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Evidence for Dual Serotonergic Projections to Neocortex: Axons from the Dorsal and Median Raphe Nuclei Are Differentially Vulnerable to the Neurotoxin *p*-Chloroamphetamine (PCA)

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Previous studies have shown that there are morphologically dissimilar serotonergic (5-HT) axon types in rat cerebral cortex which are differentially sensitive to the neurotoxic effects of certain psychotropic drugs: methylenedioxyamphetamines (MDA and MDMA) and *p*-chloroamphetamine (PCA) cause degeneration of fine 5-HT axon terminals in cortex, while sparing beaded axons. Moreover, a recent anterograde transport study suggests that fine and beaded 5-HT axons arise from the dorsal raphe (DR) and median raphe (MR) nuclei, respectively. These data led us to propose that the DR projection to neocortex is selectively vulnerable to the neurotoxic effects of PCA, while the MR projection is resistant; this hypothesis was tested in the present study by comparing retrograde axonal transport of the fluorescent tracer Fluoro-Gold in PCA-treated and control rats. Using this method, only axons that survive PCA treatment can take up and transport the injected label back to the cell bodies of origin, thus allowing us to determine which raphe–cortical projections remain intact after PCA. The results show that PCA administration produces a loss of fine 5-HT axon terminals in neocortex and a concomitant reduction in the number of retrogradely labeled neurons in the DR (77% decrease), when compared to controls. In contrast, beaded 5-HT axon terminals are spared and the number of labeled neurons in the MR remains unchanged after PCA. These results demonstrate that DR and MR projections to cortex are differentially vulnerable to PCA: fine axon terminals arise from neurons in the DR and are highly sensitive to the neurotoxic effects, whereas beaded axons from the MR are resistant. We therefore propose that there are two anatomically and functionally separate 5-HT projections to cortex having different (1) nuclei of origin, (2) axon morphology, (3) regional distributions, and (4) pharmacological properties. Since the mood-altering substances MDA, MDMA, and PCA act specifically upon 5-HT axon terminals from the dorsal raphe nucleus, DR neurons may be preferentially involved in the control of affective state.

INTRODUCTION

The cell bodies of most serotonergic (5-HT)[1] neurons are located within the midline raphe nuclei of the brainstem (14) and project directly to most areas of the brain and spinal cord (1, 2, 20). The 5-HT innervation of cerebral cortex arises primarily from the rostral-most raphe nuclei, located in the mesencephalon: the dorsal raphe (DR), median raphe (MR), and B9 cell groups (3, 5, 9, 13, 21, 32, 38, 47, 50, 54). While the B9 cell group is not well-characterized, the dorsal and median raphe nuclei form two distinct sets of 5-HT neurons with dissimilar projections. These nuclei give rise to separate ascending fiber bundles (3) and preferentially innervate different forebrain regions (2, 9, 23, 32–34, 42). However, the terminal distributions of these two ascending raphe projections converge so that most areas of cerebral cortex are innervated by both the dorsal and the median raphe, with regional differences in the relative contribution from each of these nuclei (30, 38, 53, 71). In addition to these regional differences, a recent anterograde transport study employing the lectin phaseolus vulgaris leukoagglutinin (PHA-L) indicates that axons arising from the dorsal and median raphe nuclei exhibit different patterns of laminar distribution in rat cerebral cortex and have terminals that appear morphologically dissimilar (39). Serotonergic axons arising from the dorsal raphe are of extremely fine caliber and are characterized by minute varicosities (typically less than 1 μm in diameter) that are pleomorphic in shape (generally fusiform or granular). Median raphe axons are distinguished by large, spherical varicosities (typically 2–5 μm in diameter); the intervaricose segments of MR axons are extremely thin and expand abruptly at the site of each varicosity which gives these axons a beaded appearance. For brevity, we use the terms "fine" and "beaded" to refer to the two 5-HT axon types, respectively. Similar distinctive morphologic types of 5-HT axons have also been described in entorhinal cortex (36, 37) and olfactory bulb

[1] Abbreviations used: 5-HT, serotonin; DR, dorsal raphe nucleus; FG, Fluoro-Gold; MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MR, median raphe nucleus; PCA, *p*-chloroamphetamine.

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(45) of the rat and in several neocortical areas in the cat (49); however, the origin of these 5-HT axon types has not been directly determined.

Recent evidence suggests that the two raphe projections to forebrain may be analyzed in greater detail using chemical neurotoxins that cause selective ablation of serotonergic axonal terminals. Several amphetamine derivatives including *p*-chloroamphetamine (PCA), 3,4-methylenedioxyamphetamine (MDA), and 3,4-methylenedioxymethamphetamine (MDMA) produce substantial, long-lasting reductions in biochemical markers for serotonin in forebrain suggesting that these compounds may be neurotoxic to 5-HT axons (4, 12, 16, 18, 46, 57–61, 66). Immunocytochemical studies showed that systemic administration of MDA, MDMA, or PCA causes extensive degeneration of 5-HT axon terminals in forebrain, while raphe cell bodies appear morphologically unaffected (48, 51, 52). However, the denervation caused by MDA and MDMA or PCA is subtotal: a striking feature of these neurotoxic amphetamines is that some 5-HT axon terminals are consistently spared in many forebrain regions. Immunocytochemical staining shows that these neurotoxins produce degeneration of fine 5-HT axon terminals in forebrain, while sparing the beaded 5-HT axons. Furthermore, the differential neurotoxic effect of PCA on the two types of 5-HT axon terminals is observed over a wide range of doses (2.5–40 mg/kg) and survival times (1 week to 2 months) (44). These cytopathologic findings in conjunction with the evidence that fine and beaded fibers arise from the DR and MR, respectively, raise the possibility that the DR projection to forebrain is selectively vulnerable to the neurotoxic effects of these amphetamines, while the MR projection is resistant. Evidence for such differential vulnerability would show that the projections arising from the DR and MR differ pharmacologically as well as morphologically and would provide further support for the existence of two functionally separate raphe–forebrain projection systems.

In the present study, we sought to determine directly whether the DR and MR projections to neocortex are differentially sensitive to the neurotoxic effects of PCA. We therefore conducted a retrograde axonal transport study to determine which raphe–cortical projections remain intact after PCA administration. The fluorescent tracer Fluoro-Gold (FG) was used to identify raphe neurons that project to neocortex both in control and in PCA-treated rats: only those axons which survive PCA administration should be able to take up the fluorescent label and retrogradely transport it to the cell bodies of origin; presumably, axon terminals that are damaged or eliminated by PCA will not take up or transport the label. Thus, by comparing the number and locations of cortically projecting raphe neurons in PCA-treated versus control animals, we have been able to identify the nuclei of origin of the drug-sensitive versus drug-resistant 5-HT axon terminals. Preliminary results of this study were presented in abstract form (43).

MATERIALS AND METHODS

Drug Administration

Adult male Sprague–Dawley rats (Harlan Industries, Walkersville, MD) weighing 175–200 g at the start of the experiment were used. Animals were housed four per cage, given free access to food and water, and maintained on a 12-h light–dark cycle at 70°F in a rat colony room. Immediately prior to drug treatment, animals were transferred to a cool (60°F) quiet room and housed one rat per cage. Rats were administered two intraperitoneal (ip) injections (24 h apart; 11:00 am each day) of either PCA (6 mg/kg, dissolved in normal saline and administered in a volume of 1.0 ml/200 g body wt; $n = 4$) or an equal volume of 0.9% NaCl ($n = 4$). The dose of PCA refers to the free base. Twenty-four hours after the second injection, animals were returned to the animal colony and housed as described above.

Retrograde Transport of Fluorescent Dye

One week after the second ip drug injection, rats were anesthetized with chloral hydrate (400 mg/kg ip) and the fluorescent dye Fluoro-Gold (Fluorochrome, Inc) was microinjected into fronto–parietal cortex. Supplemental doses of chloral hydrate (50 mg/kg ip) were administered as needed to maintain deep anesthesia during the entire surgical procedure. Rats were positioned in a small-animal stereotaxic apparatus (David Kopf Instruments) with the incisor bar set at 3 mm below the interaural line. Each rat received two injections of tracer into the right side of neocortex using the following stereotaxic coordinates [relative to earbar zero; derived from the Paxinos and Watson atlas (55)]: anterior site, AP +8.0, ML −2.0; posterior site, AP +6.0, ML −2.0. All injections were made 0.8 mm below the cortical surface. Fluoro-Gold (2% in sterile normal saline; 200 nl per injection) was pressure injected through a glass micropipet (50 μm tip diameter) cemented with wax to the shaft of a 1 μl Hamilton syringe which was in turn attached to the stereotaxic apparatus. A Kopf microdrive unit was used to advance the plunger. The tracer was delivered over a 10-min period and the pipet left in place an additional 5 min before being withdrawn.

Tissue Preparation and 5-HT Immunocytochemistry

Seven days after the intracortical Fluoro-Gold injections, rats were reanesthetized with chloral hydrate (400 mg/kg ip) and perfused through the aorta with 150 ml of cold (4°C) phosphate-buffered saline (PBS; pH 7.4; rate 55 ml/min) followed by 500 ml of 0.15 *M* phosphate buffer (4°C; pH 7.4; rate 50 ml/min) containing 4% para-

formaldehyde (PAF) and 0.1% glutaraldehyde. The brains were removed from the skull; tissue blocks were postfixed for 4–6 h in cold 0.15 *M* phosphate buffer with 4% PAF and then cryoprotected overnight in cold 10% dimethyl sulfoxide (DMSO) in PBS. All rats were administered a monoamine oxidase inhibitor (*trans*-2-phenyl-cyclopropylamine; 10 mg/kg ip) 2–3 h prior to sacrifice.

Frozen transverse sections (30 μm) though the midbrain and coronal sections (30 μm) through the forebrain region containing the injection sites were cut on a sliding microtome and collected in cold PBS. Some of the midbrain and forebrain sections were immediately mounted and air-dried on gelatin-chrome alum-coated slides and stored in the dark at 4°C until used for computer-assisted mapping of retrogradely labeled cells or fluorescence photography. Adjacent midbrain and forebrain sections were processed for 5-HT immunocytochemistry or stained with 0.5% cresyl violet acetate (pH 3.7) to identify cytoarchitectonic areas and landmarks.

For 5-HT immunocytochemistry, free-floating sections were incubated 48 h (with gentle agitation in a humidified chamber at 4°C) in antiserum directed against serotonin (40) diluted (1:5000) in PBS containing 1% normal goat serum and 0.2% Triton X-100. Serotonergic axons or cell bodies were visualized by using the avidin-biotin peroxidase (ABC) method of Hsu *et al.* (29) (Vector Laboratories). Stained sections were intensified with osmium (22), dehydrated, and coverslipped with Histoclad.

Data Analysis

In transverse midbrain sections (1:6 series through the DR and MR), the positions of raphe neurons retrogradely labeled with Fluoro-Gold were quantitatively mapped from digitized video images using a computer-assisted mapping system for fluorescence microscopy. Adjacent Nissl-stained sections were used to delineate raphe nuclei and other brainstem landmarks. The anatomic demarcations of the DR, MR, and the B9 serotonergic cell group were based on the studies of Taber *et al.* (67), Dahlstrom and Fuxe (14), Berman (7), Lidov and Molliver (41), and Steinbusch and Nieuwenhuys (65).

RESULTS

Fluoro-Gold Injection Sites

Examination of forebrain sections showed that all 16 injections (2 per rat) were confined unilaterally to the right side of neocortex and that Fluoro-Gold did not spread to subcortical structures or to the contralateral hemisphere in any of the cases. In each animal, the centers of the two FG injection sites were spaced about 2 mm apart in the anterior–posterior direction and were located in fronto–parietal cortex at the border between motor (AGl) and somatosensory (SI) cortex (Figs. 1b and 1c). The FG injection site was characterized by a central zone of necrosis of about 0.3–0.4 mm surrounded by an intensely fluorescent ring of staining extending

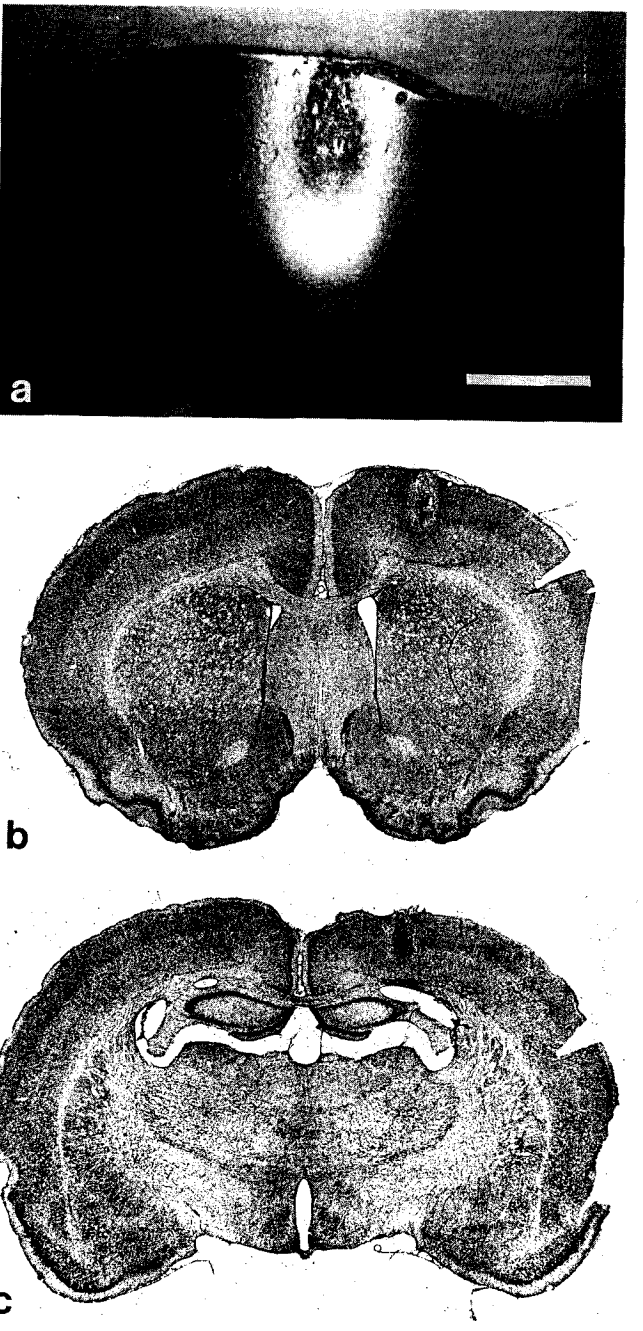

FIG. 1. Retrograde tracer injections. Each rat received two injections of Fluoro-Gold (FG) into right frontoparietal cortex; the injections were spaced 2 mm apart in the anterior–posterior direction. (a) Fluorescence photomicrograph of a typical injection site; scale bar = 1 mm. The lower two panels show the locations of FG injections in cresyl violet stained sections. (b) Anterior injection site; (c) posterior site.

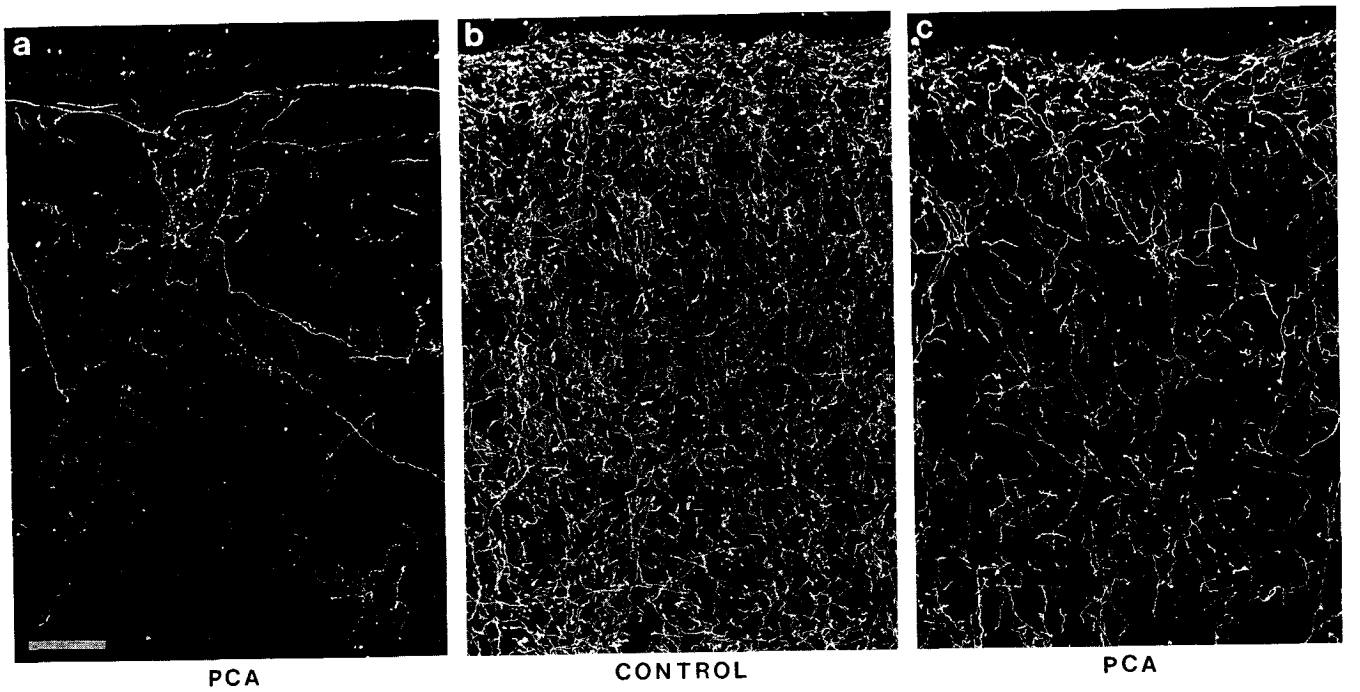

FIG. 2. The serotonergic innervation of parietal cortex in control and PCA-treated rats demonstrated by 5-HT immunocytochemistry (coronal sections through dorso–lateral SI). (b) A high density of 5-HT axons is seen in parietal cortex of a control rat. A prominent decrease in the density of 5-HT axon terminals is seen 2 weeks after PCA administration (6 mg/kg ip; two doses 24 h apart). Variation of the neurotoxic effects of PCA was observed among different animals. (a) Example of 5-HT axon density in cortex of a rat that was severely denervated by PCA. (c) Example of 5-HT axon density in cortex of a rat that was moderately affected by PCA. Darkfield photomicrographs were taken with a 16X plan apochromat objective; scale bar = 100 μm. [The morphology of 5-HT-immunoreactive axons in control and treated animals is described elsewhere (see text) and is better visualized with higher magnification, brightfield microscopy. Using brightfield (not shown), a high density of fine 5-HT axons is observed in parietal cortex of the control animal; beaded 5-HT axons are concentrated in the outer layers of cortex (I–III) and in layer VI. In the PCA-treated rats, the reduction in axon density (cf. (a) and (c)) is due to a selective elimination of fine 5-HT axons, which are almost completely lost in the severely denervated rats (a) with some remaining in the moderately affected cases (c). In both sets of treated rats, beaded axons remain intact and have the same morphology and laminar distribution as those in controls.]

another 0.4–0.6 mm; thus, each injection site was approximately 1.3 mm in diameter (Fig. 1a). The neocortical positions, size, and fluorescent intensity of the injection sites were highly consistent across animals.

The Effects of PCA on Serotonergic Axons in Neocortex

In immunocytochemically stained sections from the forebrains of control rats (non-PCA-treated; $n = 4$), a high density of 5-HT-containing axons and terminal varicosities was observed bilaterally in fronto–parietal cortex—the region containing the FG injection sites (Fig. 2b). Moreover, consistent with previous observations (8, 39, 48, 52), regional and laminar differences in 5-HT axon density and morphologically different 5-HT axon types were observed in cortex of control rats. For example, in somatosensory cortex, fine axon terminals were distributed through all layers of SI but were especially dense in the upper portion of layer V (Va). A separate group of morphologically distinct 5-HT axons, with large spherical varicosities, were found primarily in layers I–III and layer VI of SI.

Two weeks after PCA treatment ($n = 4$), there was a profound loss of serotonergic axons throughout forebrain, especially in neocortex (Figs. 2a and 2c) and striatum, as previously observed (48). Examination of immunocytochemically stained forebrain sections from PCA-treated rats showed that fine 5-HT axons and terminals were severely ablated in fronto–parietal cortex, with some variability in the degree of denervation across animals.[2] In two of the four PCA-treated rats, almost no fine 5-HT axons were found in neocortex (Fig. 2a). In the other two PCA-treated rats, a few fine fibers remained in neocortex (Fig. 2c), although their density was much lower than that in control animals. Consequently, the PCA-treated animals were divided into two groups for subsequent analyses, based on the degree of fine axon loss. All four PCA-treated rats, independent of the degree of fine axon loss, showed consistent and characteristic sparing of beaded 5-HT axons. These surviving ax-

[2] In another study (44), we find a progressive loss of fine 5-HT axons at doses of PCA ranging from 2.5 to 10 mg/kg; beaded axons are consistently spared at all doses studied (2–40 mg/kg). The dose employed in this study (6 mg/kg) lies along a steep part of the dose–response relationship where there is variation in the degree of fine axon loss among different animals.

ons were found predominantly in layers I–III and layer VI of fronto–parietal cortex.

The Effects of PCA on Retrograde Transport of Fluoro-Gold

Retrograde labeling in control animals. Fluoro-Gold injections into fronto–parietal cortex of control (non-PCA-treated; $n = 4$) rats resulted in retrograde labeling of somata located primarily in the dorsal raphe, median raphe, and the region of the B9 cell group. Numerous retrogradely labeled neurons were found in both the DR and the MR/B9 regions (Figs. 4 and 7, control animals). A few FG-labeled cells were seen in other brainstem nuclei but were not analyzed in this study. When sections through the DR and MR were compared with adjacent sections prepared for 5-HT immunocytochemistry (Fig. 3), the distribution of FG-labeled cell bodies was found to overlap with the distribution of 5-HT-containing cells in the midbrain (compare Figs. 3 and 4). The retrogradely labeled cells appear to form a subset of the 5-HT neurons in the DR and MR nuclei.

A large number of retrogradely labeled neurons were found in the dorsal raphe nucleus (Figs. 4 and 7, control animals). Within the DR, FG-labeled cell bodies were located primarily in the ventromedial part of the nucleus, ipsilateral to the FG-injection site. A smaller group of labeled cells was found in the dorsomedial division of the DR just below the cerebral aqueduct; in contrast, retrogradely labeled neurons were rarely observed in the lateral wings of the DR. Although labeled cells were distributed along the entire rostral-to-caudal extent of the dorsal raphe, they were concentrated at mid-to-rostral levels. The FG-labeled cells in the DR were typically medium-sized (20–30 μm), fusiform or oval in shape, and moderately to intensely fluorescent (Fig. 8).

Numerous retrogradely labeled cell bodies were also found bilaterally in the MR (Figs. 4 and 7, control animals), although in some animals there was a slight contralateral preference in labeling. Most of the FG-labeled cells were located in the medial part of the MR (cf. 67); however, a substantial number of labeled cells were found in the peripheral part of the MR and, ventrolaterally, in the B9 cell region. Labeled cells in the B9 region were confined to the more medial part. Since there was no clear demarcation between FG-labeled cells located in the MR and those located in the B9 region, we combined cell counts from both of these regions. The FG-labeled cells in the MR were usually larger than DR-labeled cells, fusiform or multipolar in shape, and intensely fluorescent (Fig. 8).

Retrograde labeling after PCA treatment. In animals treated with PCA followed 1 week later with Fluoro-Gold injections, the number of retrogradely labeled neurons in the dorsal raphe was markedly reduced (77% decrease), when compared to controls. There was an average of 86 ± 2 FG-labeled neurons in the DR of control rats ($n = 4$) versus 20 ± 8 labeled cells in the DR of PCA-treated animals ($n = 4$), a statistically significant difference [$t(6\ df) = 7.93$, $P < 0.001$, two-tailed]. The few remaining dorsal raphe FG-labeled cells in treated animals were concentrated in the dorsomedial division of the DR (Figs. 5 and 6). In contrast, there was no decrease in the number of FG-labeled neurons in the median raphe/B9 region after PCA treatment. There was an average of 78 ± 5 FG-labeled neurons in the MR/B9 region of control rats versus 76 ± 7 labeled cells in the MR/B9 region of PCA-treated animals which is not a significant difference [$t(6\ df) = 0.33$, $P > 0.05$, two-tailed]. In Nissl-stained sections or immunocytochemical preparations from PCA-treated rats, there was no apparent cytologic change or loss of cell bodies in the dorsal or median raphe nuclei. Moreover, after PCA treatment, the fluorescence intensity, size, and morphology of the remaining FG-labeled neurons located in either the DR or the MR/B9 region were similar to those seen in controls (Fig. 8).

Although all four PCA-treated rats demonstrated marked reductions in the number of retrogradely labeled neurons in the dorsal raphe, there was variability across animals in the magnitude of this effect. Cell counts of FG-labeled neurons in the DR of individual PCA-treated rats ranged from 1 to 36, a 58–99% decrease in labeling when compared to the mean number of labeled cells in controls. To determine whether this variation is related to the magnitude of denervation, we compared the number of retrogradely labeled cells in the two groups of rats that were separated by the degree of 5-HT axon loss in neocortex: the PCA-treated rats with a near-complete loss of fine 5-HT axons in cortex (cf. Fig. 2a) demonstrated a 92 ± 7% decrease in the number of retrogradely labeled cells in the dorsal raphe when compared to controls (Fig. 7, PCA_s; Fig. 5), while the rats with a moderate loss of fine axons (cf. Fig. 2c) demonstrated a 61 ± 3% decrease in DR labeling (Fig. 7, PCA_m; Fig. 6). In contrast, there was no decrease in the number of retrogradely labeled neurons in the MR/B9 region in either of these groups, when compared to controls.

DISCUSSION

Although systemic administration of the psychotropic amphetamines PCA, MDA, or MDMA produces extensive degeneration of serotonergic axon terminals in the forebrain, some 5-HT axons are consistently spared in particular regions and layers of cerebral cortex (48, 51, 52). The present study employs retrograde axonal transport of a fluorescent marker to determine whether the surviving 5-HT axons in neocortex arise from a distinct population of raphe neurons. The results demonstrate that, in PCA-treated animals, the number of retrogradely labeled neurons in the dorsal raphe nucleus is

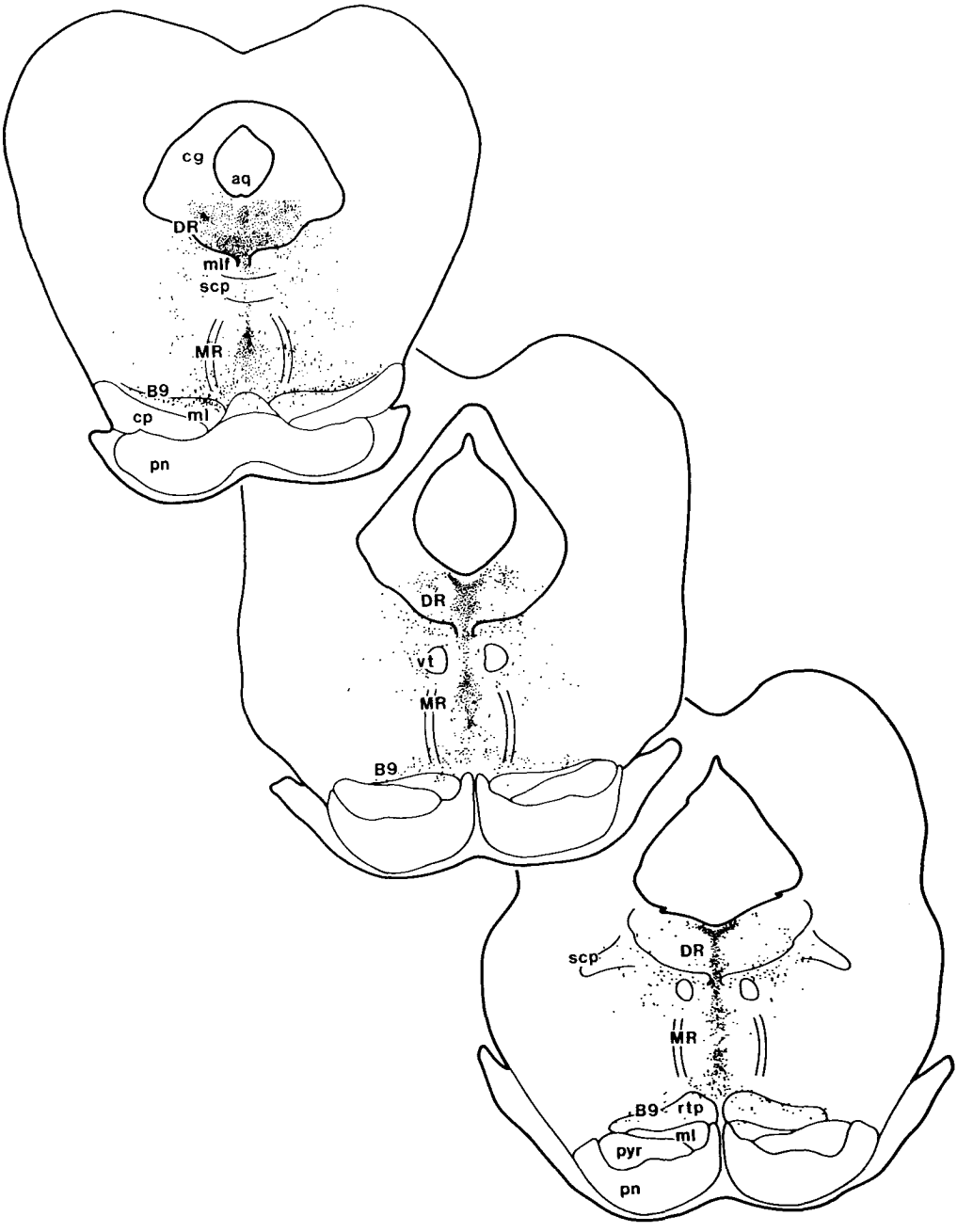

FIG. 3. Distribution of 5-HT-immunoreactive cell bodies in the midbrain of a control rat. Transverse sections through the midbrain are spaced about 0.4 mm apart from rostral (top) to caudal (bottom). Each point represents one 5-HT-positive neuron. Labeled cells from two adjacent sections are depicted at each of the levels shown. Abbreviations: aq, cerebral aqueduct; B9, 5-HT cell group of Dahlstrom and Fuxe (14); cg, central gray; cp, cerebral peduncle; DR, dorsal raphe; ml, medial lemniscus; mlf, medial longitudinal fasciculus; MR, median raphe; pn, pontine nuclei; pyr, pyramidal tract; rtp, reticulotegmental nucleus of the pons; scp, superior cerebellar peduncle; vt, ventral tegmental nucleus.

markedly reduced. The loss of retrograde labeling in the dorsal raphe provides evidence that axon terminals arising from the DR either have degenerated subsequent to PCA treatment or are damaged to the extent that they are unable to take up or retrogradely transport the injected tracer. In contrast, there is no change in the number of retrogradely labeled cells within the median raphe or B9 cell groups indicating that the ascending projections arising from these groups are not damaged by PCA. We conclude that the serotonergic projections to neocortex from the mesencephalic raphe nuclei are differentially sensitive to PCA: specifically, DR axons are highly

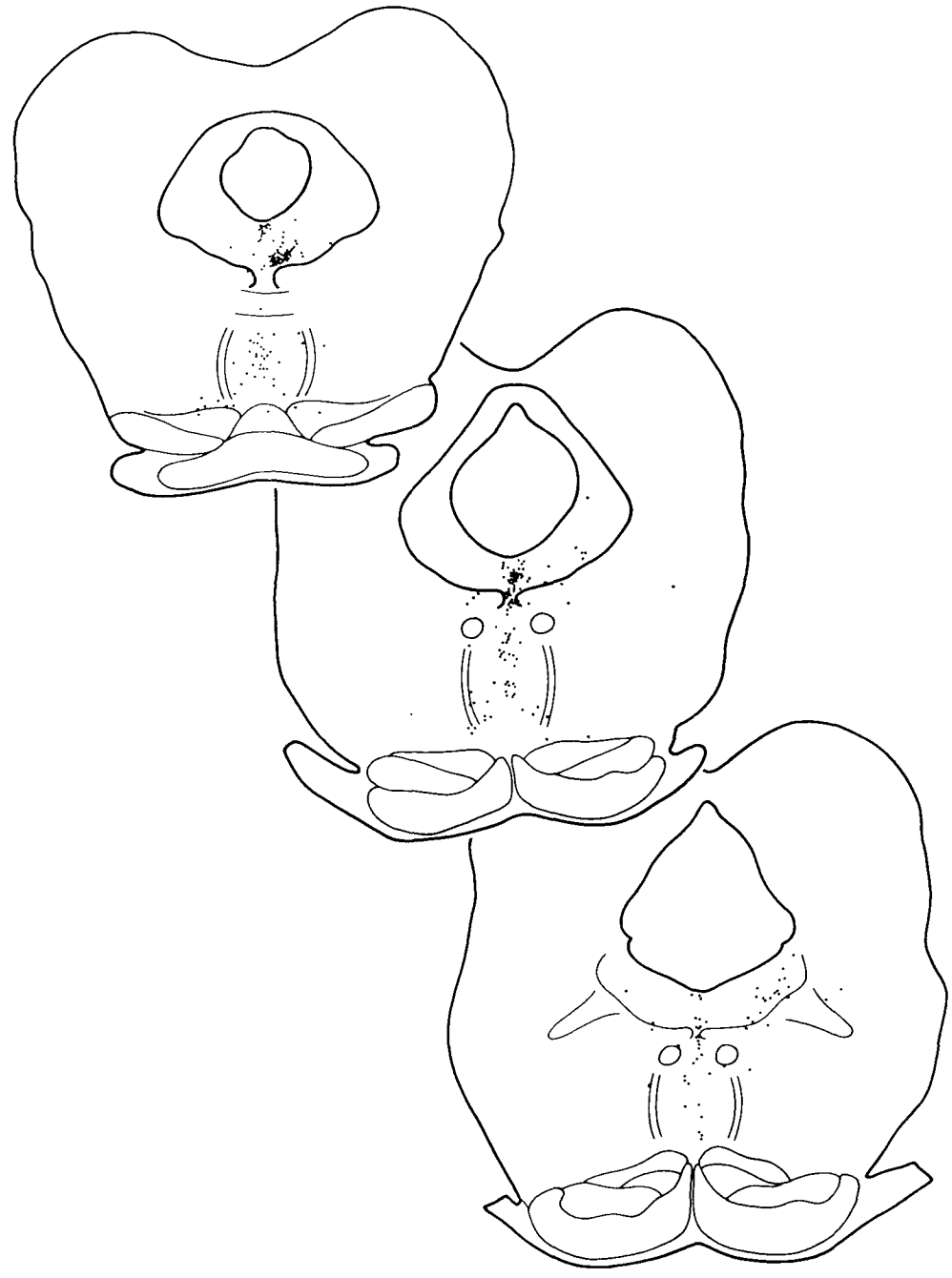

FIG. 4. Distribution of cortically projecting neurons in the midbrain of control animals. Each point represents one retrogradely labeled cell body. Data are derived from two rats: labeled cells from two adjacent sections per rat are depicted at each of the levels shown. Brainstem levels and landmarks are as shown in Fig. 3. At midbrain levels, retrogradely labeled neurons are found primarily in the dorsal raphe, median raphe, and B9 cell group.

vulnerable to PCA while MR and B9 axons are resistant to its neurotoxic effects.

The premise, based on the present data, that cortical serotonergic axons which survive PCA administration arise from neurons in the median raphe nucleus is consistent with several features of ascending raphe projections shown in previous studies. The principal argument that surviving 5-HT axons arise predominantly from the MR is based on our observation that cortical projections from median raphe neurons remain intact after PCA, while those from the DR—the other main source of 5-HT axons in cortex—are eliminated. There is no basis to suspect that the spared 5-HT axons in cortex may arise from a totally different set of 5-HT neurons since

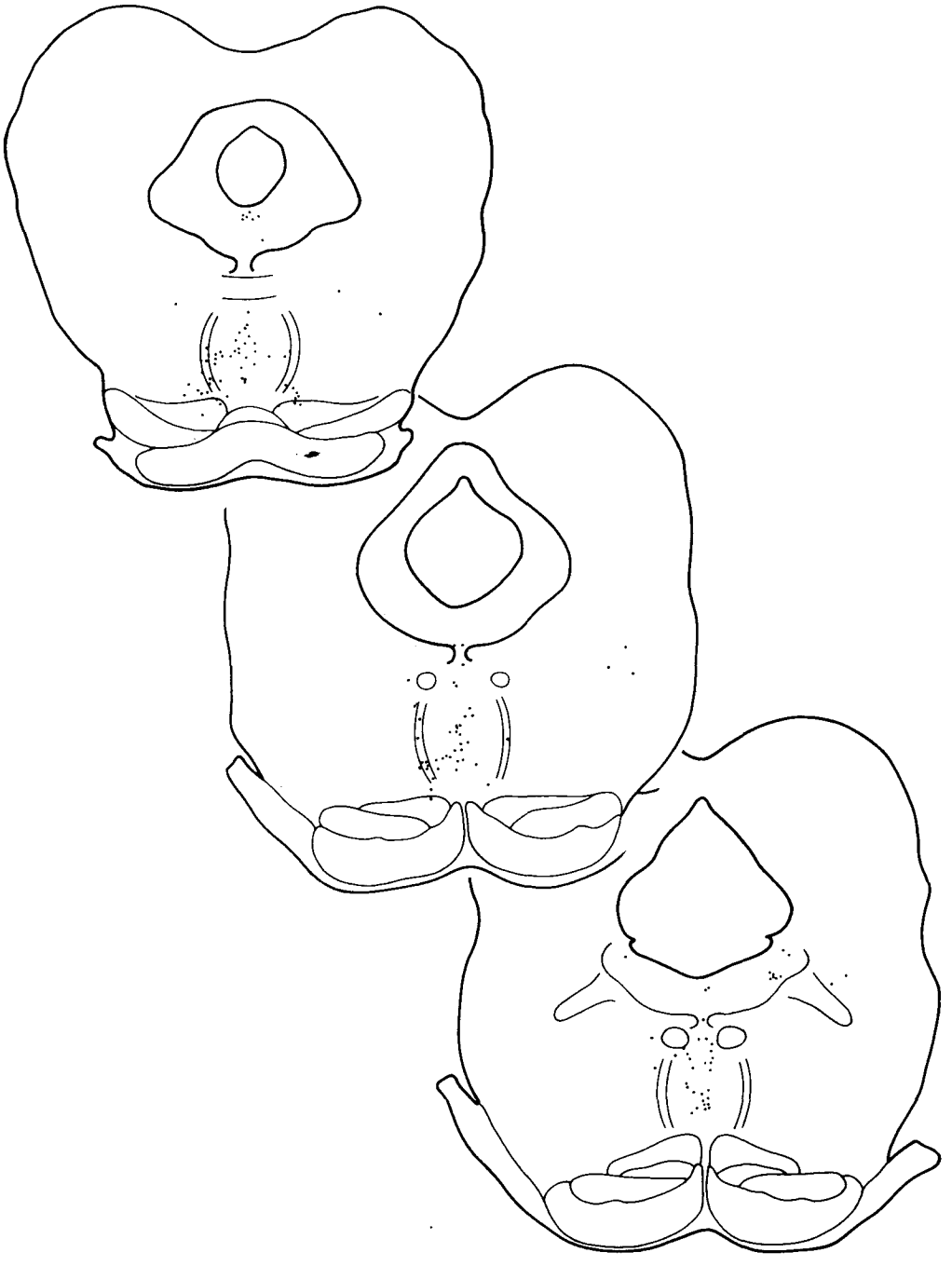

FIG. 5. The distribution of retrogradely labeled neurons in PCA-treated rats with a nearly complete loss of fine 5-HT axons in cortex (cf. Fig. 2a). Each point represents one retrogradely labeled cell body. Data are derived from two rats: labeled cells from two adjacent sections per rat are depicted at each of the levels shown. Brainstem levels and landmarks are as shown in Fig. 3. After PCA, the number of retrogradely labeled cells in the dorsal raphe nucleus is significantly decreased when compared to controls (cf. Fig. 4). In the median raphe/B9 region, the distribution and density of labeled neurons remain unchanged after PCA.

in normal animals the 5-HT innervation of neocortex arises almost exclusively from the DR and MR with a small contribution from B9 (cf. Introduction). Moreover, there are several reasons to support the conclusion that serotonergic projections from the MR are not damaged by PCA. Although the neurotransmitter of labeled cells was not directly identified in this study, the distribution of retrogradely labeled cells in the MR matches that of 5-HT-immunoreactive cells in both treated and control animals (cf. Figs. 3 through 6). It is therefore

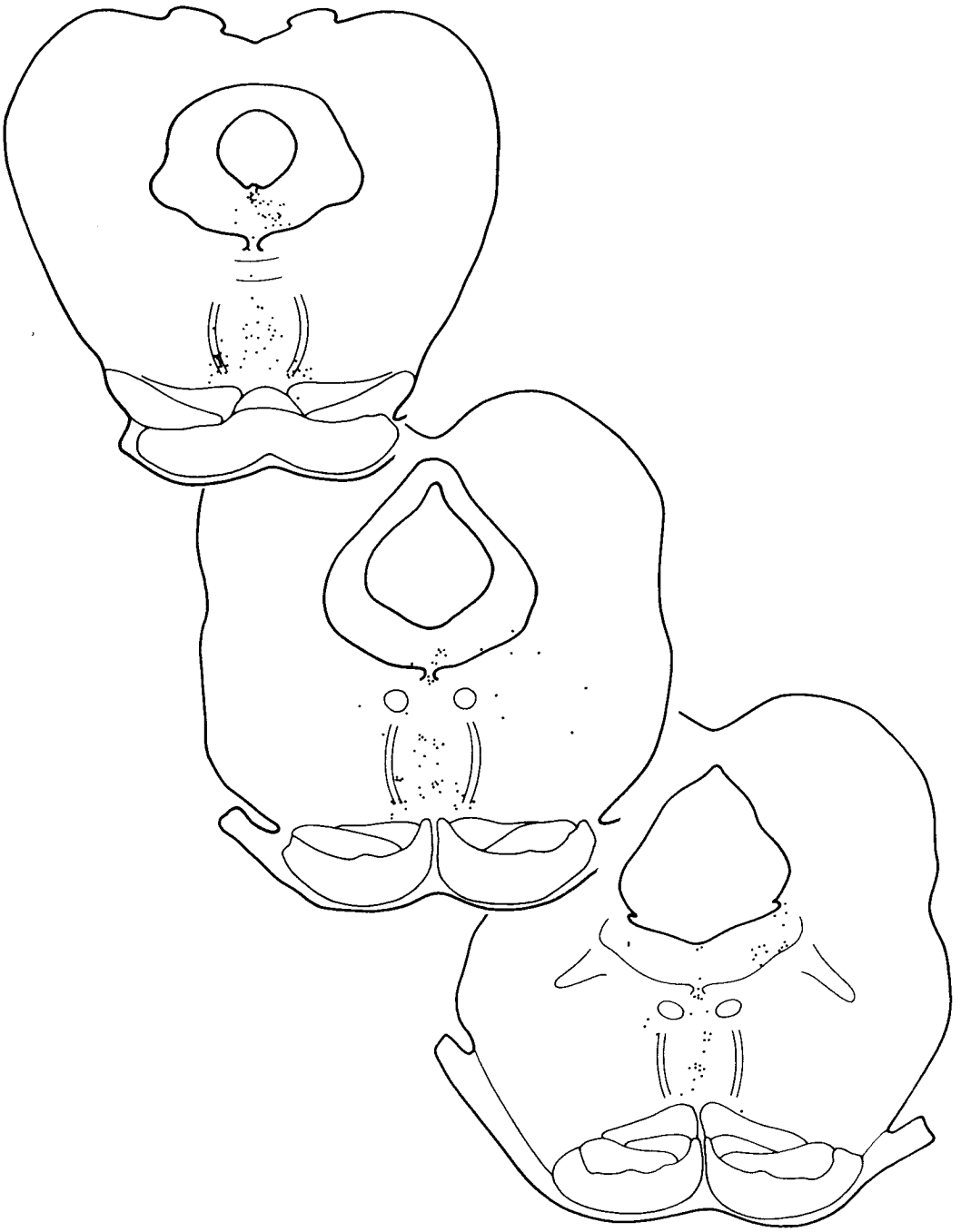

FIG. 6. The distribution of retrogradely labeled neurons in PCA-treated rats with a moderate loss of fine 5-HT axons in cortex (cf. Fig. 2c). Each point represents one retrogradely labeled cell body. Data are derived from two rats: labeled cells from two adjacent sections per rat are depicted at each of the levels shown. Brainstem levels and landmarks are as shown in Fig. 3. These maps show a moderate loss of retrograde labeling in the dorsal raphe nucleus when compared to the severe loss shown in Fig. 5; there was no loss of labeling in the median raphe/B9 region, when compared to controls.

likely that many of the cortically projecting MR cells are 5-HT-positive. In addition, previous studies report that most of the cortically projecting neurons in the MR are serotonergic (38, 45, 53); thus, a loss of 5-HT projections from this nucleus would have been readily detected with retrograde transport methods. More definitive evidence that the remaining retrogradely labeled cells in the MR are predominantly serotonergic would require the use of a method that permits simultaneous detection of the retrograde label and a marker for 5-HT. In a pilot study

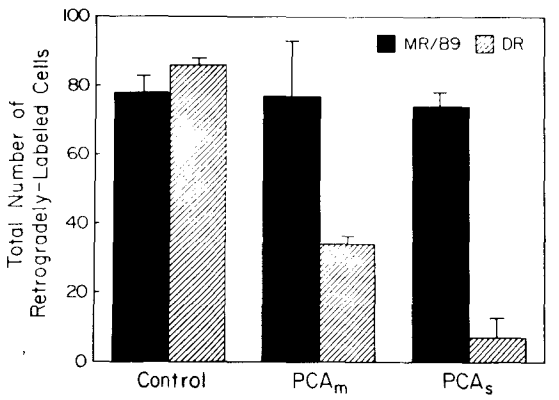

FIG. 7. The effect of PCA administration on the number of retrogradely labeled neurons in the dorsal raphe nucleus (histogram bars with cross hatching) and median raphe/B9 (black bars). Fluoro-Gold (FG)-labeled cells within these nuclei were counted in every sixth section (30 μm) through the dorsal and median raphe nuclei. Control rats (left pair of bars) exhibit many labeled cells in both raphe cell groups (control group means ± SE: MR/B9 = 78 ± 5 cells; DR = 86 ± 2; n = 4). The moderately denervated PCA-treated rats (PCA$_m$; middle bars) demonstrate a 61% decrease in the number of FG-labeled DR cells (MR/B9 = 77 ± 16; DR = 34 ± 2; n = 2). In the severely denervated PCA-treated rats (PCA$_s$; right bars), there is a 92% decrease in the number of FG-labeled cells in the DR (MR/B9 = 74 ± 4; DR = 7 ± 6; n = 2). No effects of PCA treatment on the number of MR/B9 cells with cortical projections were observed.

employing such a double-label procedure, we have preliminary evidence that a substantial number of cortically projecting MR neurons are serotonergic in both PCA-treated rats and controls.

Since the present study is based on the analysis of raphe projections to only one cortical area, dorsolateral fronto–parietal cortex, caution should be exercised in generalizing the results to other regions. Nevertheless, several lines of evidence provide grounds to predict that selective vulnerability of dorsal raphe axons to PCA is likely to be found wherever these neurons project in the brain. This generalization is supported by the finding (48, 51, 52) that neurotoxic amphetamines induce a severe loss of serotonin axons in areas that receive 5-HT innervation predominantly from the dorsal raphe nucleus (e.g., neocortex and striatum (23, 32, 53)), with sparing of serotonin axons in regions that receive a substantial 5-HT projection from the median raphe or from other raphe nuclei. Thus, hippocampus, hypothalamus, and brainstem reveal partial sparing of 5-HT axons after PCA, MDA, or MDMA, on the basis of evidence from biochemical (4, 12, 59) as well as immunocytochemical (48, 51, 52) studies. Also, in cingulate and entorhinal cortex and in the olfactory bulb there is sparing of 5-HT axons that have large, spherical varicosities which are characteristic of median raphe terminals [discussed below]. Moreover, recent evidence indicates that the dorsal raphe nucleus may be unique among raphe nuclei in its sensitivity to neurotoxic amphetamines; for example, in the trigeminal motor nucleus, dorsal raphe axons are destroyed by PCA while 5-HT projections from raphe nuclei in the caudal brainstem are spared (15). On the basis of these considerations, we propose that dorsal versus median raphe projections exhibit differential vulnerability to neurotoxic amphetamines in all brain areas that receive projections from both nuclei. To verify the validity of this proposition, retrograde transport studies are needed from other regions of the brain after neurotoxic lesions.

While the main finding of this study is the differential susceptibility to PCA among mesencephalic raphe nuclei, the data also provide information concerning the origin of different serotonergic axon types. Multiple anatomic types of 5-HT axons have been reported by several investigators (10, 11, 36, 37, 40, 45, 49). The structural heterogeneity of 5-HT axons has prompted speculation that different axon types may originate from particular raphe nuclei (10). This premise is supported by recent evidence that the dorsal and median raphe nuclei give rise to morphologically distinct axon terminals. Data derived from anterograde axonal transport of the lectin PHA-L indicate that fine axon terminals arise from dorsal raphe neurons, whereas axons from the median raphe have large, spherical varicosities (39). Those results, which were based on a small sample of neurons located in restricted parts of the raphe nuclei, are confirmed by the present findings obtained from retrograde axonal transport, a method that detects nearly all neurons which project to a single area of cortex. The experimental strategy of the present study employs neurotoxic lesions that eliminate one axon type combined with retrograde transport, allowing the origin of surviving axons to be determined directly. The concomitant loss of fine 5-HT axons and of dorsal raphe projections to cortex shown in the present study confirms the previous anterograde transport data that fine 5-HT axon terminals arise from the dorsal raphe nucleus. The complementary result that beaded 5-HT axons and median raphe projections remain intact after PCA provides verification that beaded axons originate in the median raphe nucleus. Further support for the idea that fine 5-HT axons originate in the dorsal raphe nucleus comes from comparing animals with varying degrees of PCA-induced denervation. In two sets of PCA-treated rats, the degree of fine axon loss in cortex roughly parallels the reduction in the number of retrogradely labeled DR neurons, with no change in the median raphe nucleus. Moreover, in some animals, the nearly total loss of retrograde labeling in the dorsal raphe, despite sparing of beaded 5-HT axons, provides strong evidence that these axons arise almost exclusively from the median raphe and not from cells in the dorsal raphe nucleus.

Previous studies showing differential projections of the dorsal and median raphe nuclei to separate target areas led to the proposal that serotonergic cell groups have considerable specificity in their organization. The

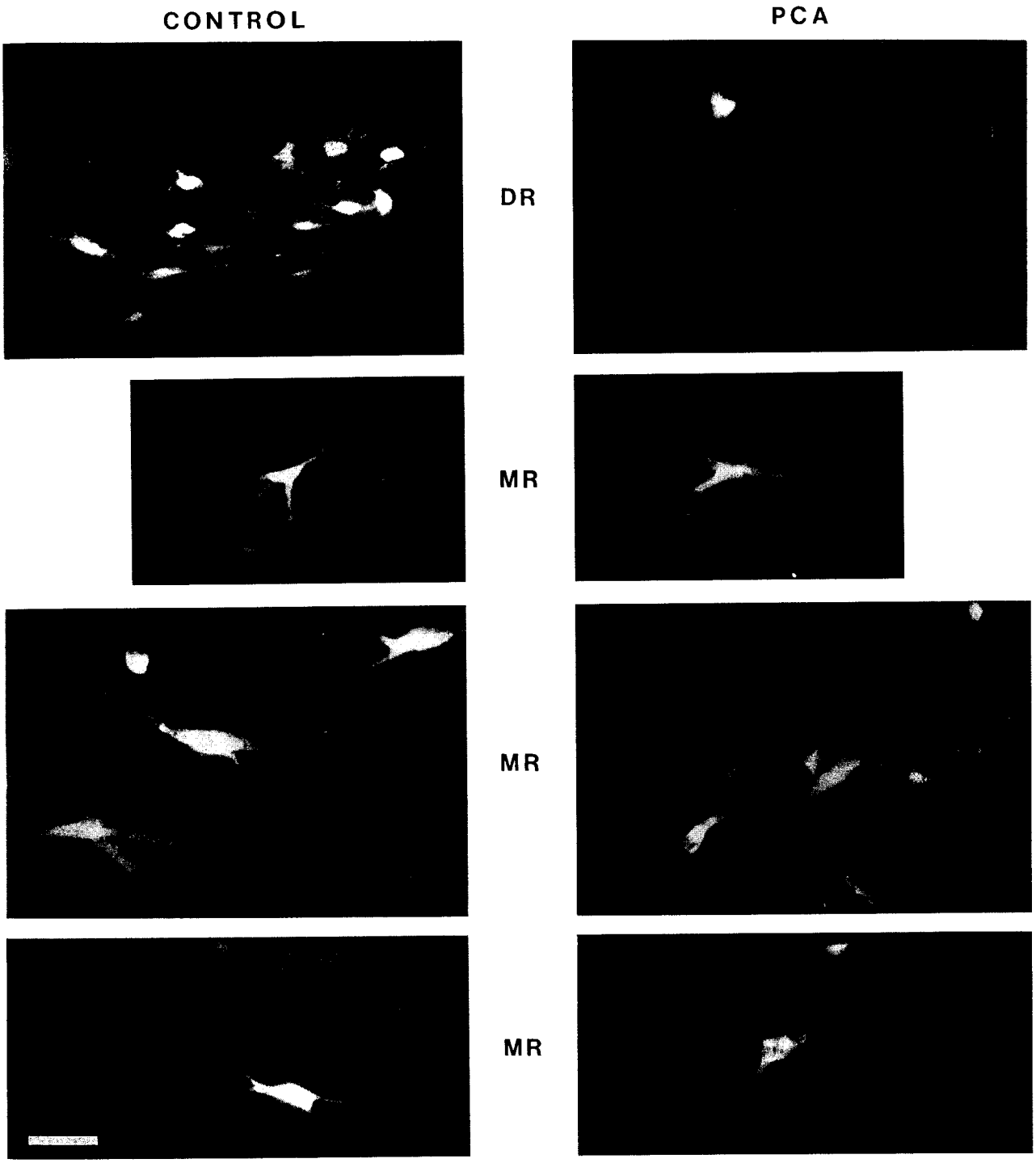

FIG. 8. Fluorescence photomicrographs of FG-labeled neurons in the dorsal raphe (DR) or median raphe (MR) nuclei. In the left-hand column are retrogradely labeled neurons from control rats; in the right-hand column are labeled neurons from PCA-treated rats. In the dorsal raphe (upper plates), clusters of closely adjacent labeled cell bodies are always found in control rats; in PCA-treated rats, FG-labeled cells are infrequent in the DR and are typically seen in isolation. Scale bar = 50 μm.

present evidence for differential vulnerability of DR and MR neurons to psychotropic amphetamines supports that view and further indicates that these two serotonergic nuclei are pharmacologically dissimilar. We therefore postulate that the dorsal and median raphe nuclei form two independent neuronal systems that differ in anatomic and functional properties and have parallel, overlapping projections to forebrain. In addition to their

separate origins in the midbrain, these two 5-HT neuronal projections differ in (i) axon morphology, (ii) regional distributions in forebrain, and (iii) pharmacological properties. Moreover, there is recent evidence that 5-HT_2 receptors, most likely located postsynaptically, may be preferentially associated with fine 5-HT axons that arise from the dorsal raphe nucleus (8). The differences between these two neuronal systems have functional implications with regard to (a) serotonin uptake mechanisms and (b) the roles of these neurons in cortical function and behavior. While the mechanism for toxicity of psychotropic amphetamine derivatives has not been elucidated, administration of serotonin uptake inhibitors provides protection of serotonin axons against the neurotoxic effects (6, 17, 19, 60). Since neurotoxicity of these compounds depends on binding to the serotonin uptake carrier, our results lead to the suggestion that there may be different 5-HT uptake receptors associated with the two axon types. Although the functional consequences of the two raphe projections remain to be characterized, there is evidence that dorsal and median raphe neurons may differentially affect a number of behavioral or functional measures including activity levels (24, 33, 63), aggression (31, 56, 70), and hormone secretion (68, 69). In humans, the psychotropic amphetamines MDA and MDMA are widely used for their mood-altering effects (26, 27, 35, 62). The selective vulnerability of DR axons to mood-elevating drugs leads us to speculate that serotonin—released from dorsal raphe axons and acting postsynaptically at 5-HT_2 receptors (8, 25, 28, 64)—may play a role in the control of affective state.

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