DARPP-32, A DOPAMINE- AND ADENOSINE 3':5'-MONOPHOSPHATE-REGULATED PHOSPHOPROTEIN ENRICHED IN DOPAMINE-INNervATED BRAIN REGIONS

III. Immunocytochemical Localization¹

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Abstract

Immunocytochemical studies have been carried out to determine the regional and cellular distribution of DARPP-32, a protein the phosphorylation of which can be regulated by dopamine and cAMP in intact cells. These immunocytochemical studies indicate that DARPP-32 is localized primarily in those brain regions enriched in dopaminergic nerve terminals. Moreover, the staining pattern supports the conclusion that the DARPP-32 is present in dopaminoceptive neurons, i.e., neurons that receive a dopamine input, and that it is absent from the dopaminergic neurons themselves.

Within the caudatoputamen, nucleus accumbens, olfactory tubercle, bed nucleus of the stria terminalis, and portions of the amygdaloid complex, all of which receive a strong dopamine input, DARPP-32 immunoreactivity is present in neuronal cell bodies and dendrites. In brain regions that are known to receive projections from these nuclei, puncta (presumed nerve terminals) are strongly immunoreactive for DARPP-32 but indigenous cell bodies and dendrites are not immunoreactive. These target areas include the globus pallidus, ventral pallidum, entopeduncular nucleus, and the pars reticulata of the substantia nigra. No immunoreactivity is detected in neuronal cell bodies or dendrites in any of the dopaminergic nuclei. Furthermore, nerve terminals immunoreactive for DARPP-32 do not resemble dopaminergic varicosities in either their morphology or their pattern of distribution.

Many neurons are weakly immunoreactive for DARPP-32 and some of these are found in areas that apparently lack a dopaminergic input: weakly labeled neuronal cell bodies and dendrites were found throughout the neocortex, primarily in layer VI, and in the Purkinje neurons of the cerebellum. DARPP-32 immunoreactivity is also present in certain glial cells, especially in the median eminence, arcuate nucleus, and medial habenula.

The present immunocytochemical studies, taken together with biochemical studies (Hemmings, H. C., Jr., A. C. Nairn, D. W. Aswad, and P. Greengard (1984) *J. Neurosci.* 4: 99-110; Walaas, S. I., and P. Greengard (1984) *J. Neurosci.* 4: 84-98) on DARPP-32, indicate that DARPP-32 is present in the subclass of dopaminoceptive neurons containing D-1 receptors (dopamine receptors coupled to adenylate cyclase). DARPP-32 may be an effective marker for certain of the actions of dopamine that are mediated through cAMP and its associated protein kinase.

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Biochemical studies of DARPP-32 (a dopamine- and cAMP-regulated phosphoprotein with an apparent M_r of 32,000) were reported in the two preceding papers (Walaas and Greengard, 1984; Hemmings et al., 1984). Those studies indicated that DARPP-32 is highly concentrated in those brain regions rich in dopaminergic nerve terminals. Moreover, the results indicated that DARPP-32 was present specifically in that subclass of dopaminergic cells containing D-1 receptors (receptors coupled to adenylate cyclase). Part of the evidence in support of this interpretation was the observation that the phosphorylation of DARPP-32 can be regulated in intact cells by dopamine and by cAMP. In this paper, we report the results of immunocytochemical studies on the regional and cellular localization of DARPP-32.

Materials and Methods

Production of monoclonal antibodies. Monoclonal antibodies to DARPP-32 were produced essentially by the method of Köhler and Milstein (1975) as described by Hemmings and Greengard (manuscript in preparation). Briefly, spleen cells from BALB/c mice, which had been immunized with DARPP-32 purified by the method of Hemmings et al. (1984), were fused with SP2/0 Ag14 myeloma cells using polyethylene glycol. Hybridomas secreting antibodies reactive with DARPP-32 were selected using a co-precipitation antigen-binding assay by screening culture supernatants for their ability to precipitate ^{125}I -labeled DARPP-32 with protein A-bearing *Staphylococcus aureus* cells as an immunoadsorbent. Positive cultures were cloned by limiting dilution and were injected intraperitoneally into BALB/c mice primed with pristane (2,6,10,14-tetramethylpentadecane) for the production of ascites fluid. Ascites fluid was collected and the antibodies were partially purified by precipitation with 45% ammonium sulfate. The ammonium sulfate precipitate was collected by centrifugation, dissolved in one-tenth the original volume of 150 mM NaCl, 10 mM potassium phosphate, pH 7.4 (PBS), dialyzed overnight against PBS, and stored in aliquots at -70°C . This procedure resulted in five stable anti-DARPP-32 antibody-producing hybridomas. Three of these (C24a-5a, C24a-6a, and C24a-4D7) produced the monoclonal antibodies used in this study.

Characterization of monoclonal antibodies. Crude cytosol was prepared from bovine caudate nucleus and rat caudatoputamen and subjected to endogenous phosphorylation and SDS-polyacrylamide gel electrophoresis as described (Hemmings et al., 1984). Immunoprecipitation of DARPP-32 from phosphorylated crude cytosol prepared from bovine caudate nucleus or rat caudatoputamen was performed using each of the three monoclonal antibodies and *S. aureus* cells as a solid phase immunoadsorbent (H. C. Hemmings, Jr., and P. Greengard, manuscript in preparation). Specificity of immunoprecipitation of DARPP-32 by antibody C24a-4D7 is illustrated in Figure 1A. Immunoprecipitation by antibodies C24a-5a and C24a-6a yielded similar results (data not shown).

For the immunoblotting studies, whole rat brain or dissected rat caudatoputamen was homogenized in 25 vol of 1% SDS using 10 up-and-down strokes in a Teflon-

Figure 1. A, Immunoprecipitation of DARPP-32 from endogenously phosphorylated cytosol. Crude cytosol was prepared from bovine caudate nucleus and subjected to endogenous phosphorylation (lane 2) followed by immunoprecipitation with 5 μl of antibody C24a-4D7 (lane 3). Similarly, crude cytosol prepared from rat caudatoputamen was phosphorylated (lane 4) and immunoprecipitated (lane 5). Lane 1 is standard bovine [^{32}P]phospho-DARPP-32. Phosphorylated proteins were separated on 10% SDS-polyacrylamide gels and analyzed by autoradiography. B, Antibody binding to DARPP-32 in crude brain extracts. Rat caudatoputamen (lane 2) or rat whole brain (lane 3) was homogenized in 1% SDS and 150 μg of protein subjected to electrophoresis on 10% SDS-polyacrylamide gels followed by electrophoretic transfer to a nitrocellulose sheet. The nitrocellulose sheet was labeled with a 1:500 dilution of each of the three antibodies (C24a-5a, C24a-6a, and C24a-4D7) followed by ^{125}I -protein A, and analyzed by autoradiography after exposure of the film for 7 days. Lane 1 is 5 pmol of standard purified bovine DARPP-32.

glass homogenizer. Aliquots containing 150 μg of protein were then separated on 10% SDS-polyacrylamide slab gels using the buffer system of Laemmli as described (Hemmings et al., 1984). The proteins were then electrophoretically transferred to nitrocellulose sheets (0.2 μm pore size, Schleicher & Schuell) at 200 mA for 8 hr using the buffer system of Towbin et al. (1979). Following the protein transfer, the nitrocellulose sheets were quenched by soaking for 12 hr in 5% bovine serum albumin in 150 mM NaCl, 50 mM Tris-HCl, 0.1% sodium azide, pH 7.4 (TBS), at room temperature. Following quenching, the sheets were incubated in a solution of antibodies C24a-5a, C24a-6a, and C24a-4D7, each diluted 1:500 into TBS containing 3% bovine serum albumin, for 2 to 6 hr at room temperature. The antibody solution was removed and the sheets were washed in five changes of TBS over 1 hr. Radiolabeling of the bound antibodies was performed by incubating the washed sheets with ^{125}I -labeled protein A (5×10^5 cpm/ml; New England Nuclear) in TBS containing 3% bovine serum albumin for 2 hr at room temperature. The protein A solution was removed and the sheets were washed in five changes of TBS over 1 hr. The sheets were blotted dry and analyzed by auto-

radiography at -70°C using Kodak X-Omat AR X-ray film and a DuPont Lightning Plus intensifying screen. Specificity of the binding of DARPP-32 antibodies to DARPP-32 in crude brain extracts is illustrated in Figure 1B.

Immunocytochemical procedures. Male Sprague-Dawley rats (150 to 250 gm) were anesthetized with chloral hydrate (500 mg/kg) and perfused transcardially with 200 ml of sodium phosphate buffer (0.12 M, pH 7.4, 4°C) followed by 300 to 400 ml of periodate/lysine/formaldehyde fixative at 4°C (McLean and Nakane, 1974). This solution contained 2% formaldehyde (freshly prepared from paraformaldehyde), 0.075 M lysine, and 0.01 M sodium periodate in sodium phosphate buffer (final pH 6.9). Brains were then removed, cut into slabs 2 mm thick, and placed in the fixative (4°C) overnight. On the following morning, the brain slabs were passed through a series of sucrose solutions (12 to 20% in 0.12 M sodium phosphate buffer, pH 7.4) and left in the 20% solution for at least 48 hr. Sucrose-impregnated brain slabs were covered with OCT embedding medium (Tissue Tek) and frozen onto brass specimen chucks by immersion in isopentane that had been chilled to viscosity in liquid nitrogen. Cryostat sections 10 μm thick were then cut and mounted on gelatin-coated microscope slides.

The mounted sections were immunostained with primary antibody (1:500 dilution) overnight at room temperature and subsequently were washed with modified phosphate-buffered saline (modified PBS; 150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4) for 1 hr. The sections were then incubated for 1 hr at room temperature with fluorescein isothiocyanate (FITC)-labeled secondary antibody (N. L. Cappel Laboratories) diluted 1:50. Goat anti-rabbit IgG was used to label rabbit serum antibodies and goat anti-mouse IgG was used to label mouse monoclonal antibodies. The sections were again washed with PBS for 1 hr, coverslipped with glycerol phosphate (95% glycerol, 5% 0.12 M phosphate buffer, pH 7.4), and examined with a Zeiss Photomicroscope III equipped with an epifluorescence system.

All antibodies were diluted in 10% normal goat serum in modified PBS or in 0.12 M sodium phosphate buffer containing 0.45 M NaCl (pH 7.4). Triton X-100 (0.3%) could not be used to enhance tissue permeability to immunohistochemical reagents because it reduced staining intensity and made fine processes difficult to resolve by causing the neuropil in immunoreactive areas to fluoresce diffusely. In some experiments, mounted sections were preincubated with normal goat serum prior to incubation with primary antibody, but this did not change the immunostaining pattern. Therefore, this preincubation step was omitted from the standard procedure.

Five antisera were useful in the staining protocol described above. Three of these antisera were the monoclonal antibodies discussed above and two were polyclonal antibodies raised in rabbits. All five antisera gave the same staining pattern, but background fluorescence was lower with the monoclonal antibodies. For this paper, a mixture of the three monoclonal antibodies was used because the mixed monoclonal antibodies gave the brightest signal and lowest background. With the exception of the altered signal-to-noise ratio, the staining

produced when each monoclonal antibody was used separately did not differ from that produced by the mixture.

The following alterations were used to assess specificity of the staining protocol. When either (a) modified PBS, (b) "undirected" monoclonal IgG solutions (diluted 1:20), (c) antibodies against DARPP-32 preabsorbed batchwise with purified DARPP-32, or (d) flowthrough from DARPP-32 affinity columns was used in place of anti-DARPP-32 antibodies in the staining protocol, no staining was observed. Affinity purification of the partially purified antibodies did not alter the staining pattern. Antibodies directed against antigens other than DARPP-32 gave different staining patterns. Omission of FITC-labeled secondary antibodies provided a measure of autofluorescence. Data were taken from 45 rat brains.

Results

Immunostaining for DARPP-32 was region specific. Some brain regions showed virtually no labeling, in other regions labeled cell bodies and processes were observed, and in still other regions the label was confined to axons and/or fine puncta only. Furthermore, neurons and/or neuronal processes were brightly fluorescent in some brain regions but weakly fluorescent in others. Areas characterized by bright immunofluorescence and by weak immunofluorescence are discussed separately below. In Figures 3, A and B, and 6, brightly and weakly immunofluorescent neurons are juxtaposed in the same photographic field and demonstrate the relative difference between bright and weak staining intensity. Strongly immunofluorescent areas are diagrammed on the schematic drawings of coronal sections through the rat brain in Figure 2.

Telencephalon

Bright immunofluorescence. Intensely labeled neurons were observed in the caudatoputamen, olfactory tubercle, anterior olfactory nucleus, nucleus accumbens, bed nucleus of the stria terminalis, and portions of the amygdaloid complex. These areas (see Fig. 2) contained many brightly fluorescent neuronal cell bodies and dendrites. Fine puncta representing nerve terminals may also have been present in these regions but were difficult to identify unequivocally because of the high density of heavily labeled somata and dendrites.

Labeled neurons were more or less uniformly distributed throughout the caudatoputamen but nests of labeled neurons could also be found (Fig. 3, A and C). The staining intensity of individual neurons and of the neuropil was greatest in the dorsomedial quadrant of this nucleus. When the caudatoputamen was viewed in horizontal or parasagittal sections, a subtle rostrocaudal gradient of staining intensity in neurons and neuropil was also apparent. The staining was brightest rostrally and weakest caudally. Crude estimates of labeled versus unlabeled neurons in 10- μm sections (counterstained with 0.00002% ethidium bromide in modified PBS (Schmued et al., 1982) to reveal all neurons) showed that minimally 60% of the caudatoputamen neurons are immunoreactive. The remaining 40% were clearly unlabeled. Most of the fiber bundles of the internal capsule contained one or two labeled axons.

Figure 2. Diagrams of coronal sections through the rat brain at the following levels: A, forceps minor; B, rostral caudatoputamen; C, septum; D, globus pallidus; E, arcuate nucleus; F, substantia nigra. Only the strongly immunoreactive cells and processes are represented. *Stippled areas*, labeled neuronal cell bodies and processes; Δ (E only), labeled glial cells; *densely packed lines*, high density of labeled axons and puncta; *less densely packed lines* (F only), low density of labeled axons and puncta. A, nucleus accumbens; AC, anterior commissure; ACe, central amygdaloid nucleus; ALP, lateral posterior amygdaloid nucleus; AR, arcuate nucleus; CC, corpus callosum; CP, caudatoputamen; GP, globus pallidus; HI, hippocampal formation; IC, internal capsule; ICa, islands of Calleja; IP, interpeduncular nucleus; LOT, lateral olfactory tract; LV, lateral ventricle; MG, medial geniculate nucleus; MH, medial habenula; OT, olfactory tubercle; PC, substantia nigra-pars compacta; PR, substantia nigra-pars reticulata; Rh, rhinal fissure; SC, superior colliculus; VP, ventral pallidum.

The staining patterns in the nucleus accumbens (Fig. 4) and in parts of the bed nucleus of the stria terminalis were identical to that in the caudatoputamen except that gradients in fluorescent intensity were not observed. Bridges of stained neurons and their processes connected the ventral caudatoputamen to the underlying olfactory tubercle (Fig. 5). In the tubercle itself, neurons in the polymorph and pyramidal layers were intensely fluorescent as were their dendrites (Figs. 4A and 6). The DARPP-32 staining pattern in the caudatoputamen, nucleus accumbens, olfactory tubercle, and bed nucleus of the stria terminalis made these nuclei appear confluent with each other at their borders (see, for example, Fig. 4, A and C).

Brightly fluorescent neurons and their processes were also observed in the central (Fig. 7), lateral posterior, and cortical amygdaloid nuclei and in the massa intercalata. Bright patches of fluorescent neurons were also

observed external to the lateral edge of the lateral posterior amygdaloid nucleus.

Brightly fluorescent fibers and puncta were observed in the globus pallidus, entopeduncular nucleus, and ventral pallidum, but neuronal cell bodies and dendrites in these regions were unstained. The difference between this staining pattern and that seen in the neostriatum is clearly illustrated in Figure 8, a photomicrograph of the border between the caudatoputamen and the globus pallidus. The DARPP-32 staining pattern clearly marked this border. Furthermore, the staining pattern sharply delineated a ventral pallidal region which extended rostrally, dividing into finger-like projections immediately dorsal to the islands of Calleja (for a cross-section of one such "finger," see Fig. 4B). An exception to this organization was the insula magna (the large medial island of Calleja), which was bordered dorsally and ventrally by brightly stained neuronal cell bodies and processes that were confluent with the olfactory tubercle (Fig. 4C).

Weak immunofluorescence. Weakly immunofluorescent neurons were found in neocortex, cingulate cortex, piriform cortex, entorhinal cortex, and the hippocampal formation. In neocortex, most of the fluorescent neurons were present in layer VI. The majority of the labeled neurons were pyramidal cells, and in general, fluorescence could be detected only in the cell body region and in the apical dendrites (Fig. 9A). Thus, if label was present in basal dendrites and small branches of the apical dendrites, it was too weak to detect. The apical dendrites from layer VI pyramidal cells formed fascicles (Fig. 9B) that could be traced toward the pial surface at least to layer IV (in 10- μ m sections). Pyramidal neurons, more weakly fluorescent than those in layer VI, were also present in much of layer III. Although the staining pattern for DARPP-32 in layers VI and III was typical for most of the neocortex, regional variations were observed. For example, in areas 4 and 41, no immunofluorescent neurons were present in layer VI.

Neurons in prefrontal (Fig. 9C), cingulate, and deep perirhinal cortex were more brightly labeled than other cortical neurons. In prefrontal and cingulate cortex, most of the labeled neurons were in layer VI, whereas in piriform and entorhinal cortex, weakly fluorescent neurons were present primarily in the superficial layers. Within the anterior regions of entorhinal cortex, the more brightly labeled neurons often occurred in clusters (Fig. 9D).

In the hippocampal formation, the granule cells of the dentate gyrus were weakly immunofluorescent. Weakly fluorescent puncta were present in the outer two-thirds of the molecular layer of the dentate gyrus and in regions CA3 and CA4.

Weakly fluorescent neurons were observed in the lateral septum and in the anterior hippocampal rudiment.

Diencephalon

Bright immunofluorescence. No brightly labeled neuronal cell bodies or terminal fields were observed in diencephalic structures. Many brightly fluorescent fibers, however, were present in the internal capsule (Fig. 10).

Weak immunofluorescence. In the epithalamus, the neurons of the medial habenula were weakly labeled (Fig. 11) as were the fibers of the fasciculus retroflexus.

Figure 3. Photomicrograph of coronal sections through the caudatoputamen. **A**, Photomicrograph of the caudatoputamen (CP), corpus callosum (CC), and prefrontal cortex (PFC). Dorsal is to the right and medial is up. Note the cluster of labeled neurons (arrow) in the caudatoputamen, fluorescent astrocytes in the corpus callosum, and weakly labeled neurons in the deep layers of the prefrontal cortex. **B**, Comparison of staining intensity in the caudatoputamen and in layer VI (VI) of the parietal cortex. Dorsal is to the left and lateral is up. **C**, Two clusters of labeled neurons in the caudatoputamen are enclosed by dashed lines. **D**, High power photomicrograph of labeled caudatoputamen neurons. Arrows point out unlabeled cells. IC, a fiber fascicle of the internal capsule. Calibration bars = 100 μ m.

Figure 4. Photomicrographs of coronal sections through the islands of Calleja. **A**, A low power photomicrograph of the olfactory tubercle (OT), an island of Calleja (asterisk), and the rostral extension of the ventral pallidal area (arrow). Dorsal is up and medial is to the left. NAcc, ventral portion of the nucleus accumbens. **B**, A higher power photomicrograph showing the relationship between a rostral extension of the ventral pallidal

Figure 5. Photomicrograph of a coronal section through a bridge (Br) connecting the ventrolateral edge of the caudato-putamen (CP) to the olfactory tubercle (not included in the photographic field). Dorsal is up and lateral is to the right. Calibration bar = 100 μ m.

Weakly labeled fibers were present in the external medullary lamina, and fine weakly fluorescent puncta were present in the neuropil of most of the dorsal thalamic nuclei. No fluorescent puncta, however, were observed in the ventral lateral geniculate nucleus, nucleus reticularis, nucleus periventricularis, or zona incerta. In the hypothalamus, weakly fluorescent neurons were observed in the ventromedial and suprachiasmatic nuclei.

Mesencephalon

Bright immunofluorescence. Labeled fibers were present in the crus cerebri at levels rostral but not caudal to the substantia nigra. The pars reticulata of the substantia nigra contained a dense population of fine labeled puncta and fibers (Fig. 12). In contrast, the pars compacta and the pars compacta pars lateralis contained fewer labeled puncta and fibers. A very small number of brightly fluorescent fibers ramified in the ventral tegmental area of Tsai. Labeled fibers in the region of the pars compacta extended in a dorsocaudal direction to the caudal border of the mesencephalon. Neuronal cell bodies were not labeled in any regions of the substantia nigra or ventral tegmentum.

Weak immunofluorescence. Many neurons in the periaqueductal grey matter were weakly fluorescent. Weakly fluorescent puncta were present in the interpeduncular nucleus.

area (arrow) and an island of Calleja (asterisk). In the ventral pallidal region, neurons are unstained and many fine fluorescent puncta are present. Labeled elements were not observed within the islands of Calleja. **C**, A low power photomicrograph of the ventral septal area. Dorsal is up and the arrow marks the midline. Extensions of the olfactory tubercle surround the insula magna (asterisk) on each side. NAcc, nucleus accumbens. Calibration bar = 100 μ m.

Metencephalon

Bright immunofluorescence. No brightly immunofluorescent neurons were observed in the pons or cerebellum.

Weak immunofluorescence. Cerebellar Purkinje cell bodies, dendrites, and axons were weakly immunofluorescent as were the granule cell bodies (Fig. 13). Labeled axons were present in the cerebellar white matter and weakly fluorescent puncta were observed in the deep cerebellar nuclei.

Myelencephalon and spinal cord

Bright immunofluorescence. No brightly fluorescent neurons or neuronal processes were found in the medulla or spinal cord.

Weak immunofluorescence. Weakly stained puncta and fibers were observed in the vestibular nuclei, especially in the lateral nucleus where the cell bodies were surrounded by immunoreactive puncta.

Figure 6. Photomicrograph of a coronal section through the olfactory tubercle (OT), piriform cortex (PC), ventral pallidum (VP), and lateral olfactory tract (LOT). Dorsal is up and lateral is to the right. An unlabeled neuron (black arrow) in the ventral pallidal area stands out against a background of very fine fluorescent puncta. The relative difference between the terms "bright immunofluorescence" and "weak immunofluorescence" as used in this paper is illustrated by comparing the brightly immunofluorescent neurons in the olfactory tubercle with the weakly immunofluorescent neurons in the piriform cortex (white arrows). Calibration bar = 100 μ m.

Figure 7. Photomicrograph of a coronal section through the central amygdaloid nucleus. Some neurons are brightly labeled and others are not stained or are only weakly stained. Dendrites, such as the one illustrated by the arrow, could often be followed for some distance from the neuronal cell bodies. Calibration bar = 100 μ m.

Non-neuronal immunoreactivity

Many glial cells were immunoreactive. Some astrocytes were weakly immunoreactive in the white matter throughout the central nervous system (see for example, Fig. 3A). Astrocytes were very brightly fluorescent in the medial habenula, where many of them were in intimate apposition with the more weakly labeled habenular neurons (Fig. 11). Tanycytes were strongly immunoreactive in the arcuate nucleus and median eminence and their labeled processes often formed rings around blood vessels (Fig. 14). In the spinal cord, radial glial cells were brightly labeled. No dilution of the primary antibody was found at which the glial staining disappeared but neuronal staining remained.

Discussion

In the first paper of this series (Walaas and Greengard, 1984), evidence was presented suggesting a close association between DARPP-32 and dopaminergic pathways in the brain. The concentration of DARPP-32 in various regions of the brain correlated well with the density of dopaminergic innervation in those regions. Studies of the cellular localization of DARPP-32 indicated that it was present in dopaminoceptive rather than in dopaminergic neurons. Moreover, DARPP-32 appeared to be present in that subpopulation of dopaminoceptive neurons that contains the D-1 class of dopamine receptor (dopamine receptors coupled to adenylate cyclase). No evidence was obtained for the presence of DARPP-32 in the subclass of dopaminoceptive neurons containing D-2 receptors. The immunocytochemical studies reported in this paper are fully compatible with the data obtained in the biochemical studies of DARPP-32 and their interpretations (Walaas and Greengard, 1984).

In the present immunocytochemical study, prominent immunoreactivity for DARPP-32 was demonstrated in most of the brain regions that are heavily innervated by dopamine fibers: the caudatoputamen, nucleus accumbens, olfactory tubercle, bed nucleus of the stria terminalis, massa intercalata, and the cortical, lateral posterior, and central amygdaloid nuclei. In these nuclei, where dopamine is present in nerve terminals (for review see Lindvall and Björklund, 1978), immunoreactivity for DARPP-32 was seen in neuronal cell bodies and dendrites.

Somata, dendrites, axons, and presumed terminals of labeled neurons were all immunoreactive for DARPP-32. The fluorescent material appeared evenly distributed throughout the cytoplasm, suggesting that DARPP-32 immunoreactivity is not primarily associated with discrete organelles such as mitochondria, microtubules, synaptic vesicles, or plasma membranes. This conclusion is consistent with the results of subcellular fractionation studies in which DARPP-32 was found to be present in the soluble rather than the particulate fraction (Walaas et al., 1983; Walaas and Greengard, 1984).

In many brain regions, immunoreactivity was found exclusively in nerve terminals and axons. These areas included the globus pallidus, entopeduncular nucleus, ventral pallidal area, substantia nigra, interpeduncular nucleus, ventral tegmental area, dorsal thalamus, hip-

pocampal CA3 and CA4 regions, deep cerebellar nuclei, and the vestibular nuclei. Our interpretation of this pattern of immunoreactivity is that it represents fields of labeled nerve terminals that arise from distant groups of DARPP-32-containing cell bodies, an interpretation consistent with known anatomical relationships. Thus, the caudatoputamen projects to the globus pallidus (Voneida, 1960; Szabo, 1962, 1970; Cowan and Powell, 1966; Nagy et al., 1978; Tulloch et al., 1978; Graybiel et al., 1979), to the entopeduncular nucleus (Adinolfi, 1969; Nagy et al., 1978), and to the substantia nigra (Voneida, 1960; Niimi et al., 1970; Szabo, 1970; Grofova, 1975; Bunney and Aghajanian, 1976; Nagy et al., 1978; Tulloch et al., 1978; Walaas and Greengard, 1984), and DARPP-32-containing neurons of the caudatoputamen are probably the source of DARPP-32-containing nerve terminals in those nuclei. Similarly, the nucleus accumbens projects to the ventral tegmental area and to a dorsomedial portion of the substantia nigra (Swanson and Cowan, 1975; Conrad and Pfaff, 1976; Nauta et al., 1978; Phillipson, 1978, 1979), and DARPP-32-containing neurons of the nucleus accumbens are a probable source of labeled terminals in those nuclei.

By analogy with our interpretation of the relationships between strongly immunoreactive cell bodies and terminals, groups of lightly labeled neuronal cell bodies probably give rise to distant fields of lightly labeled nerve

Figure 8. Photomicrograph of a coronal section through the border between the caudatoputamen (CP) and the globus pallidus (GP). In the caudatoputamen, neuronal cell bodies and dendrites are labeled. In contrast, in the globus pallidus, neuronal cell bodies and dendrites are unlabeled but are surrounded by labeled puncta. Long arrows, cell bodies; short arrows, dendrites. Calibration bar = 100 μ m.

Figure 9. Photomicrographs of sections through cortical areas. For A and C of this figure, photographic parameters were held constant to illustrate the greater fluorescence intensity of the prefrontal cortex than of the parietal cortex. (The apparently greater brightness of neurons in the prefrontal cortex in part C of this figure as compared to that seen in Fig. 3A is attributable to differences in photographic conditions including magnification factor.) **A**, Photomicrograph of a coronal section through layers V (V) and VI (VI) of parietal cortex. Many neurons in layer VI, but only a few in layer V, are labeled. Layer VI neurons give rise to fascicles of labeled dendrites (arrows) that can be traced through layer IV in sections 10 μm thick. The pial surface is up. Calibration bar = 100 μm . **B**, Photomicrograph of a section taken through layer VI of parietal cortex tangential to the white matter. Weakly stained neurons (black arrows) and dendritic fascicles (white arrows) from more deeply situated neurons can be seen. **C**, Photomicrograph of a coronal section through the deep layers of prefrontal cortex. Medial is up. These neurons and those in the cingulate cortex are more brightly fluorescent than those in any other cortical region. **D**, Photomicrograph of a coronal section through the anterior entorhinal cortex. Dorsal is up and medial is on the left. Weakly labeled neurons are present in the superficial layers of entorhinal cortex and clusters of more brightly immunofluorescent neurons (enclosed by dashed lines) are observed in anterior regions of entorhinal cortex. Calibration bars = 100 μm .

Figure 10. Photomicrograph of a cross-section through the internal capsule. Fascicles of unlabeled fibers (white arrows) are present but most of the fascicles contain at least some labeled fibers. Some unlabeled neurons of the entopeduncular nucleus (black arrow) surrounded by fluorescent puncta are also present. Calibration bar = 100 μ m.

Figure 11. Photomicrograph of a coronal section through the medial habenula showing weakly fluorescent neurons (small white arrows) and brightly fluorescent astrocytes (large black arrow). Note the intimate spatial association between some of the astrocytes and neurons (large white arrow). Ependymal cells in this region were also immunofluorescent. Calibration bar = 100 μ m.

Figure 12. Photomicrograph of a coronal section through the substantia nigra. Dorsal is up and medial is to the left. Fine puncta cover the pars reticulata (PR). A much smaller population of puncta (asterisk) is also present dorsal to the lateral aspect of the pars reticulata. At this level, immunoreactive axons are seen only rarely in the crus cerebri (CC). Calibration bar = 100 μ m.

Figure 13. Photomicrograph of a parasagittal section through the cerebellum. Purkinje neurons and their dendrites are immunoreactive. In the granule cell layer (G), very faint immunofluorescence is associated with the thin layer of granule cell cytoplasm (white arrows) surrounding the nucleus. M, molecular layer. Calibration bar = 100 μ m.

terminals. For instance, layer VI of neocortex projects to the dorsal thalamus (see Jones, 1981, for review), and the immunoreactive layer VI neurons may be the source of the weakly labeled axons in the thalamic external medullary lamina and of the weakly labeled terminals in the dorsal thalamus. Similarly, the weakly labeled neu-

rons in the medial habenula may give rise to the weakly labeled axons in the fasciculus retroflexus and terminals in the interpeduncular nucleus. Granule cells in the dentate gyrus send mossy fibers to the CA3 and CA4 regions of the hippocampus (Blackstad et al., 1970) and are probably the source of the weakly fluorescent terminals in that area. Layers II and III of the entorhinal cortex project to the outer two-thirds of the molecular layer of the dentate gyrus and to the outer molecular layer of the hippocampus (Blackstad, 1958) and are probably the source of labeled terminals in those regions. Likewise, the cerebellar Purkinje cells are probably the source of labeled nerve terminals in the deep cerebellar nuclei and vestibular nuclei.

Nature of the neurotransmitter in DARPP-32 neurons. It will be of interest to determine the nature of the neurotransmitter(s) present in DARPP-32-containing neurons. A major part of the projections from the caudatoputamen and nucleus accumbens to the target areas described above is GABAergic (Hattori et al., 1973; Brownstein et al., 1977; Fonnum et al., 1974, 1978; Grofova, 1975; Jessel et al., 1978; Nagy et al., 1978; Walaas and Fonnum, 1979, 1980). This raises the possibility that GABAergic neurons in the caudatoputamen and in the nucleus accumbens contain DARPP-32. However, GABAergic neurons, as revealed by immunocytochemical labeling, are much more widely distributed throughout the brain than neurons that are immunoreactive for DARPP-32 (see, for example, McLaughlin et al., 1974, 1975; Saito et al., 1974; Barber and Saito, 1976; Ribak et al., 1976, 1977; Wood et al., 1976). Many striatonigral fibers are believed to contain substance P (Hong et al., 1977; Jessel et al., 1978), and striatopallidal fibers (Cuello

and Paxinos, 1978) and mossy fibers of the hippocampal formation (Gall et al., 1981) are immunoreactive for enkephalin. Thus it is also possible that DARPP-32 is present in neurons that contain substance P and/or enkephalin. As is the case for GABA, however, the general distribution of neurons and neuronal processes immunoreactive for substance P and for enkephalin is not identical to that of DARPP-32 neurons and their processes.

Are all DARPP-32 neurons dopaminergic? The distribution of neuronal cell bodies and dendrites weakly immunoreactive for DARPP-32 was wider than that of known dopaminergic terminals. Neurons in layers III and VI were weakly immunoreactive throughout most of the cerebral cortex. It is generally believed that the dopamine input to the cerebral cortex is restricted to the prefrontal, cingulate, and perirhinal regions. (It is interesting that DARPP-32 immunoreactivity was stronger in these three regions than elsewhere in the cortex.) Also, cerebellar Purkinje cells were immunoreactive, but a dopaminergic projection to the cerebellum has not been demonstrated.

There are several possible explanations for these apparent discrepancies. The simplest is that the distribution of dopamine receptors may be wider than that of dopaminergic terminals. For example, there are neurons in the parietal neocortex that respond to the iontophoretic application of dopamine (for review, see Bunney, 1979). In addition, dopamine-sensitive adenylate cyclase has been found in cat cerebellum, indicating the presence of D-1 receptors there (Dolphin et al., 1979). It is also possible that, in some brain regions, a minor dopaminergic input has escaped detection. Moreover, it is conceivable that DARPP-32 can act as an effector for neurotransmitter agents, other than dopamine, that increase cAMP levels. For instance, in the Purkinje cells, where DARPP-32 immunoreactivity is weak and where a dopaminergic input has not been demonstrated, cAMP levels can be increased by norepinephrine (see, for example, Kakiuchi and Rall, 1968).

Do all dopaminergic neurons contain DARPP-32? A variety of evidence suggests that DARPP-32 is present in dopaminergic cells containing D-1 receptors, and that it is absent from dopaminergic cells containing D-2 receptors. Part of this evidence is discussed in a preceding paper (Walaas and Greengard, 1984). In addition, most of the brain regions found in the present study to be intensely immunofluorescent for DARPP-32 have been shown to contain D-1 receptors. Thus, D-1 receptors have been reported in the olfactory tubercle, nucleus accumbens, caudatoputamen, amygdala, and pars reticulata of the substantia nigra (Kebabian et al., 1972; Clement-Cormier et al., 1974; Horn et al., 1974; Mishra et al., 1974; Kebabian and Saavedra, 1976; Philipson and Horn, 1976; Premont et al., 1976; Seeber and Kuschinsky, 1976; Spano et al., 1976; Clement-Cormier and Robison, 1977; Gale et al., 1977; Kreiger et al., 1977). Conversely, DARPP-32 immunoreactivity was absent from the nigrostriatal neurons, which have autoreceptors but no detectable D-1 receptors (Kebabian and Saavedra, 1976). Further work will be required to establish whether DARPP-32 is present in all neurons containing D-1

Figure 14. Low power photomicrograph of a coronal section through the arcuate nucleus and median eminence. Labeled tanyocyte processes often surround capillaries (arrows). Calibration bar = 100 μ m.

receptors and whether it is absent from all neurons containing D-2 receptors.

DARPP-32 in non-neuronal cells. DARPP-32 immunoreactivity was present in certain glial cells. It is interesting that some glial cells contain dopamine-sensitive adenylate cyclase (Schubert et al., 1976; Henn et al., 1977), although the function of dopamine and DARPP-32 in these cells has not been established. The observation that DARPP-32-immunoreactive astrocytes were wrapped around habenular neurons (Fig. 11) is suggestive of a glial-neuronal interaction between these two cell types. The relationship of labeled tanycytes to blood vessels in the arcuate nucleus and median eminence suggests that it would be worthwhile to study the possible relationship of dopamine, DARPP-32, and regulation of the blood-brain barrier in this region.

Is all "DARPP-32 immunoreactivity" attributable to DARPP-32? There are several reasons for believing that the immunocytochemical staining observed in the present study is attributable to DARPP-32. The results of immunoprecipitation experiments (Fig. 1A) and of transfer immunoblot experiments (Fig. 1B) indicate a high degree of specificity of the antibodies used in the present study. A number of other control experiments, outlined under "Materials and Methods," also support the specificity of the staining procedure used. In addition, the distribution of DARPP-32 as determined qualitatively by immunocytochemistry in the present study correlates well with the distribution observed in biochemical studies (Walaas and Greengard, 1984). For instance, the Purkinje cells in the cerebellum show weak staining for DARPP-32 (see Fig. 13), and biochemical studies (Walaas and Greengard, 1984) indicate the presence of a low concentration of DARPP-32 in the cerebellum. Although DARPP-32 has been shown to share several properties in common with G-substrate and phosphatase inhibitor-1 (Hemmings et al., 1984), we have found only minimal ($<10^{-3}$) immunological cross-reactivity of these two proteins with antibodies against DARPP-32 (H. C. Hemmings, Jr., and P. Greengard, unpublished observations). Therefore, it seems unlikely that any significant amount of the immunocytochemical staining seen in the present study is attributable to cross-reactivity of the DARPP-32 antibodies with antigens other than DARPP-32 itself.

Concluding remarks. We anticipate that the immunocytochemical study of neurons postsynaptic to identified neurotransmitter agents may prove generally useful in the identification of neurotransmitter-related neuronal networks. Thus, it may be possible to identify neurons responding to neurotransmitter agents partly on the basis of their specific phosphoproteins rather than solely on the basis of synaptic connections. This can be illustrated in the case of DARPP-32, which appears to be useful for the identification of dopaminoceptive cells containing the D-1 class of dopamine receptor. The use of DARPP-32 as a marker should be valuable in the identification of both the afferent and efferent neurons with which DARPP-32 neurons interact.

In the case of monoaminergic neurotransmitter systems specifically, the neurotransmitters can diffuse a considerable distance through the brain (Reader et al., 1979). Neurons possessing the molecular machinery to

respond to those monoamines are "physiologically" postsynaptic, even in the absence of direct synaptic contact. In the case of the D-1 subclass of dopaminoceptive neurons, it should now be possible, through the use of DARPP-32 antibodies, to identify these "postsynaptic" cells. A detailed study of such dopaminoceptive cells may show an anatomical organization that cannot presently be inferred from the apparently diffuse arrangement of the monoaminergic fibers themselves.

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