. Injection of colchicine enhanced the periinfarct increase in neuropeptide Y but did not alter the increase in tyrosine hydroxylase. The neuropeptide Y increase was observed in local cortical neurons. Colchicine prevented the increases in immunoreactivity for the neuropeptides in the amygdala on the side of stroke, although there were small perikarya that showed immunoreactivity for these neuropeptides within the amygdala on both sides. We conclude that local cortical neurons are responsible for the increase in neuropeptide Y in the periinfarct region, that the cortical increase in tyrosine hydroxylase is not dependent on fast axonal transport, and that axonal transport of signals from the insular cortex to the amygdala is critical in mediating the amygdalar neuropeptide changes seen after stroke. J. Comp. Neurol. 387:27–41, 1997.

Indexing terms: insular cortex; tyrosine hydroxylase; neuropeptide Y; endogenous opioids; neurotensin

Recently, we reported a striking increase in the immunoreactivity (ir) for neuropeptide Y (NPY) and tyrosine hydroxylase (TH; a cytosolic catecholamine-synthesizing enzyme) in the insular cortex (IC) surrounding the focal infarction 5 days after middle cerebral artery occlusion (MCAO; Allen et al., 1995; Cheung et al., 1995a). The NPY-ir also increased in the ipsilateral basolateral nucleus of the amygdala (BLA). In addition, there were unilateral increases in the ir for leucine-enkephalin (l-ENK), dynorphin (DYN), and neurotensin (NT) in the central nucleus of the amygdala (ACE) on the side of MCAO. The changes of NPY, l-ENK, DYN, and NT in the IC, BLA, or ACE followed a similar time course, being maximal 3–5 days after MCAO and subsiding by day 10, although the increased NPY-ir in the IC preceded the amygdalar neurochemical changes, starting to increase at 6 hours after MCAO and becoming significant by day 1 (Cheung et al., 1995a). Excitotoxic lesion localized to the IC, but not to the adjacent primary somatosensory cortex, elicited similar neurochemical changes in the ipsilateral amygdala 5 days after an excitotoxic insult (Cheung and Cechetto, 1995). However, the NPY-ir increased locally within the IC or primary somatosensory cortex following the localized exci-

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*Correspondence to: Dr. David F. Cechetto, John P. Robarts Research Institute, 100 Perth Drive, P.O. Box 5015, London, Ontario N6A 5K8, Canada. E-mail: cechetto@julian.uwo.ca

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totoxic insults to either cortical site. These results suggest that the cortical increase in NPY is a local response of the nervous tissue to injury and that the amygdalar neurochemical changes are remote effects specific to the involvement of the IC in the damaged region.

The local cortical increase in NPY-ir and/or TH-ir has been postulated to mediate a neuroprotective response, whereas the remote amygdalar neurochemical changes have been hypothesized to be related to stroke-induced autonomic disturbances (Allen et al., 1995; Cheung et al., 1995a). Supportive evidence is now available for both hypotheses. Knockdown of NPY-Y1 receptor binding sites in the IC by using antisense oligodeoxynucleotide resulted in a doubling of the infarct volume; this suggests that interfering with NPY actions via Y1 receptors is deleterious during focal cerebral ischemia (Cheung and Cechetto, 1996a). Rats with MCAO were found to have exaggerated cardiovascular responses to stress in a time-course comparable to that of the amygdalar neurochemical changes (Cheung et al., 1995b). At present, there is little information on the molecular mechanisms responsible for the local and remote neurochemical changes seen after MCAO.

The increases in ir were observed in nerve terminals and fibers in our previous studies (Allen et al., 1995; Cheung and Cechetto, 1995; Cheung et al., 1995a). It is not known whether these terminals and fibers originate from local intrinsic neurons or from remote neurons projecting to these sites. In the present study, we investigated the effects of intracerebroventricular injection of colchicine, an axonal transport inhibitor, on the immunohistochemical changes of TH, NPY, l-ENK, DYN, and NT over the IC and amygdala at 3 days after MCAO. Colchicine disrupts microtubules and blocks the fast axonal transport of secretory vehicles, which contain the neuropeptides, but leaves the slow axoplasmic flow of cytosolic enzymes and cytoskeletal elements relatively unaffected (Dahlström, 1968; Kreutzberg, 1969; Ceccatelli et al., 1991; Liu et al., 1991; Schwaber, 1991; Boyer et al., 1994). Thus, interruption of axonal transport with colchicine would cause accumulation of NPY in the perikarya of local neurons in the IC and similar perikaryal accumulation of neuropeptides in the local neurons within the respective subnuclei of the amygdala. In addition, the colchicine treatment should not affect the TH response in the IC. Preliminary results of this study have been reported previously (Cheung and Cechetto, 1996b).

MATERIALS AND METHODS

Surgical procedures

Adult male Wistar rats (n = 23; 290–400 g) were anesthetized with sodium pentobarbital (65 mg/kg i.p.) and placed in a David Kopf stereotaxic apparatus. A small burr hole 1 mm in diameter was made by using a low speed drill in the parietal bone to allow stereotaxic microinjection into the left lateral ventricle. The microinjection was made through a 10 µl Hamilton syringe (Hamilton Company, Reno, NV) whose needle tip was 0.9 mm behind and 1.4 mm lateral to Bregma and 3.6 mm beneath the dural surface. Seventeen rats received colchicine (100 µg in 10 µl of phosphate buffered saline; PBS), and six rats received 10 µl of PBS. Each injection was made slowly over 4–5 minutes, and the needle was left in situ for another 5–6 minutes before withdrawal. While the rats were under anesthesia, the right middle cerebral artery (MCA) was accessed as previously described in detail (Cechetto et al., 1989; Hachinski et al., 1992a,b; Butcher et al., 1993; Allen et al., 1995). Briefly, two incisions were made into the skin: one from the corner of the right eye to the right ear and the other from the ear to the lower jaw. The frontalis and temporalis muscles were reflected to expose the underlying squamosal bone. A burr hole around 3 mm in diameter was made in the rostroventral part of the squamosal bone at about 3–4 mm dorsal to the foramen ovale and 1–2 mm anterior to the junction of the zygoma and squamosal bone. The overlying dura mater was retracted to expose the MCA. In 12 colchicine-treated rats and in all six PBS-treated rats, the MCA was occluded at two points: one above and the other below the rhinal fissure. The remaining five colchicine-treated rats had sham MCAO in which the MCA was exposed without occlusion. Dental impression material (Perfourm; Miles Laboratories, Berkeley, CA) was used to cover the opening in the skull. Sutures and surgical clips were used to close the muscle and skin incisions, respectively. Benzathine penicillin G (Penlong S; 20,000 iu; Rogar-STB Inc., London, Ontario, Canada) was given as a prophylactic antibiotic. Rectal temperature was monitored and maintained constant at 37.5°C by a heating pad and lamp while the rats were under anesthesia. Following the surgery, all rats were allowed to recover with food and water provided ad libitum. Experimental procedures and animal welfare were approved by the Council on Animal Care of the University of Western Ontario.

Tissue preparation

Three days after MCAO or sham MCAO, the rats were deeply reanesthetized with sodium pentobarbital and perfused transcardially with PBS (10 mM, pH 7.4), 4% paraformaldehyde in sodium acetate (0.1 M, pH 6.5), and finally 4% paraformaldehyde in sodium borate (0.1 M, pH 9.5–11). The brains were taken out for postfixation in a solution composed of 10% sucrose, 4% paraformaldehyde, and 0.1 M sodium borate overnight and then in 30% sucrose-phosphate buffer for 1–3 days. By using a freezing microtome, 30–40 µm coronal sections of brains were obtained and distributed sequentially into four or five series for immunohistochemical reactions. In addition, 20-µm brain sections stained for hematoxylin and eosin or thionin at 1-mm intervals from ten rats that had been treated with both MCAO and colchicine and from all six PBS-treated MCAO rats were used to assess the areas of infarction between the Bregma levels of +3 mm (anterior) to −4 mm (posterior).

Immunohistochemical methods

Each series was stained for ir to one of the peptides, TH, NPY, l-ENK, DYN, or NT, by using the corresponding antisera (dilution 1:500 for TH antiserum; Eugene Tech Inc., Allendale, NJ; dilution 1:1,000 for the other antisera; INCSTAR Corp., Stillwater, MN). Brain sections were processed according to the peroxidase-antiperoxidase (PAP) technique of Sternberger and collaborators (Sternberger, 1979). Briefly, sections were mixed with 10% normal sheep serum containing 0.3% Triton X-100 and 2% bovine serum albumin for 45 minutes before they were incubated at 4°C for 48 hours in one of the above antisera containing 1% normal sheep serum and 0.3% Triton X-100. Then, the sections were rinsed in Tris-buffered saline (TBS; 0.05 M, pH 7.6), incubated with sheep anti-rabbit antiserum (1:50) in 1% normal sheep serum and 0.3% Triton X-100 for 90

minutes, rinsed with TBS, incubated with PAP complex (1:150) in 1% normal sheep serum and 0.3% Triton X-100 for 90 minutes, rinsed in TBS, reacted with 3,3′-diaminobenzidine tetrahydrochloride (0.5 mg/ml in TBS) and H_2O_2 (0.3 µl/ml in distilled H_2O) for 15–30 minutes, rinsed, mounted on glass slides, and coverslipped. All incubations were performed in a slowly moving shaker bath. Specificity of the antisera was confirmed by omission of the antisera prior to the PAP reaction and by prior absorption with excess antigen.

Analysis of sections

The method of analysis of the immunostaining was identical to that used in our previous MCAO experiments (Allen et al., 1995; Cheung and Cechetto, 1995; Cheung et al., 1995a). Briefly, the brain sections were first examined by using a microscope (Leitz Diaplan; Ernst Leitz Wetzlar GMBH, Postfach, Wetzlar, Germany) under brightfield and darkfield illumination. Five consecutive and equally spaced sections starting at the level of the anterior commissure were selected for further analysis and photomicrography to determine the change in immunostaining in the IC. Similarly, five consecutive and equally spaced sections containing the ACE or BLA were selected for analysis and photomicrography to determine the change in immunostaining in the amygdala. No counterstaining was performed on these selected sections to avoid introducing a nonspecific and variable staining. Nevertheless, adjacent sections were counterstained with thionin to provide guidance in choosing the areas for measurement in the computer-assisted image analysis.

Digitized images of the sections under brightfield illumination were obtained by using a video camera for computer-assisted image analysis. A computer-assisted image-analysis system (Mocha; Jandel Scientific, San Rafael, CA) was used to quantify the average intensity over three specific regions, namely, the IC, ACE, and BLA on both sides, as in our previous studies (Allen et al., 1995; Cheung and Cechetto, 1995; Cheung et al., 1995a). The background illumination was adjusted to be identical for all sections by subtracting the relative intensity measure from immediately outside the tissue section but nearest to the brain region that was being analyzed, so that reliable measurement of the staining intensity could be obtained on both sides of the section and from section to section. The measured average intensity reflected the amount of immunocytochemical staining produced in the PAP reaction. Because the reaction conditions were identical within the same series of brain sections, the side-to-side comparison was considered appropriate. During the image analysis, anatomical landmarks, such as the rhinal fissure, piriform cortex, external capsule, and claustrum (Paxinos and Watson, 1986), that were seen in the background staining within the brain sections were used to define the boundary of the IC, and guidance was also derived from the adjacent thionin-counterstained sections. Similarly, anatomical landmarks, such as the optic tract, internal capsule, caudate putamen, and external capsules, and reference to the adjacent counterstained sections were used to define the boundaries of the ACE and BLA (Paxinos and Watson, 1986). Measurement was made over the entire cross-sectional area of the IC, ACE, and BLA instead of an arbitrarily chosen area within the region to avoid introducing a sampling error due to nonuniform immunostaining.

The same image-analysis system was used to measure the areas of infarction and hemispheric areas between +3 and −4 mm Bregma levels by using the hematoxylin and eosin- or thionin-stained sections. The boundary between normal and infarcted brain tissue was determined under light microscopy, and the measurement of infarction areas was made in the MCAO rats without knowledge of whether colchicine or PBS was injected intracerebroventricularly. The hemispheric volumes and volume of infarction between the above Bregma levels were obtained from integrating the respective area measurements (Butcher et al., 1993).

Data analysis and statistical test

Concerning the relative change in immunostaining, a mean was initially derived from the intensity measurement for the five selected sections that contained a specific region of the brain in each rat. Next, the mean from a discrete region on the right side was expressed as a percentage of the mean intensity of the respective region on the left side of the same section. To indicate a significant side-to-side difference in staining intensity for rats of the same group, the two-tailed Student's t test was applied to the data in percentage to challenge the null hypothesis that the data in percentage did not differ from 100%. In addition, an ordinary analysis of variance (ANOVA) with the Bonferroni post-hoc test was used to compare among the three groups of rats the relative change in intensity of immunostaining and the integrated volume of the infarct and hemispheres, but the two-tailed Student's t test was used for comparison when there were two groups only. A probability level of 0.05 or below was taken as significant. Data were expressed in mean ± standard error of mean.

RESULTS

Infarct site and size

Cerebral infarcts in the MCAO rats with intracerebroventricular injection of colchicine did not differ in location or appearance from infarcts in the MCAO rats with PBS injection, and there was no appreciable damage after sham MCAO and colchicine injection. In fact, location and appearance of the infarct were the same as previously observed (Cechetto et al., 1989; Hachinski et al., 1992a,b; Butcher et al., 1993; Allen et al., 1995; Cheung et al., 1995a). The infarct was primarily centered in the right IC. All cortical layers of the three regions of the IC, the granular, dysgranular, and agranular cortices, were contained within the infarct zone at the level of the greatest extent of damage, with variable involvement of the adjacent cortices, such as the primary and secondary somatosensory cortices and the piriform cortex. Although the claustrum was commonly affected, damage to the IC and the caudate-putamen was minimal or absent. In brain sections anterior and posterior to the greatest extent of infarct, increasing portions of the IC were spared. The computer-assisted image analysis revealed infarct volume and right and left hemispheric volumes in the colchicine-treated MCAO rats of 25.7 ± 7.6, 255.6 ± 6.2, and 239.7 ± 6.2 mm^3 (n = 10), respectively, between the Bregma levels of +3 and −4 mm. The corresponding data for the PBS-treated MCAO rats were 20.6 ± 4.4, 228.9 ± 9.3, and 212.1 ± 7.4 mm^3 (n = 6). There was no significant difference in the infarct volume between the two MCAO groups, but the hemispheric volumes were greater in the colchicine-

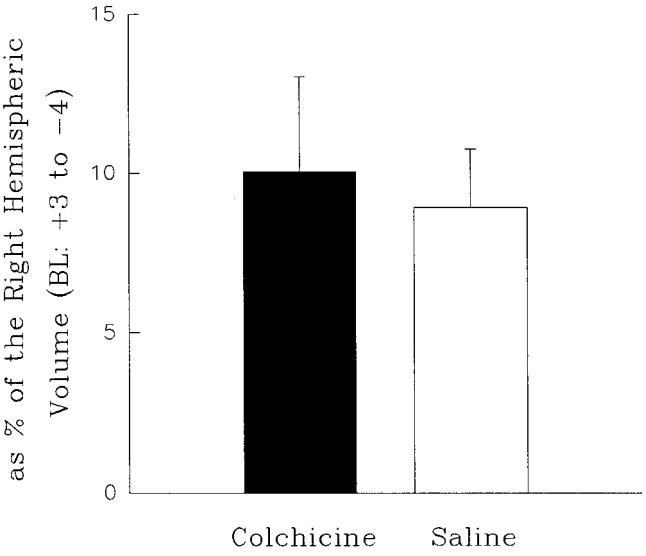

Fig. 1. Bar graph showing the relative volume of infarction after right-sided middle cerebral artery occlusion and injection of colchicine (n = 10) or saline (n = 6) into the left lateral ventricle. Initially, the volume of infarction was integrated from the area measurements obtained by a computer-assisted image analysis system between the Bregma levels of +3 to −4 mm. Next, the relative volume of infarction was obtained as a percentage of the right hemispheric volume between the same Bregma levels. No significant difference was found between the two groups. BL, Bregma level; ICV, intracerebroventricular; MCAO, middle cerebral artery occlusion.

treated MCAO group than in the PBS-treated MCAO group (an ordinary ANOVA revealed significant differences in the hemispheric volumes with $P = 0.002$; the Bonferroni post-hoc test revealed significant differences in the left or right hemispheric volumes of the two MCAO groups with $P < 0.05$). To correct for differences in the hemispheric volumes, the infarct volumes were expressed as percentages of the right hemispheric volumes, and, again, there was no significant difference between the two MCAO groups (Fig. 1).

Immunohistochemistry of the IC

The results of the immunohistochemistry of the IC from the PBS-treated MCAO rats were the same as those seen and described previously (Allen et al., 1995; Cheung et al., 1995a). In brief, ir for TH (Fig. 2D) and NPY (Fig. 3B) was extremely dense in neuronal axons and terminal-like swellings in and around the ischemic core. The increase in ir for TH and NPY was nonhomogeneous, being more marked toward the ischemic core, with a variable spread into the adjacent regions. In addition, increased ir was not confined to any individual cortical layer of the right IC. In contrast, TH-ir (Fig. 2C) and NPY-ir (Fig. 3A) of the left IC were very light, with only occasional labeled fibers.

The colchicine injection enhanced the MCAO-induced local increase in NPY-ir within the right IC (Fig. 4A,B) but did not alter the unilateral increase in TH-ir within the right IC (Fig. 2A,B). Unlike the saline treatment (Fig. 5D), the colchicine treatment resulted in localization of NPY-ir within cell bodies as well as axons and terminals in the right IC on the side of MCAO (Fig. 5B). On the other hand, colchicine treatment in the sham-MCAO rats did not cause any significant side-to-side difference in NPY-ir within the IC (Fig. 6A,B), even though abundant cell bodies with NPY-ir were present throughout the cortex (Fig. 5E,F). TH was not studied in the colchicine-treated sham-MCAO group.

Computer-assisted image analysis of the staining intensity of TH-ir and NPY-ir in the IC generated semiquantitative measurements for statistical testing to confirm our visual analysis. By using the left side as the denominator, the relative intensities of TH-ir in the right IC of the colchicine-treated and saline-treated MCAO groups were 188.3 ± 30.3% (n = 5) and 208.2 ± 17.1% (n = 6), respectively (Fig. 7A). There was no significant difference between the two groups. The relative intensities of NPY-ir in the right IC of the colchicine-treated and saline-treated MCAO groups and in the colchicine-treated sham-MCAO group, respectively, were 241.6 ± 13.1% (n = 12), 171.3 ± 10.6% (n = 6), and 91.4 ± 7.42% (n = 5; Fig. 7B; an ordinary ANOVA revealed significant differences among groups with $P < 0.0001$; the Bonferroni post-hoc test revealed a significant difference between any two groups with $P < 0.01$).

Immunohistochemistry of the amygdala

The results of the immunohistochemistry of the amygdala from the PBS-treated MCAO rats were the same as those seen and described previously in MCAO rats (Allen et al., 1995; Cheung et al., 1995a). In brief, NPY-ir was most dense in neuronal axons and terminal-like swellings in the right BLA, with somewhat less labeling in the lateral nucleus of the amygdala on the side of MCAO (Fig. 3C,D). The increase in the NPY-ir in the right BLA was nonhomogeneous, being more marked toward the ACE and around the external capsule. Colchicine injection prevented the MCAO-induced unilateral increase in NPY-ir within the right BLA (Fig. 4C,D). Colchicine treatment in the sham-MCAO rats did not cause any significant side-to-side difference in NPY-ir within the BLA (Fig. 6C,D). In both groups of rats with colchicine treatment, cell bodies with low levels of NPY-ir were seen throughout the BLA on both sides.

Computer-assisted image analysis of the staining intensity of NPY-ir in the BLA generated semiquantitative measurements for statistical testing. By using the left side as the denominator, the relative intensities of NPY-ir in the right BLA of the colchicine-treated and saline-treated MCAO groups and in the colchicine-treated sham MCAO group, respectively, were 101.7 ± 7.3% (n = 12), 133.9 ± 6.7% (n = 6), and 86.5 ± 15.8% (n = 5; Fig. 8A; an ordinary ANOVA revealed significant differences among groups with $P < 0.02$; the Bonferroni post-hoc test revealed a significant difference between the saline-treated MCAO group and the colchicine-treated sham-MCAO group with $P < 0.05$).

Following saline injections and MCAO, l-ENK-ir, DYN-ir, and NT-ir were most dense in neuronal axons and terminal-like swellings in the right ACE on the side of MCAO (Allen et al., 1995; Cheung et al., 1995a). The increase in ir in the right ACE was nonhomogeneous, being more marked in the lateral part of the ACE. Colchicine injection prevented the MCAO-induced unilateral increase in l-ENK-ir, DYN-ir, and NT-ir within the right ACE (Fig. 9). Colchicine treatment enhanced immunostaining within the cell bodies relative to the fibers and terminals.

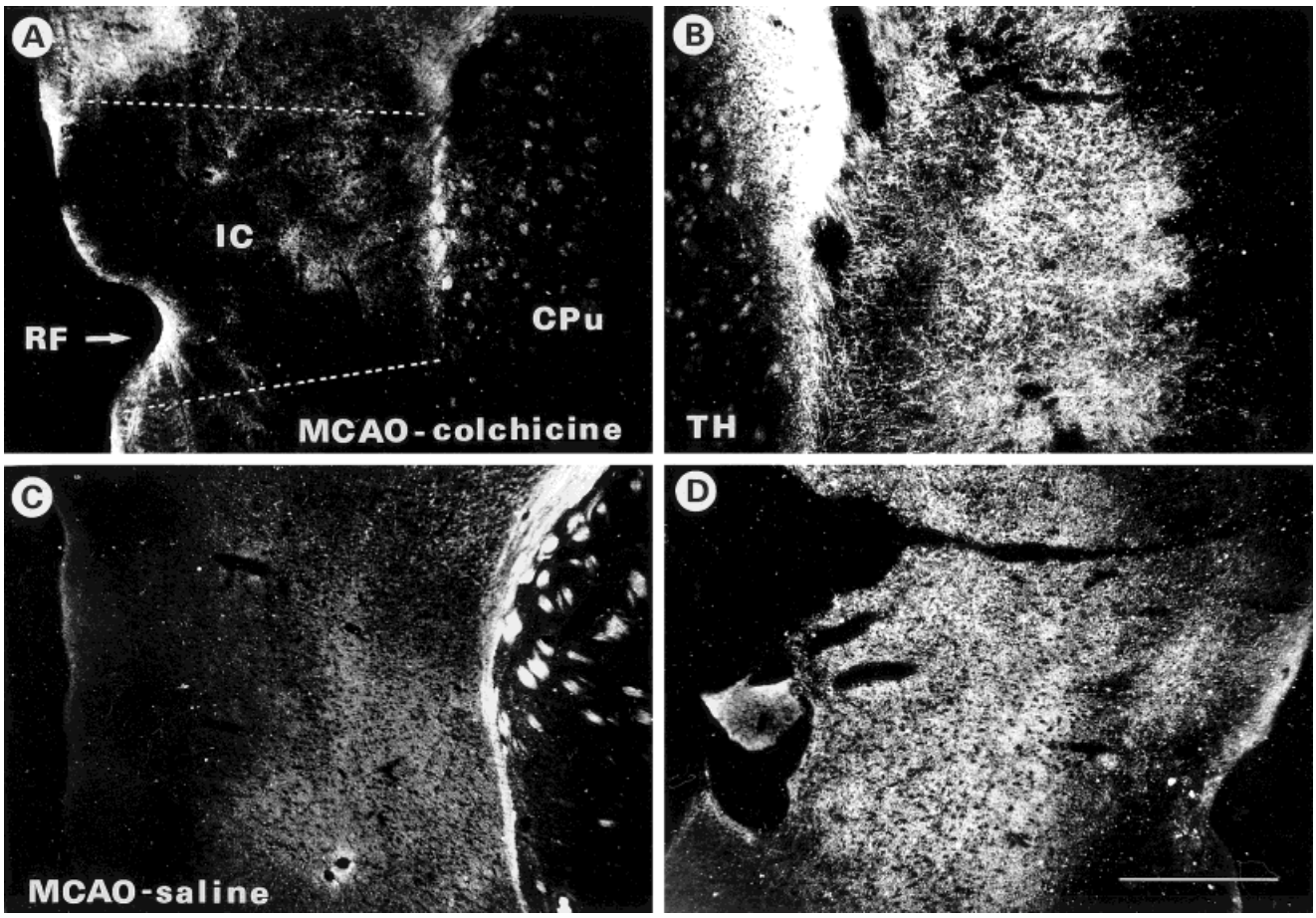

Fig. 2. Darkfield photomicrographs of coronal brain sections showing the immunoreactivity for tyrosine hydroxylase (TH) in the fibers and terminals within right (B,D) and left (A,C) insular cortex (IC) 3 days after right-sided MCAO and injection of colchicine (**A,B**) or saline (**C,D**) into the left lateral ventricle to illustrate the increase in staining in the right IC (B,D). The immunostaining of TH in (B) is more patchy and localized, whereas the immunostaining of TH in (D) is more diffuse and extensive. Some tissue loss is evident in the right IC (B,D) because of MCAO and tissue processing for immunohistochemistry. The upper and lower boundaries of the IC are outlined by the dashed lines in A. CPu, caudate putamen; MCAO-colchicine, MCAO and intracerebroventricular injection of colchicine; MCAO-saline, MCAO and intracerebroventricular injection of saline; RF, rhinal fissure. Scale bar = 500 μm.

In both the MCAO and the sham-MCAO rats treated with colchicine, there was a suggestion of a relative decrease in l-ENK-ir, DYN-ir, and NT-ir within the right ACE ipsilateral to the right-sided MCAO but contralateral to the injection of colchicine into the left lateral ventricle (Figs. 9,10). However, as indicated below, there was no significant side-to-side difference in the staining intensity.

Computer-assisted image analysis of the staining intensity of l-ENK-ir in the ACE generated semiquantitative measurements for statistical testing. By using the left side as the denominator, the relative intensities of l-ENK-ir in the right ACE of the colchicine-treated and saline-treated MCAO groups and in the colchicine-treated sham-MCAO group, respectively, were 112.6 ± 10.1% (n = 12), 147.0 ± 14.1% (n = 6), and 80.3 ± 8.0% (n = 5; Fig. 8B; an ordinary ANOVA revealed significant differences among groups with $P < 0.01$; the Bonferroni post-hoc test revealed a significant difference between the saline-treated MCAO group and the colchicine-treated sham MCAO group with $P < 0.01$). Similarly, the relative intensities of DYN-ir in the right ACE of the colchicine-treated and saline-treated MCAO groups and of the colchicine-treated sham-MCAO group, respectively, were 107.7 ± 7.5% (n = 10), 152.2 ± 4.1% (n = 3), and 79.9 ± 8.3% (n = 5; Fig. 11A; an ordinary ANOVA revealed significant differences among groups with $P = 0.001$; the Bonferroni post-hoc test revealed a significant difference between the colchicine-treated MCAO group and the saline-treated MCAO group with $P < 0.05$ and a significant difference between the saline-treated MCAO group and the colchicine-treated sham-MCAO group with $P < 0.001$). Finally, the relative intensities of NT-ir in the right ACE of the colchicine-treated and saline-treated MCAO groups and of the colchicine-treated sham-MCAO group, respectively, were 89.2 ± 8.6% (n = 12), 129.7 ± 10.1% (n = 6), and 74.6 ± 10.0% (n = 5; Fig. 11B; an ordinary ANOVA revealed significant differences among groups with $P < 0.007$; the Bonferroni post-hoc test revealed a significant difference between the colchicine-treated MCAO group and the saline-treated MCAO group with $P < 0.05$ and a significant difference between the saline-treated MCAO group and the colchicine-treated sham-MCAO group with $P < 0.01$).

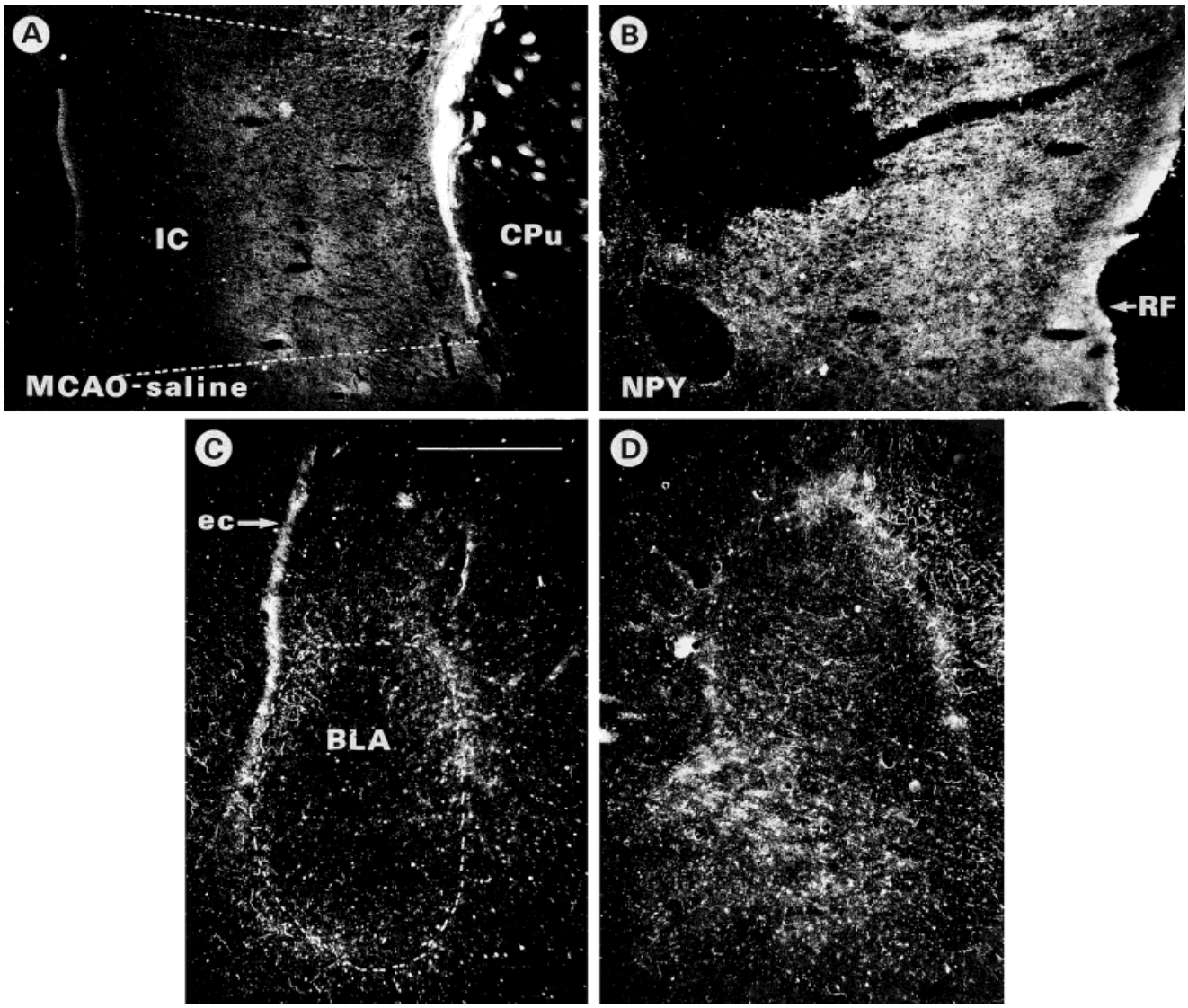

Fig. 3. Darkfield photomicrographs of coronal brain sections showing the immunoreactivity for neuropeptide Y (NPY) in the fibers and terminals within the right (B,D) and left (A,C) IC (**A,B**) and basolateral nucleus of the amygdala (BLA; **C,D**) 3 days after right-sided MCAO and injection of saline into the left lateral ventricle to illustrate the marked increase in staining in the right IC (B) and BLA (D). Some tissue loss is evident in the right IC (B) because of MCAO and tissue processing for immunohistochemistry. The boundaries of the IC and BLA are outlined by the dashed lines in A and C, respectively. ec, External capsule. Scale bar = 500 μm.

DISCUSSION

General considerations

In the saline-treated MCAO rats, we confirmed that neurochemical changes of TH, NPY, l-ENK, DYN, and NT occurred in the IC, BLA, and ACE at 3 days after right-sided MCAO and that adding the left-sided intracerebroventricular injection of the saline vehicle did not affect the neurochemical changes compared with the results of our previous studies (Allen et al., 1995; Cheung et al., 1995a). In addition, injection of colchicine alone into the left lateral ventricle did not produce any significant side-to-side neurochemical changes. Intracerebroventricular injection of colchicine into the MCAO rats, however, enhanced the local NPY increase in the right IC, did not alter the local TH response in the right IC, and prevented the unilateral neurochemical changes in the right amygdala. These differential effects of colchicine, an axonal transport inhibitor, in the MCAO rats suggest different mechanisms for these neurochemical changes following stroke. To appreciate these putative mechanisms, we must address some technical aspects of this study and the effects of colchicine on neurochemical levels.

Technical considerations

As discussed in detail elsewhere (Allen et al., 1995; Cheung et al., 1995a), the combination of immunohistochemistry and computer-assisted image analysis permits only a semiquantitative measurement of specific anatomical regions within brain sections. In addition, there is no information on the rate of synthesis and release of the

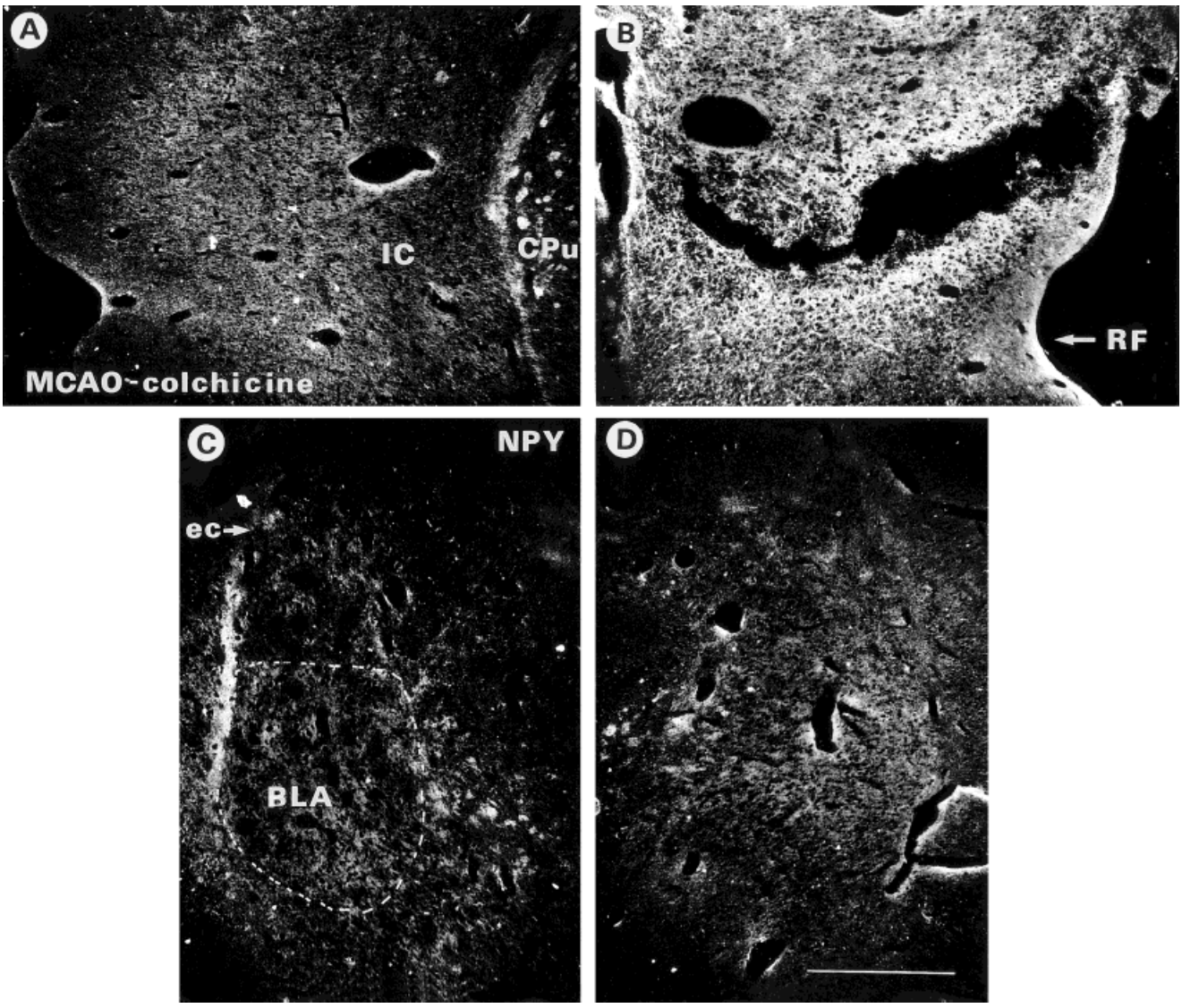

Fig. 4. Darkfield photomicrographs of coronal brain sections showing the immunoreactivity for NPY within the fibers and terminals in the right (B,D) and left (A,C) IC (**A,B**) and BLA (**C,D**) 3 days after right-sided MCAO and injection of colchicine into the left lateral ventricle to illustrate the marked increase in staining in the right IC (B) but not in the right BLA (D). Some tissue loss is evident in the right IC (B) because of MCAO and tissue processing for immunohistochemistry. The boundary of the BLA is outlined by the dotted lines in C. Scale bar = 500 μm.

neuropeptides. Furthermore, measurement of staining density within subdivisions of the ACE is not reliable without counterstaining the sections. Although radioimmunoassay is quantitative, it does not allow any correlation to be made between the neurochemical changes and the anatomical localization. More quantitative methods should be used in future studies. In this study, we adopted the same methods that were used in the analysis of sections and data analysis in our previous studies (Allen et al., 1995; Cheung and Cechetto, 1995; Cheung et al., 1995a) to allow comparison between the present and previous results.

A survival time longer than 3 days is not practical, because the rats treated with the current dosage of colchicine were relatively immobilized by 3 days, whereas a shorter interval is undesirable, because colchicine takes 24–48 hours to achieve its effects (Ceccatelli et al., 1991; Liu et al., 1991; Boyer et al., 1994). Furthermore, these MCAO-induced neurochemical changes peak around 3–5 days after stroke (Cheung et al., 1995a). The colchicine-treated MCAO rats constituted the "experimental" group, but two "control" groups were included to control for the effects of colchicine treatment and the nonspecific effects of making an intracerebroventricular injection. Finally, the infarct volume was also determined in the two MCAO groups. On the one hand, we did not intend to investigate the influence of colchicine on the infarct volume. On the other hand, a significant change in the infarct volume after colchicine treatment might have affected the resulting neurochemical change and made interpretations difficult. Intracerebroventricular injection of colchicine increased the hemispheric volumes significantly but did not affect

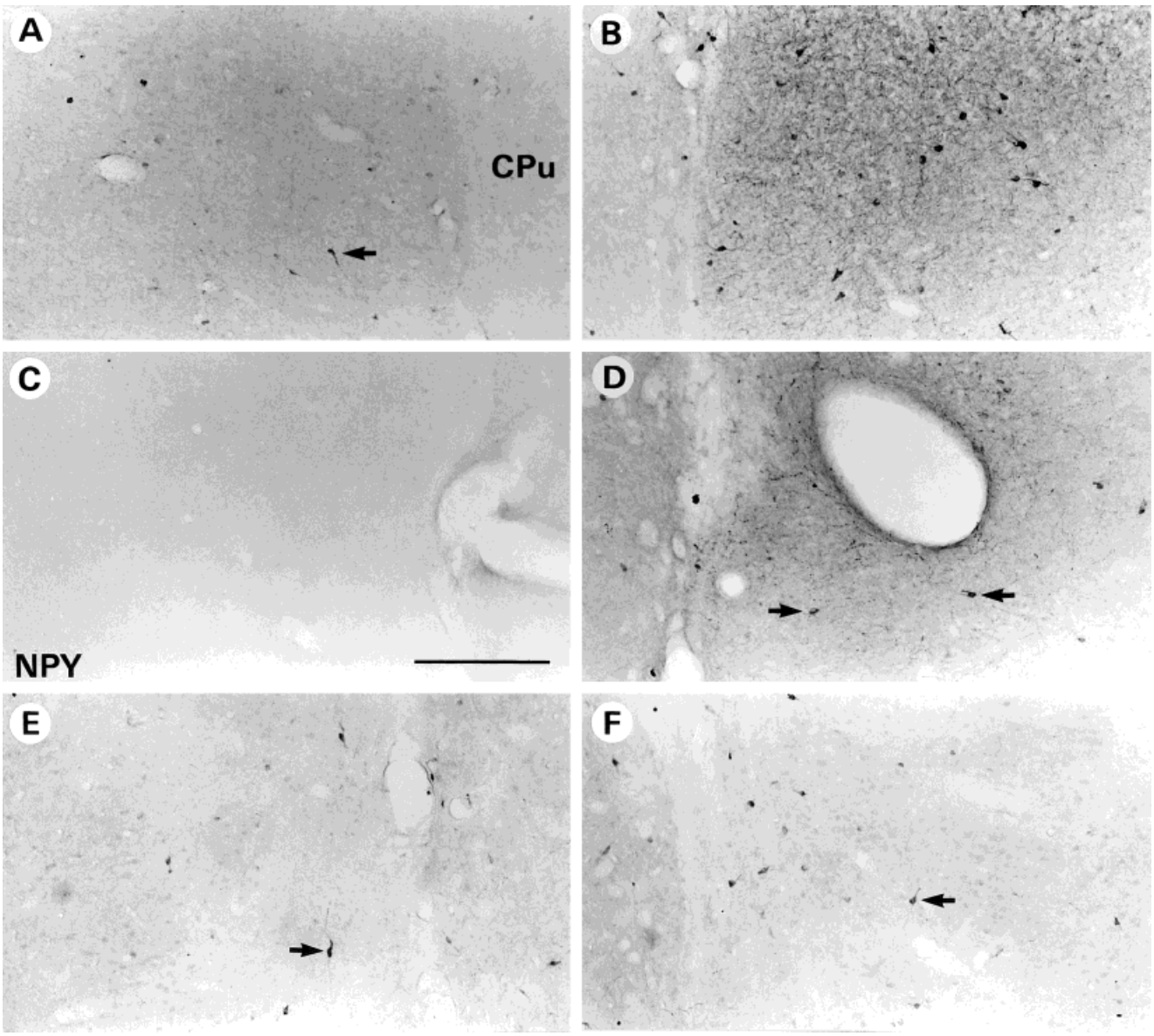

Fig. 5. Brightfield photomicrographs of coronal brain sections showing the immunoreactivity for NPY within the cell bodies, fibers, and terminals in the right (B,D,F) or left (A,C,E) IC 3 days after right-sided MCAO and injection of colchicine (**A,B**) or saline (**C,D**) into the left lateral ventricle or after right-sided sham MCAO and injection of colchicine into the left lateral ventricle (**E,F**). There is increased immunostaining within fibers and terminals of the right IC after MCAO (B,D). However, more labeled cell bodies are seen in the IC after colchicine treatment (A,B). Arrows point to the labeled cell bodies. Scale bar = 250 μm.

the absolute or relative infarct volume compared with the saline-treated MCAO rats.

Effects of colchicine on the level of neurochemicals

Macromolecular synthesis takes place in the neuronal cell body. Axonal transport controls the distribution of membranes, proteins, and other macromolecules in the neuron. Bidirectional axonal transport is mediated by fast anterograde axonal transport (at about 400 mm per day), fast retrograde axonal transport (at about 200–270 mm per day), and slow axoplasmic flow (which transports the cytosol; Schwaber, 1991). Fast anterograde axonal transport, which carries synaptic vesicles and secretary granules, is dependent on microtubules and is highly sensitive to colchicine. Slow axoplasmic flow, which is not affected directly by colchicine, consists of two components (Schwaber, 1991). The slower component flows at about 0.2–2.5 mm per day and carries the cytoskeletal proteins. The faster component, which is twice as fast, carries actin, clathrin, and many cytosolic enzymes.

Local application of colchicine to lumbar sympathetic ganglia and axons produced an interruption of the fast anterograde axonal transport of amine storage granules (Dahlström, 1968). Subepineurial injection of colchicine into the sciatic nerve of male Sprague-Dawley rats re-

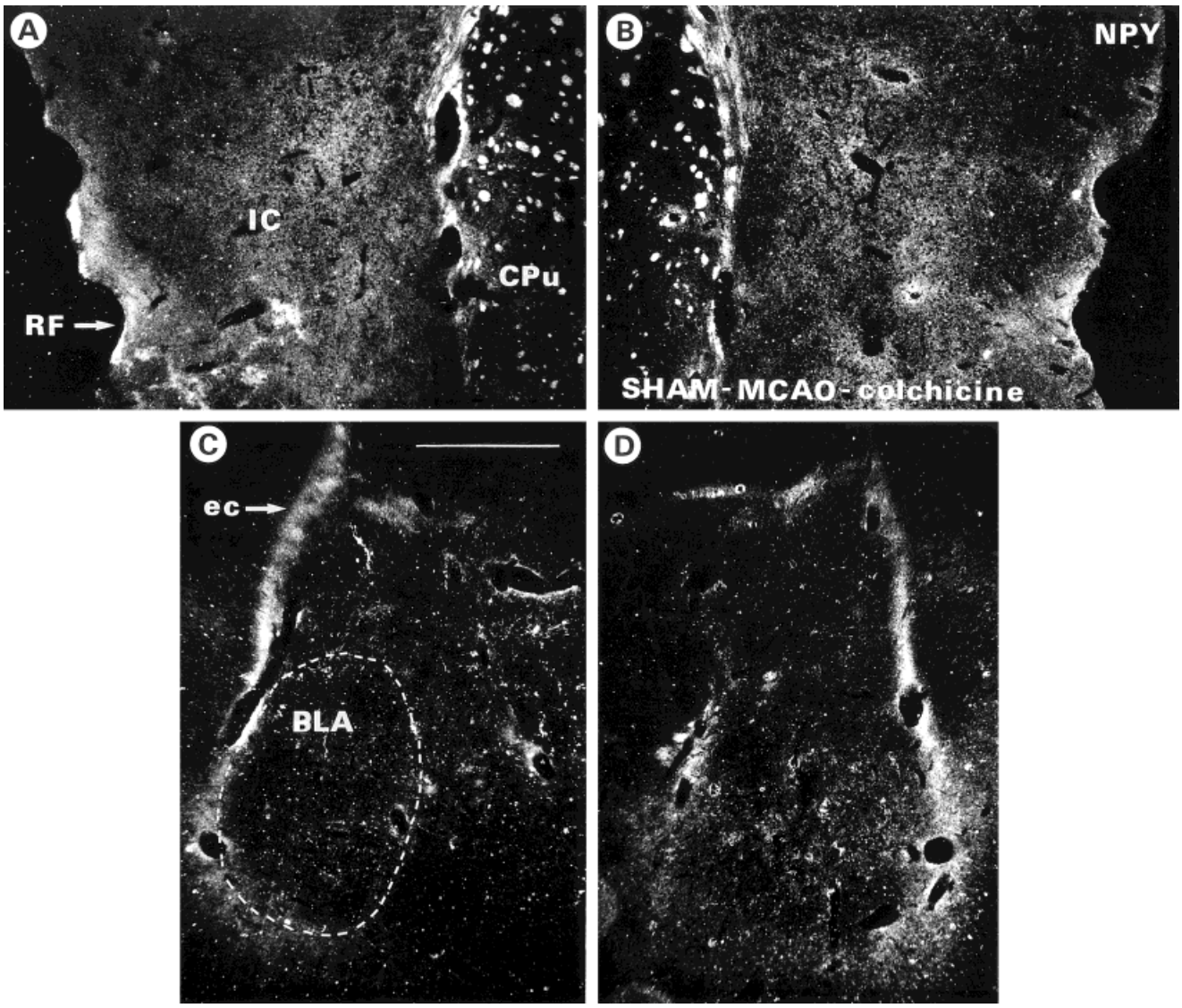

Fig. 6. Darkfield photomicrographs of coronal brain sections showing the immunoreactivity for neuropeptide Y within the fibers and terminals in the right (B,D) and left (A,C) IC (**A,B**) and BLA (**C,D**) 3 days after right-sided sham MCAO and injection of colchicine into the left lateral ventricle to illustrate the absence of any change in staining in the right IC (B) and BLA (D). The boundary of the BLA is outlined by the dashed line in C. Scale bar = 500 μm.

sulted in a dose-dependent blockade of axonal transport of acetylcholinesterase and a mitochondrial enzyme (Kreutzberg, 1969). Since these early reports, colchicine has often been used to improve the histochemical visualization of peptides in the neuronal cell body, and this effect has been attributed to its blockade of fast axonal transport alone. In general, many peptidergic perikarya are not commonly seen without local or systemic use of colchicine (Hökfelt et al., 1977).

More recent studies of neuropeptides in the brain revealed multiple effects of colchicine. For example, Ceccatelli and colleagues (1989) suggested that colchicine acted as a stress stimulus to differentially increase the synthesis of stress-related neuropeptides. In addition, Liu and colleagues (1991) proposed that colchicine could be used to provide an index of biosynthesis for neuropeptides that have a high basal rate of synthesis. Furthermore, Boyer and colleagues (1994) reported the "postsynaptic" and "presynaptic" effects of intranigral injections of colchicine on the gene expression for methionine-enkephalin, substance P, and NPY in the forebrain limbic structures of the rat. The authors concluded that the postsynaptic effects of colchicine in the forebrain limbic structures were due to the blockade of anterograde physiological actions of the ascending nerve fibers; the forebrain limbic structures are postsynaptic to the nigrolimbocortical fibers. The authors also suggested that the presynaptic effects in the substantia nigra and other regions could be attributed to the blockade of axonal transport, resulting in proximal accumulation of peptides and negative feedback regulation of gene expression within these regions (Boyer et al., 1994).

Despite the limitations imposed by the multiple effects of colchicine, the objective of this investigation has been fulfilled by our experiments for the following reasons.

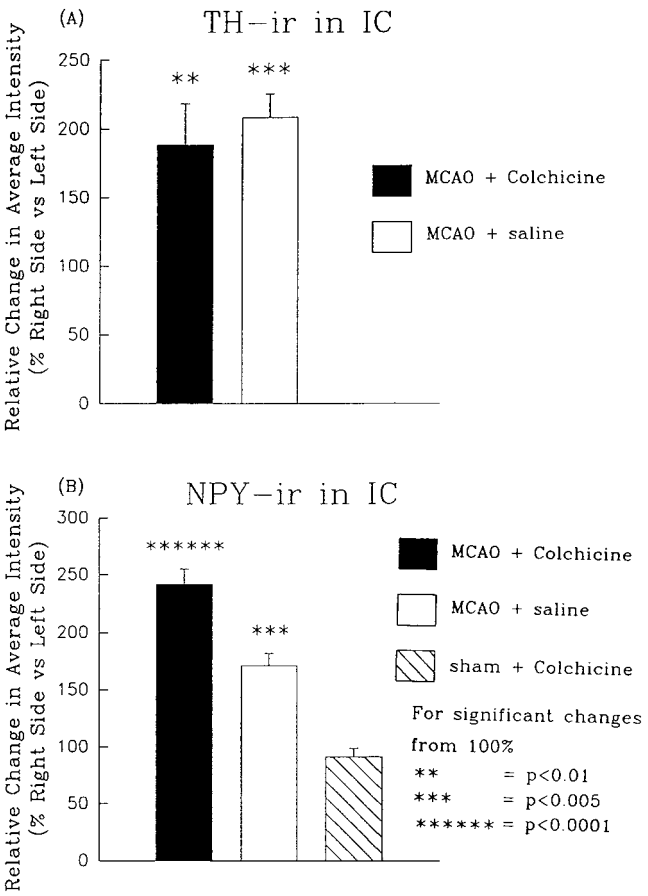

Fig. 7. **A,B:** Bar graphs showing the relative change in average intensity of the immunoreactivity (ir) for TH and NPY in the IC. Measurement from the right side is expressed as a percentage of that from the left side. A shows the relative staining for TH in the right IC after right-sided MCAO and injection of colchicine (n = 5) or saline (n = 6) into the left lateral ventricle. Significant right-sided increase in TH-ir occurs in both groups. B shows the relative intensity of NPY-ir in the right IC after right-sided MCAO and injection of colchicine (n = 12) or saline (n = 6) into the left lateral ventricle or after right-sided sham MCAO and injection of colchicine into the left lateral ventricle (n = 5). Significant right-sided increase in NPY-ir occurs in rats with MCAO but not in rats with sham MCAO.

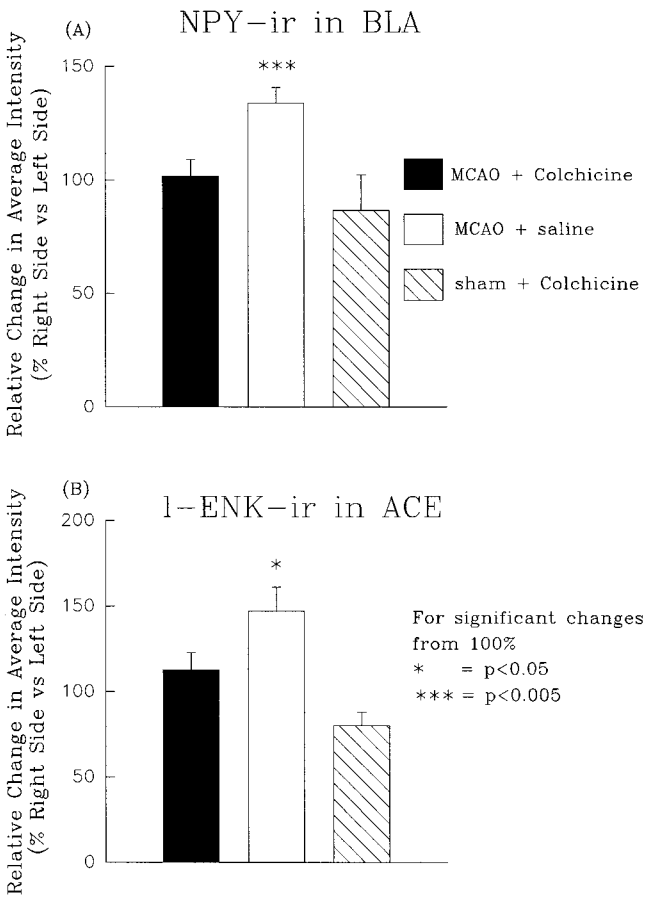

Fig. 8. **A,B:** Bar graphs showing the relative change in average intensity of ir for NPY in the BLA and for leucine-enkephalin (l-ENK) in the central nucleus of the amygdala (ACE). Measurement from the right side is expressed as a percentage of that from the contralateral site. A shows the relative staining for NPY in the right BLA 3 days after right-sided MCAO and injection of colchicine (n = 12) or saline (n = 6) into the left lateral ventricle or after right-sided sham MCAO and injection of colchicine into the left lateral ventricle (n = 5). B shows the relative staining for l-ENK in the right ACE 3 days after right-sided MCAO and injection of colchicine (n = 12) or saline (n = 6) into the left lateral ventricle or after right-sided sham MCAO and injection of colchicine into the left lateral ventricle (n = 5). Significant increase in NPY-ir within the right BLA or l-ENK-ir within the right ACE occurs only in the saline-treated MCAO rats but not in the colchicine-treated MCAO or sham-MCAO rats.

First, proper controls were included, and the colchicine injections, per se, did not produce any significant side-to-side difference in the staining intensity over the IC, BLA, and ACE. Second, results obtained in the colchicine-injected MCAO rats were consistent with a blockade of fast axonal transport. Third, colchicine was used to examine its effects on specific MCAO-induced neurochemical responses that were defined by our previous studies (Allen et al., 1995; Cheung et al., 1995).

Local neurochemical changes in the IC

We previously observed an increased TH immunostaining in the IC within the periinfarct zone 5 days after MCAO (Allen et al., 1995). The present results indicated that a similar TH response in the right IC was detectable 3 days following the right-sided MCAO and that the response was not affected by injection of colchicine into the left lateral ventricle. Both the enzymatic activity and the synthetic rate of TH are tightly regulated according to the ongoing level of neurotransmission at the catecholaminergic synapse (Hall, 1992). Thus, the MCAO-induced local increase in TH immunostaining most likely represents an enhanced synthesis and release of catecholamines in the local axon terminals of the cortex. Although the catecholamine-containing synaptic vesicles are synthesized de novo in the cell body and are transported by fast axonal transport to the axon terminals, the local axon terminals are responsible for local recycling of the synaptic vesicles and for replenishing the vesicles with catecholamines (Hall, 1992). TH, a cytosolic enzyme, is most likely transported by the faster component of the axoplasmic flow, so its transport should not be affected by colchicine, and this is consistent with our results.

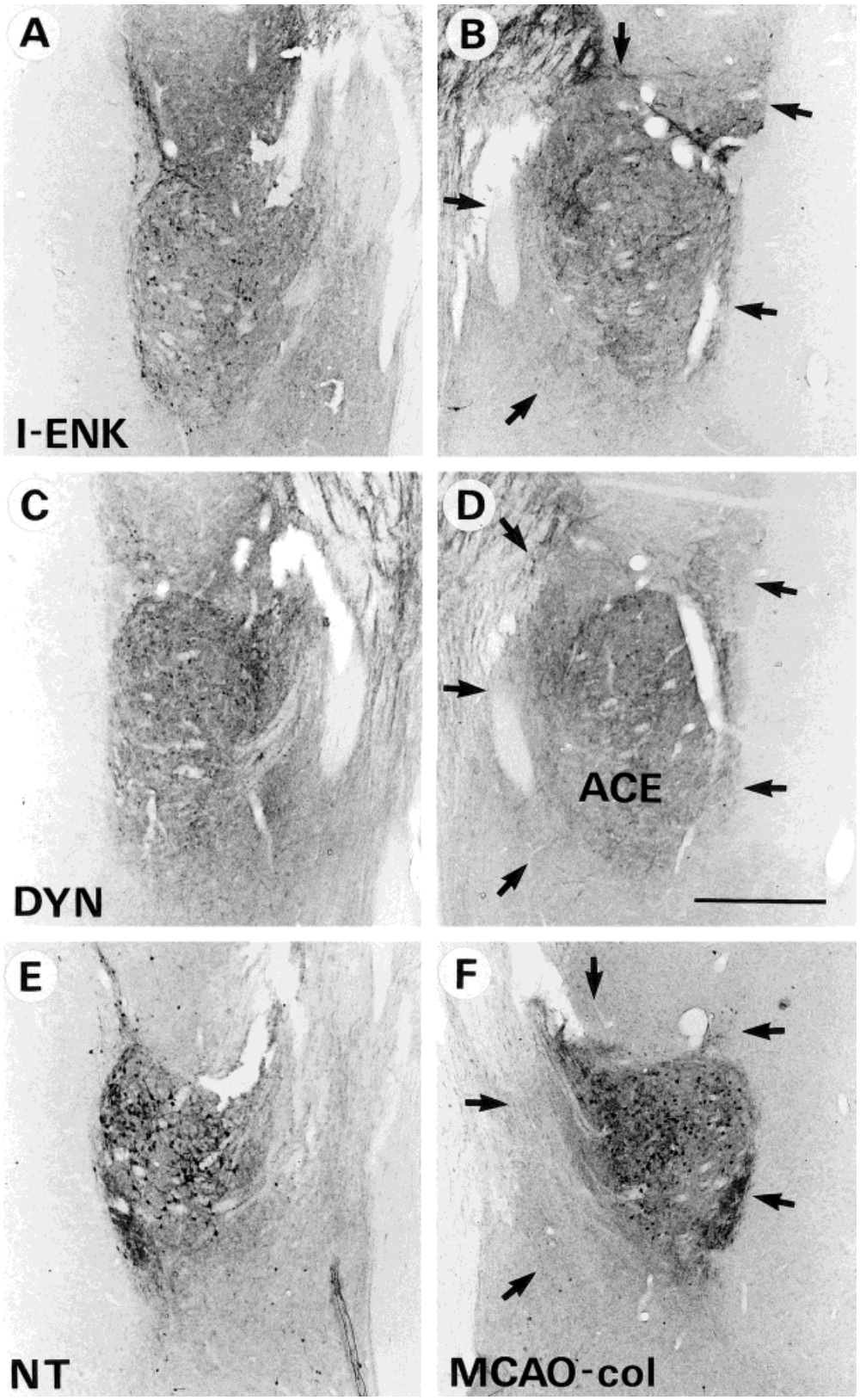

Fig. 9. Brightfield photomicrographs of coronal brain sections obtained from a rat that had right-sided MCAO and injection of colchicine into the left lateral ventricle to illustrate the absence of any increase in staining in the right ACE (B,D,F) for l-ENK (**A,B**), dynorphin (DYN; **C,D**), or neurotensin (NT; **E,F**). The arrows (B,D,F) outline the boundary of the ACE. Scale bar = 500 μm.

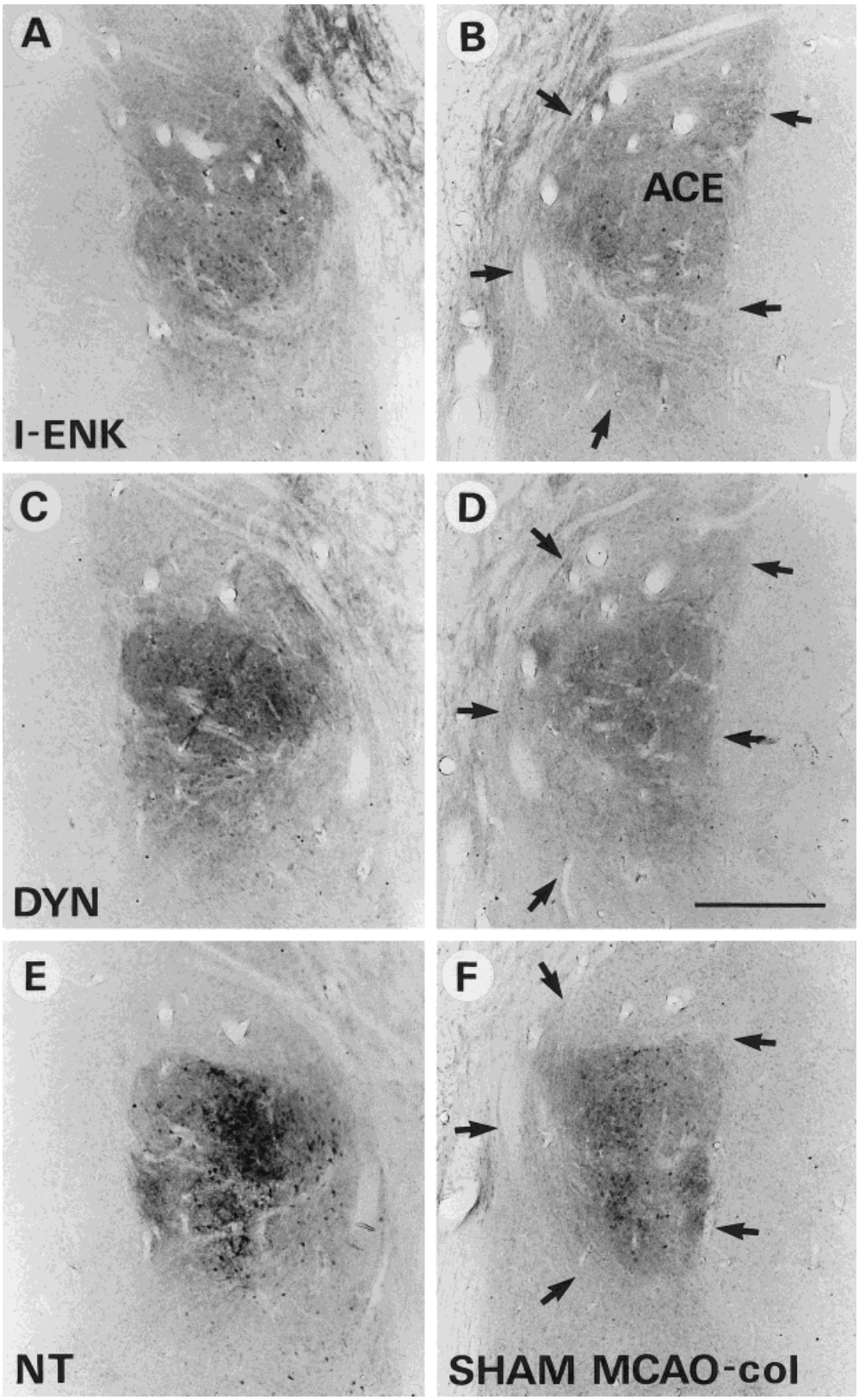

Fig. 10. Brightfield photomicrographs of coronal brain sections obtained from a rat that had right-sided sham MCAO and injection of colchicine (col) into the left lateral ventricle to illustrate the absence of any increase in staining in the right ACE (B,D,F) for l-ENK (**A,B**), DYN (**C,D**), or NT (**E,F**). The arrows (B,D,F) outline the boundary of the ACE. Scale bar = 500 μm.

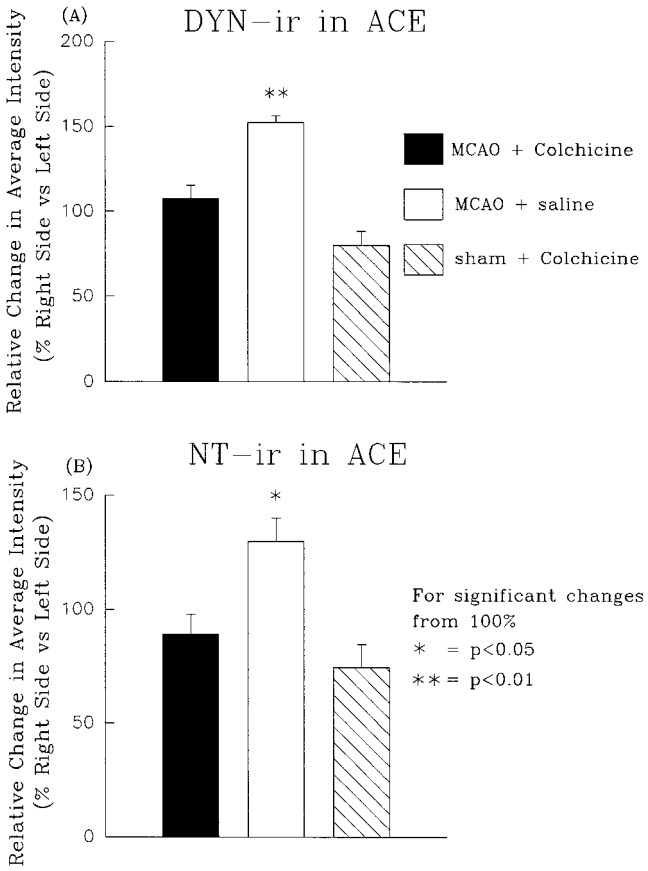

Fig. 11. **A,B:** Bar graphs showing the relative change in average intensity of ir for DYN and NT in the ACE. Measurement from the right side is expressed as a percentage of that from the left side. A shows the relative staining for DYN in the right ACE 3 days after right-sided MCAO and injection of colchicine (n = 10) or saline (n = 3) into the left lateral ventricle or after right-sided sham MCAO and injection of colchicine into the left lateral ventricle (n = 5). B shows the relative staining for NT in the right ACE 3 days after right-sided MCAO and injection of colchicine (n = 12) or saline (n = 6) into the left lateral ventricle or after right-sided sham MCAO and injection of colchicine into the left lateral ventricle (n = 5). Significant right-sided increase in DYN-ir or NT-ir occurs only in the saline-treated MCAO rats but not in the colchicine-treated MCAO or sham-MCAO rats.

Because TH is the first enzyme in the synthetic pathway of catecholamine (Hall, 1992), it is not known whether the TH response after MCAO occurs in the epinephrinergic, norepinephrinergic, and/or dopaminergic neurons. However, norepinephrinergic neurons in the locus coeruleus are the most likely origin of the TH response in the IC because of some strong but indirect evidence (for review, see Allen et al., 1995). In the lateral frontal cortex, norepinephrine concentration is higher than concentrations of the other catecholamines (Role and Kelly, 1991; Kozuka and Iwata, 1995). Both focal and global cerebral ischemia have been shown to rapidly increase the rates of local release and turnover of norepinephrine in the ischemic regions (Robinson et al., 1975; Gaudet et al., 1978; Robinson, 1979; Gustafson et al., 1991). The locus coeruleus provides most of the norepinephrinergic inputs to the cerebral cortex (Robinson et al., 1975; Blomqvist et al., 1985; Role and Kelly, 1991; Mizuki et al., 1995), and these inputs have been found to mediate a neuroprotective effect following transient global ischemia (Blomqvist et al., 1985). In addition, systemic infusion of norepinephrine and epinephrine was found to be protective in transient global ischemia (Koide et al., 1986).

In this study, we did not observe a side-to-side difference in the immunostaining of TH in the locus coeruleus 3 days following MCAO with or without colchicine, suggesting that immunohistochemistry is not sensitive enough to reveal a small change, and/or that the TH response occurs rapidly in the cell body, or the ceiling effect of immunohistochemistry prevented the demonstration of additional increases. Thus, in situ hybridization study of messenger ribonucleic acid (mRNA) specific for TH at 24–48 hours after MCAO is needed to confirm the cellular origin of the TH response. Alternatively, our results suggest that the TH response may be confined to the catecholaminergic terminals of the cortex. However, it is difficult to propose a mechanism whereby ischemia would induce an increase in TH within terminals without involving changes in axoplasmic transport. It is more likely that the TH response occurs through an increase in slow axoplasmic flow of TH from the locus coeruleus, because slow axoplasmic flow is relatively unaffected by colchicine. In addition, studying the immunohistochemistry of dopamine β-hydroxylase and phenylethanolamine N-methyltransferase in the IC following MCAO may confirm whether the TH response is confined to norepinephrinergic neurons.

Unlike the TH response, our previous MCAO studies have provided some insights into the molecular mechanisms of the NPY response in the IC within the periinfarct zone (Allen et al., 1995; Cheung and Cechetto, 1995; Cheung et al., 1995a). The observed increase in NPY immunostaining in nerve terminals and fibers in the periinfarct region, as discussed above, may be the result of increased synthesis, decreased release, or enhanced axonal transport (Cheung et al., 1995a). The present results indicate that the intracerebroventricular injection of saline did not affect the local NPY response of MCAO and that the injection of colchicine alone did not result in any significant side-to-side change in NPY immunostaining within the IC. However, our results in the colchicine-treated MCAO rats reveal that blockade of axonal transport by colchicine enhances the local increase in NPY. In these rats, numerous cell bodies were seen in the periinfarct cortex. Thus, our results suggest that increased synthesis of NPY in local cortical neurons is responsible for the NPY response in the periinfarct area following MCAO. This enhanced local increase in NPY may be secondary to a simple concentration of the peptide within the cell bodies and proximal axons following colchicine treatment. In addition, this local NPY increase was postulated to be a neuroprotective response to ischaemia (Allen et al., 1995; Cheung et al., 1995a; Cheung and Cechetto, 1996a). Blocking the axonal transport of NPY with colchicine will lessen the availability of NPY in the periischemic zone. If this prolongs the ischemic stimulus, then more NPY will be synthesized.

We have also shown that terminals and fibers with NPY-ir increased within the IC or the primary somatosensory cortex when localized excitotoxic damage was made into either region, suggesting that the NPY response is a local reaction of the cortex to ischemia or excitotoxicity (Cheung and Cechetto, 1995). Although NPY colocalizes with norepinephrine in many locus coeruleus neurons (Everitt et al., 1984; de Quidt and Emson, 1986), the locus

coeruleus neurons do not appear to contribute much to the local NPY response after MCAO. Lesion of the locus coeruleus by 6-hydroxydopamine markedly reduces the cortical concentration of norepinephrine but does not significantly affect the cortical concentration of NPY (Schon et al., 1986). In addition, this study shows that colchicine treatment enhances rather than reduces the NPY response in the IC despite axonal blockade and that side-to-side differences in NPY immunostaining are not seen in brainstem regions, such as the locus coeruleus.

In situ hybridization studies on mRNA levels of NPY are required to confirm an increased rate of NPY synthesis after MCAO. At present, the molecular mechanisms of increased NPY synthesis following MCAO or excitotoxicity and the relationships between the changes in the TH and NPY immunostaining are unknown.

Remote neurochemical changes in the amygdala

Collectively, our previous studies have shown that the remote unilateral increase in fibers and terminals, with ir for NPY in the BLA and with ir for l-ENK, DYN, and NT in the ACE, is a specific response to damage of the ipsilateral IC by either ischemia or excitotoxicity (Allen et al., 1995; Cheung and Cechetto, 1995; Cheung et al., 1995a). In particular, excitotoxic damage to the IC, but not to the adjacent primary somatosensory cortex, elicits the ipsilateral amygdalar neurochemical changes that are seen following MCAO (Cheung and Cechetto, 1995). However, there is no information to indicate which of the following mechanisms is/are responsible: increased synthesis, enhanced axonal transport, and/or reduced release in terminals (Allen et al., 1995; Cheung et al., 1995a). We also suggested that the amygdalar neurochemical changes may be the consequence of a loss of input from the IC (Cheung and Cechetto, 1995; Cheung et al., 1995a).

In this study, left-sided intracerebroventricular injection of colchicine was used to locate the cellular origin of the amygdalar neurochemical changes following right-sided MCAO. Nevertheless, the colchicine treatment completely prevented the amygdalar neurochemical changes. This is in contrast to the right IC, in which the TH response to MCAO is preserved and the NPY response to MCAO is enhanced following colchicine treatment. Failure of our immunohistochemical technique is not applicable, because staining was seen in specific brain regions in all three groups of rats and because the stroke-induced amygdalar neurochemical changes were reproduced in the saline-treated MCAO rats. After colchicine treatment in both MCAO and sham-MCAO rats, cell bodies containing l-ENK, DYN, or NT were seen in the ACE bilaterally, and some cell bodies containing NPY were found in the BLA on both sides.

Our results appear to be inconsistent with the hypothesis that the local amygdalar neurons respond to the ischemic or excitotoxic insult to the ipsilateral IC by increasing their immunohistochemical staining, because local increases in ir within the cell bodies were not observed. In fact, the present results tend to support the possibility of some remote sources of axonal input to the amygdala. It is possible that neurons in these remote sites are activated by MCAO or excitotoxic lesion to the IC, resulting in an increased immunostaining of their terminals within the amygdala. This increase in staining of terminals would be prevented by colchicine. In our previous study, however, neurochemical changes were not observed in the brainstem or in other potential sites of input to the amygdala (Allen et al., 1995). In the present study, no significant side-to-side change in the immunostaining of NPY, l-ENK, DYN, or NT was seen in the brainstem.

An alternative explanation for our results is that the colchicine treatment prevents the amygdala from responding to the ipsilateral focal ischemia. If a loss of action potentials from the IC is responsible for the ipsilateral amygdalar neurochemical changes, then blockade of axonal transport inhibitor should not interfere with the generation of these changes. On the other hand, colchicine can block the axonal transport of signalling molecules within the degenerating axons that project ipsilaterally from the ischemic IC to the amygdala and, in turn, will prevent any increase in staining of the peptidergic terminals within the amygdala. This hypothesis is most consistent with our observations. Thus, the present results suggest that the axonal transport of signals from the ischemic IC to the ipsilateral amygdala is crucial in producing the amygdalar neurochemical changes of MCAO. This is analogous to the anterograde or post synaptic effect of colchicine, as suggested by Boyer and colleagues (1994).

CONCLUSION

Results from the present study provide further information on the mechanisms of the MCAO-induced neurochemical changes in the IC, BLA, and ACE. The local neurochemical changes in the IC, which may be neuroprotective after focal ischemia, are produced by different neurons in different locations. On the one hand, local cortical NPY neurons are responsible for the increase in NPY-ir in the periinfarct region. On the other hand, increase in TH-ir over the periinfarct zone, which is not affected by axonal transport blockade, probably originates from the brainstem catecholaminergic cell groups. Finally, ipsilateral axonal transport of signals from the IC to the amygdala may be critical in mediating the amygdalar neuropeptide changes seen after MCAO.

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