

# DARPP-32, A Dopamine- and Adenosine 3':5'-Monophosphate-Regulated Phosphoprotein: Regional, Tissue, and Phylogenetic Distribution

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Rabbit antisera and mouse monoclonal antibodies have been prepared to bovine DARPP-32 (dopamine- and adenosine 3':5'-monophosphate-regulated phosphoprotein,  $M_r = 32,000$ ), and used to study its regional, tissue, and phylogenetic distributions. The antibodies, none of which distinguished between dephospho-DARPP-32 and phospho-DARPP-32, were characterized and used to develop a sensitive and specific radioimmunoassay for DARPP-32. The radioimmunoassay, in conjunction with immunolabeling of SDS/PAGE transfers and immunoprecipitation of phosphorylated tissue extracts, was used to measure immunoreactive DARPP-32 in microdissected regions of rat CNS, in peripheral nervous and non-nervous tissues, and in CNS tissue from various animal species.

The distribution of DARPP-32 was generally consistent with the interpretation that it is localized primarily to dopaminergic cells that possess dopamine-sensitive adenylate cyclase (D-1 dopamine receptors coupled to adenylate cyclase). Within the rat CNS, DARPP-32 was most highly concentrated in the basal ganglia. DARPP-32 was present in neostriatum from all six mammalian species tested (mouse, rat, guinea pig, rabbit, cow, and rhesus monkey) at concentrations of from 96 to 144 pmol/mg total protein, which constituted from 0.22 to 0.32% of the total protein. DARPP-32 was also identified at low levels in several peripheral tissues, including choroid plexus, parathyroid cells, adrenal chromaffin cells, posterior pituitary gland, pineal gland, and superior cervical sympathetic ganglion. A phylogenetic survey was carried out of proteins immunologically related to DARPP-32 in nervous tissue from nonmammalian species. DARPP-32-like proteins were identified in dopaminergic brain regions from representative members of the amniote vertebrate classes (birds and reptiles), while none was identified in dopaminergic brain regions from representative members of the anamniote vertebrate classes (bony fishes and amphibians).

or in nervous tissue from representative members of several invertebrate classes.

The stimulation of adenylate cyclase activity by dopamine acting at D-1 dopamine receptors (Kebabian and Calne, 1979) is believed to result in physiological changes that are mediated by the activation of cAMP-dependent protein kinase and the concomitant phosphorylation of specific substrate proteins (for reviews, see Hemmings et al., 1986a; Nairn et al., 1985; Nestler et al., 1984). A marked variation has been demonstrated in the regional distribution of a distinct subset of neuronal substrate proteins for cAMP-dependent protein kinase in rat CNS (Walaas et al., 1983b, c). At least seven of these substrate proteins, six soluble and one particulate, were found to be highly enriched in the forebrain basal ganglia, regions of the brain that receive many dopamine-containing axon terminals (for review, see Lindvall and Björklund, 1978) and that contain relatively high dopamine-sensitive adenylate cyclase activities (for reviews, see Iversen, 1975, 1977; Miller and McDermed, 1979). The state of phosphorylation of one of the soluble phosphoproteins, which exhibited a relative molecular weight ( $M_r$ ) of 32,000 on SDS/PAGE, was found to be regulated by dopamine and by the cAMP analog 8-bromo-cAMP in slices prepared from rat caudatoputamen (Walaas and Greengard, 1984; Walaas et al., 1983a). For these reasons, this phosphoprotein was termed DARPP-32 for dopamine- and cAMP-regulated phosphoprotein,  $M_r = 32,000$  (for reviews, see Hemmings et al., 1986a-c; Walaas et al., 1985).

The purification and biochemical characterization of DARPP-32 from bovine caudate nucleus has recently been reported (Hemmings et al., 1984a-d; Williams et al., 1986). Enzymological studies have indicated that DARPP-32 is a potent inhibitor of the catalytic subunit of the broad specificity enzyme protein phosphatase-1 (Hemmings et al., 1984a). Inhibition of protein phosphatase-1 by the dopamine- and cAMP-stimulated phosphorylation of DARPP-32 may be an important component of the biochemical mechanisms involved in producing some of the neurophysiological effects of dopamine acting at D-1 dopamine receptors on dopaminergic cells.

We now report the production and characterization of rabbit antiserum and mouse monoclonal antibodies to DARPP-32 and the use of these antibodies to develop a detergent-based radioimmunoassay (RIA) for DARPP-32. This RIA has been used to determine quantitatively the regional distribution of DARPP-32 in rat brain and to study its tissue and species distribution. The results obtained using the RIA have been substantiated qualitatively by immunolabeling and by immunoprecipitation techniques. The regional, tissue, and species distributions of DARPP-32 appear to be highly restricted, and to correlate with the distributions of dopaminergic axon terminals and of D-1 dopamine receptors.

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## Materials and Methods

### Materials

$\gamma$ - $^{32}\text{P}$ -ATP ( $5\text{--}10 \times 10^7$  cpm/nmol) was synthesized by the method of Glynn and Chappell (1964) from dipotassium ATP and carrier-free  $^{32}\text{P}$ -orthophosphate (New England Nuclear) or purchased from New England Nuclear. Carrier-free  $^{125}\text{I}$ -sodium iodide ( $\sim 17$  Ci/mg),  $^{125}\text{I}$ -labeled goat anti-rabbit Ig's ( $2\text{--}10 \mu\text{Ci}/\mu\text{g}$ ), and  $^{125}\text{I}$ -labeled goat anti-mouse Ig's ( $2\text{--}10 \mu\text{Ci}/\mu\text{g}$ ) were purchased from New England Nuclear. The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart as described by Kaczmarek et al. (1980). Bovine DARPP-32 was purified to apparent homogeneity by the method of Hemmings et al. (1984b). G-substrate, purified from rabbit cerebellum (Aswad and Greengard, 1981), and inhibitor-1, purified from rabbit skeletal muscle (Hemmings et al., 1984c; Nimmo and Cohen, 1978), were gifts from Dr. Angus Nairn.

Formalin-fixed protein A-bearing *Staphylococcus aureus* cells (SAC) (Pansorbin), phenylmethylsulfonyl fluoride, and HEPES were purchased from Calbiochem-Behring. Triton X-100 was from J. T. Baker Chemical Co. ATP, NP-40, BSA (Cohn fraction V powder), 8-bromo-cAMP, sodium deoxycholate, hypoxanthine, aminopterin, and thymidine were from Sigma Chemical Co. Leupeptin was from Chemicon (Los Angeles). Chloramine-T was from Eastman Chemical Co. Aprotinin (Trasylol) was from Mobay Chemical Corp. (New York). Dulbecco's modified Eagle's medium and fetal calf serum were from Gibco. Pristane (2,6,10,14-tetramethylpentadecane) was from Aldrich Chemical Co. Polyethylene glycol 1500 was from Gallard-Schlesinger. Freund's adjuvant (complete and incomplete) and *S. aureus* V8 protease were from Miles Laboratories. SDS, Tween 20, and gelatin were from Bio-Rad Laboratories. Nitrocellulose membranes were from Schleicher and Schuell (Keene, NH). Sephadex G-25 (fine) was from Pharmacia. Falcon 3911 Microtest III flexible 96-well assay plates were from Becton Dickinson.

Brains, superior cervical sympathetic ganglia, and adrenal glands from 1- to 3-week-old calves were obtained, packed on ice, from a local slaughterhouse. Normal goat serum and frozen mature rabbit superior cervical sympathetic ganglia were from Pel-Freez Biologicals (Rogers, AK). Male Sprague-Dawley rats (150–200 gm) and BALB/c mice (20–25 gm) were from Charles River Laboratories. *Pseudomys scripta* and *Rana pipiens* were from Connecticut Valley (Southampton, MA). *Cyprinus carpio* was from a local fish market. *Serinus canarius* was a gift of Dr. Gail Burd, *Macaca mulatta* brain a gift of Dr. Tamas Bartfai, rabbit vascular tissues a gift of Dr. Eliot Ohlstein, *Drosophila melanogaster* a gift of Dr. Suzanne Garen, *Aplysia californica* a gift of Dr. Leonard Kaczmarek, and *Homarus americanus* a gift of Dr. Michael Goy. Bovine parathyroid glands and isolated bovine parathyroid cells were a gift of Dr. Edward Brown. Isolated bovine adrenal chromaffin cells were a gift of Dr. John Haycock.

### Radioiodination of DARPP-32

DARPP-32 was radioiodinated by the chloramine-T method of Greenwood et al. (1963). To a vial containing 2 mCi of  $^{125}\text{I}$ -sodium iodide, 10  $\mu\text{l}$  of 0.5 M sodium phosphate, pH 7.4, was added, followed by the addition of 10  $\mu\text{g}$  of purified DARPP-32 in 10  $\mu\text{l}$  of 50 mM sodium phosphate, pH 7.4. The reaction was initiated by the addition of 10  $\mu\text{l}$  of 0.5 mg/ml chloramine-T in 50 mM sodium phosphate, pH 7.4, allowed to proceed for 3 min at room temperature, and terminated by the addition of 10  $\mu\text{l}$  of 0.5 mg/ml sodium metabisulfite and 100  $\mu\text{l}$  of 20 mg/ml sodium iodide, both in 50 mM sodium phosphate, pH 7.4. To this solution, 60  $\mu\text{l}$  of column buffer [50 mM sodium phosphate, pH 7.4, 0.5% (vol/vol) NP-40, 2.5 mg/ml gelatin] was added, and the reaction mixture removed. The reaction vial was then washed with 100  $\mu\text{l}$  of column buffer; the wash was combined with the reaction mixture and then transferred to a  $0.7 \times 20$  cm Sephadex G-25 (fine) column equilibrated in column buffer.  $^{125}\text{I}$ -labeled DARPP-32 was separated from the reaction mixture by elution with column buffer. Fractions of 0.25 ml were collected, aliquots of each fraction were analyzed for  $^{125}\text{I}$  radioactivity using a Micromedex Systems 2/2000 gamma counter, and the peak of  $^{125}\text{I}$ -labeled DARPP-32 pooled. The  $^{125}\text{I}$ -labeled DARPP-32 was stored in 50  $\mu\text{l}$  aliquots at  $-20^\circ\text{C}$ . This procedure resulted in a preparation of  $^{125}\text{I}$ -labeled DARPP-32 in which 65–85% of the total radioactivity was contained in intact DARPP-32; the remainder consisted largely of lower-molecular-weight breakdown products. The specific activity was about 50–100  $\mu\text{Ci}/\mu\text{g}$  as determined by SDS/PAGE or trichloroacetic acid precipitation. From 80–90% of the radioactivity con-

tained in intact  $^{125}\text{I}$ -labeled DARPP-32 was precipitated by anti-DARPP-32 antisera or monoclonal antibodies using the immunoprecipitation procedure described below, suggesting that the iodination procedure had no substantial effect on the immunoreactivity of DARPP-32 (data not shown). DARPP-32 radioiodinated using  $^{125}\text{I}$ -labeled Bolton-Hunter reagent (Goetz et al., 1981) gave results in the RIA similar to those obtained with DARPP-32 radioiodinated using the chloramine-T method (unpublished observations).

### Antibody detection assay

Rabbit and mouse sera, hybridoma culture supernatants, and ascites fluid were tested for anti-DARPP-32 antibodies by their ability to precipitate  $^{125}\text{I}$ -labeled DARPP-32 using SAC as an immunoadsorbent (Kessler, 1975). To samples of 70  $\mu\text{l}$  in plastic 96-well flexible assay plates were added 10  $\mu\text{l}$  of  $10 \times$  RIA buffer (0.1 M sodium phosphate, pH 7.6, 2 M NaCl, 0.1 M EDTA), 10  $\mu\text{l}$  of 10% (vol/vol) NP-40, and 10  $\mu\text{l}$  of  $^{125}\text{I}$ -labeled DARPP-32 (containing about 10,000 cpm), followed by a 2–16 hr incubation at  $4^\circ\text{C}$ . Bound  $^{125}\text{I}$ -labeled DARPP-32 was precipitated with 10  $\mu\text{l}$  of a freshly washed 10% (wt/vol) suspension of protein A-bearing SAC prepared as described previously (Goetz et al., 1981) in NET buffer [50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 0.02% (wt/vol) sodium azide] containing 1% (vol/vol) NP-40 and 25 mg/ml BSA. Following a 1–2 hr incubation at  $4^\circ\text{C}$ , samples were centrifuged at  $1000 \times g$  for 10 min and the supernatants removed. The SAC pellets were resuspended in NET buffer containing 1% (vol/vol) NP-40, centrifuged again, and the amount of immunoprecipitated  $^{125}\text{I}$ -labeled DARPP-32 determined. A sample was considered positive for anti-DARPP-32 antibodies when the precipitated radioactivity was greater than twice the nonspecifically precipitated radioactivity (about 100 cpm).

### Preparation of rabbit anti-DARPP-32 antiserum

Antiserum to DARPP-32 was raised in three female New Zealand white rabbits (1.5–2 kg). After bleeding each rabbit to obtain preimmune serum, each was immunized intradermally at multiple sites over the abdomen with a total of 200  $\mu\text{g}$  purified DARPP-32 (0.2 mg/ml in PBS) emulsified in an equal volume of Freund's complete adjuvant. At 2 and 4 weeks following the first injection, a 100  $\mu\text{g}$  dose of DARPP-32 emulsified in Freund's incomplete adjuvant was injected as before. The rabbits were bled by cardiac puncture 10 d following the final injection. The blood was allowed to clot for 2 hr at room temperature and then overnight at  $4^\circ\text{C}$ , and the serum was recovered by centrifugation at  $1000 \times g$  for 10 min. The antisera obtained were stored at  $-70^\circ\text{C}$  in small aliquots. The antisera were screened for anti-DARPP-32 antibodies by the antibody-detection assay. The antiserum containing the highest antibody titer (antiserum R2) was used in the present studies.

### Preparation of mouse anti-DARPP-32 monoclonal antibodies

Monoclonal antibodies were produced essentially by the method of Köhler and Milstein (1975). BALB/c, NZB, and AJ mice (two mice each) were immunized with 100  $\mu\text{g}$  of purified bovine DARPP-32 in 0.1 ml PBS emulsified with an equal volume of Freund's complete adjuvant by subcutaneous injections into foot pads and over the abdomen. Twelve days following immunization, mice were bled and their sera assayed for anti-DARPP-32 antibodies. Twenty-one days after the initial immunization, mice exhibiting a positive response were boosted intravenously with 100  $\mu\text{g}$  purified DARPP-32 in PBS. The BALB/c mice were found to exhibit the greatest immune response and were used for the boost and fusion. Three days after the boost, the spleens were removed and the fusion performed by incubating  $10^8$  spleen cells with  $10^7$  Sp2/0-Ag14 myeloma cells (Shulman et al., 1978) in 50% polyethylene glycol 1500 for 5 min at  $37^\circ\text{C}$ . Following the fusion, cells were plated into flat-bottom microtiter plates in HT medium [Dulbecco's modified Eagle's medium containing 100  $\mu\text{M}$  hypoxanthine, 16  $\mu\text{M}$  thymidine and 15% (vol/vol) fetal calf serum] at a density of  $10^5$  cells/well in 0.12 ml. After 1 d, HAT medium (HT medium containing 0.8  $\mu\text{M}$  aminopterin) was substituted for HT medium in the cultures, and was replaced at 3 d intervals. After two changes of HAT medium, cells were again fed HT medium at 3 d intervals. Two weeks after fusion, culture supernatants were assayed for anti-DARPP-32 antibody. Hybridoma cultures secreting antibodies specific for DARPP-32 were expanded and were then cloned by limiting dilution in flat-bottomed microtiter plates using BALB/c thymocytes as a feeder layer. This procedure resulted in

the production of five stable anti-DARPP-32 antibody-producing clonal hybrid cell lines designated C24-4D7, C24-5a, C24-6a, C24-7a, and C24-13a. The antibody-producing hybrid cell lines were subcloned and expanded, and  $2 \times 10^6$  cells (in HT medium without serum) per animal were then injected intraperitoneally into BALB/c recipients primed with pristane (0.5 ml per mouse). In 1–3 weeks ascites developed and was collected by paracentesis. Cells were removed by centrifugation at  $1000 \times g$  for 10 min, and the gamma globulin fraction was obtained by precipitation with 45% saturated ammonium sulfate at 4°C. After centrifugation at  $15,000 \times g$  for 20 min, the protein pellet was dissolved in PBS containing 0.02% (wt/vol) sodium azide, and dialyzed overnight against the same buffer. The resulting high-titered ascites monoclonal antibodies were stored at  $-70^\circ\text{C}$  in small aliquots.

#### Immunolabeling of electrophoretic transfers from SDS/PAGE

Immunolabeling of proteins solubilized in SDS, separated by SDS/PAGE, and transferred to nitrocellulose membranes was performed by a modification of the procedure described previously (Ouimet et al., 1984). Tissue samples were prepared by homogenization in 20 vol (ml/gm) of an SDS solution [1% (wt/vol) SDS, 10  $\mu\text{g}/\text{ml}$  leupeptin, and 100  $\mu\text{M}$  phenylmethylsulfonyl fluoride] using 10 up-and-down strokes in a glass/Teflon or glass/glass homogenizer. Purified proteins were dissolved in 1% (wt/vol) SDS. Aliquots of the samples were then subjected to SDS/PAGE and electrophoretically transferred to nitrocellulose membranes (0.2  $\mu\text{m}$  pore size) at 200 mA for 8–12 hr in 20 mM Tris-HCl, 150 mM glycine, pH 8.6, containing 20% (vol/vol) methanol (Towbin et al., 1979). Following the protein transfer, excess protein binding sites on the nitrocellulose membranes were quenched at  $45^\circ\text{C}$  in 50 ml of TBS [50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% (wt/vol) sodium azide] containing 10% (vol/vol) normal goat serum and 1% (wt/vol) BSA for 8–12 hr. Membranes were then incubated at room temperature in 50 ml of TBS containing 1% (vol/vol) normal goat serum, 0.5% (wt/vol) BSA, and various dilutions of either the antiserum or monoclonal antibodies for 2–4 hr. Membranes were washed in 50 ml of TBS containing 0.05% (vol/vol) Tween 20 (Batteiger et al., 1982) for 20 min, followed by two washes in 50 ml of TBS alone for 20 min each. Radiolabeling of the bound antibodies was performed by incubating the washed membranes in 50 ml of  $^{125}\text{I}$ -labeled goat anti-rabbit or goat anti-mouse Ig's ( $10^5$  cpm/ml) in TBS containing 1% (vol/vol) normal goat serum and 0.5% (wt/vol) BSA for 2 hr. The sheets were then washed  $3 \times 20$  min with 50 ml of TBS containing Tween 20, followed by a single wash in 50 ml of TBS alone. The sheets were then dried and exposed at  $-70^\circ\text{C}$  to Kodak X-Omat AR film using a DuPont Lighting Plus intensifying screen.

#### Immunoprecipitation of phosphorylated tissue extracts

Crude cytosol was prepared and endogenously phosphorylated using 8-bromo-cAMP and  $\gamma\text{-}^{32}\text{P}$ -ATP as described previously by Hemmings et al. (1984b), and acid extracts were prepared and phosphorylated using exogenous catalytic subunit of cAMP-dependent protein kinase and  $\gamma\text{-}^{32}\text{P}$ -ATP as described by Walaas and Greengard (1984). Phosphorylation reactions were terminated by the addition of SDS to a final concentration of 1% (wt/vol), followed by heating to  $95^\circ\text{C}$  for 2 min. Immunoprecipitation was performed at  $4^\circ\text{C}$  in polypropylene microfuge tubes. An equal volume of a  $2\times$  concentrated buffer solution was added to the SDS-containing samples to yield final concentrations of 20 mM Tris-HCl, pH 7.6, 125 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM EDTA, 0.5% SDS, and 2.5% (vol/vol) NP-40. A 5  $\mu\text{l}$  aliquot of a 10% (wt/vol) SAC suspension in NET buffer containing 25 mg/ml BSA was added to the phosphorylated mixture, and incubated for 15 min. The SAC was removed by centrifugation in a Beckman Microfuge 12 at  $12,400 \times g$  for 5 min, and the "precleared" supernatant transferred to a second microfuge tube containing anti-DARPP-32 antibody. This solution was vortexed and incubated for 30–90 min. A 50  $\mu\text{l}$  aliquot of SAC was then added, incubated for 15 min, and the suspension centrifuged for 5 min. The supernatant was removed and the pellet resuspended in 200  $\mu\text{l}$  of 1% (wt/vol) SDS, heated to  $95^\circ\text{C}$  for 5 min, and the suspension centrifuged for 5 min. The supernatant was removed and subjected to a second cycle of immunoprecipitation beginning with the addition of an equal volume of the  $2\times$  concentrated buffer solution as described above. The resulting SAC pellet was washed with 0.5 ml of NET buffer, resuspended in 125  $\mu\text{l}$  of SDS-containing "stop solution," subjected to SDS/PAGE, and analyzed by autoradiography (Hemmings et al., 1984b).

#### Competitive nonequilibrium radioimmunoassay for DARPP-32

Formalin-fixed SAC were employed as a solid-phase immunoabsorbent in the RIA. Prior to each RIA, the  $^{125}\text{I}$ -labeled DARPP-32 was pre-cleared with SAC. This was accomplished by adding 100  $\mu\text{l}$  of RIA buffer (10 mM sodium phosphate, pH 7.6, 200 mM NaCl, 10 mM EDTA) containing 1% (vol/vol) NP-40 to an aliquot of  $^{125}\text{I}$ -labeled DARPP-32, followed by 20  $\mu\text{l}$  of freshly washed SAC in NET buffer containing 25 mg/ml BSA. This solution was incubated for 30 min at  $4^\circ\text{C}$ . After centrifugation at  $12,400 \times g$  in a Beckman Microfuge 12 for 5 min, the "precleared" supernatant was removed and added to an appropriate volume of RIA buffer containing 1% (vol/vol) NP-40 to give  $5\text{--}10 \times 10^3$  cpm/10  $\mu\text{l}$ . Using this amount of  $^{125}\text{I}$ -labeled DARPP-32, various antiserum and monoclonal antibody dilutions were tested to determine the amount of antibody that precipitated 50–60% of the label in the competition state of the RIA. The final dilution of antiserum R2 used in the RIA was 1:200 and that of monoclonal antibody C24-13a was 1:100,000.

The RIA was performed in plastic 96-well flexible assay plates at  $4^\circ\text{C}$  in a final volume of 100  $\mu\text{l}$ . All standards and samples were assayed in triplicate. The individual solutions of standard DARPP-32, of pre-cleared  $^{125}\text{I}$ -labeled DARPP-32, and of anti-DARPP-32 antibodies were diluted with RIA buffer containing 1% (vol/vol) NP-40. Standards and samples both contained final concentrations of 0.1% (wt/vol) SDS and 1% (vol/vol) NP-40 (Dimitriadis, 1979). The samples and standards were brought to a final volume of 80  $\mu\text{l}$  and the precompetition stage of the RIA initiated by the addition of 10  $\mu\text{l}$  of an appropriate dilution of the anti-DARPP-32 antibodies followed by gentle shaking of the assay plate for several seconds. After a 12 hr precompetition, 10  $\mu\text{l}$  of pre-cleared  $^{125}\text{I}$ -labeled DARPP-32 was added, again with gentle shaking. After a 1 hr competition, 10  $\mu\text{l}$  of pre-washed SAC in RIA buffer containing 25 mg/ml BSA was added, the assay plates were shaken gently, and the samples were incubated for a further 20 min. The assay plates were then centrifuged at  $1000 \times g$  for 10 min. The supernatants were removed by inverting and flicking the plates, and the pellets softened by vortexing the plates. The pellets were washed once in 100  $\mu\text{l}$  of RIA buffer containing 1% (vol/vol) NP-40. The wells containing the SAC pellets were cut from the assay plates, and the  $^{125}\text{I}$  radioactivity measured using a Micromedex Systems 2/2000 gamma counter. Data were analyzed using linear logit-log plots of the competition curves (data not shown).

#### Preparation of samples for RIA

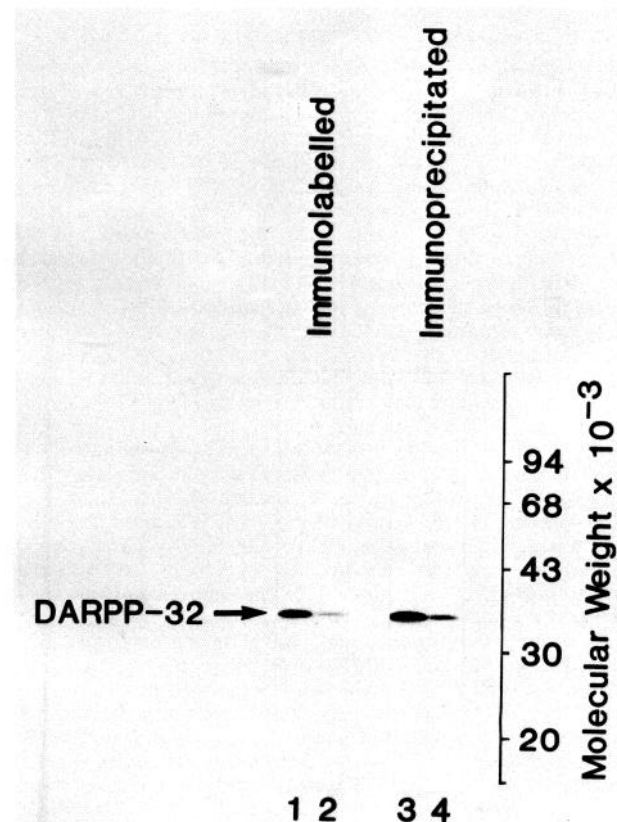
Rat brain was dissected as described (Walaas et al., 1983b), and the microdissected regions immediately homogenized in an SDS solution [1% (wt/vol) SDS, 10  $\mu\text{g}/\text{ml}$  leupeptin, and 100  $\mu\text{M}$  phenylmethylsulfonyl fluoride]. For determination of the levels of DARPP-32 in the neostriatum of various mammalian species, and in the nervous systems of various vertebrate and invertebrate species, samples were obtained from freshly killed animals and immediately frozen or homogenized in SDS solution. In some experiments various peripheral tissues were also analyzed. Freshly dissected or quick-frozen tissue samples were homogenized with a glass/Teflon homogenizer in 20 vol of SDS solution. The homogenates were then heated to  $95^\circ\text{C}$  for 2 min and sonicated, and insoluble material was removed by centrifugation at  $12,400 \times g$  for 10 min. Protein concentrations were determined by a detergent-based Folin reagent assay, using BSA as a standard, after precipitation of protein with sodium deoxycholate and trichloroacetic acid (Peterson, 1977).

#### Phosphorylation of purified DARPP-32

DARPP-32 was phosphorylated by incubating purified DARPP-32 in a reaction mixture containing 50 mM HEPES, pH 7.4, 10 mM magnesium acetate, 1 mM EGTA, 2 ng purified catalytic subunit of cAMP-dependent protein kinase and 50  $\mu\text{M}$  ATP in a final volume of 100  $\mu\text{l}$  at  $30^\circ\text{C}$  for 60 min (Hemmings et al., 1984b, c). As a control, a sample of DARPP-32 was treated identically except that the catalytic subunit was omitted.

#### Miscellaneous methods

One-dimensional phosphopeptide mapping using *S. aureus* V8 protease, according to the method of Cleveland et al. (1977), and SDS/PAGE, according to the method of Laemmli (1970), were carried out as described previously (Hemmings et al., 1984b). Two-dimensional phosphopeptide mapping of thermolytic digests and phospho-amino acid



**Figure 1.** Characterization of specificity of anti-DARPP-32 monoclonal antibody C24-13a by immunolabeling and immunoprecipitation. *Lanes 1 and 2*, Samples of rat caudatoputamen and neocortex were dissected from the brain, homogenized in SDS, subjected to SDS/PAGE (9% acrylamide), and electrophoretically transferred to a nitrocellulose membrane as described in Materials and Methods. The nitrocellulose membrane was labeled with a 1:1000 dilution of monoclonal antibody C24-13a, followed by  $^{125}\text{I}$ -labeled goat anti-mouse Ig's, and analyzed by autoradiography. *Lanes 3 and 4*, Crude cytosol was prepared from samples of rat caudatoputamen and neocortex and subjected to endogenous phosphorylation with 8-bromo-cAMP and  $\gamma\text{-}^{32}\text{P}$ -ATP, followed by immunoprecipitation with 2  $\mu\text{l}$  of monoclonal antibody C24-13a. Immunoprecipitated proteins were subjected to SDS/PAGE (9% acrylamide) and analyzed by autoradiography. For the caudatoputamen samples (lanes 1 and 3) 75  $\mu\text{g}$  of total protein were used, while for the neocortex samples (lanes 2 and 4) 150  $\mu\text{g}$  of total protein were used.

analyses were performed as described previously (Hemmings et al., 1984c).

## Results

### Characterization of DARPP-32 antibodies

All three immunized rabbits and all six immunized mice produced antibodies to DARPP-32. The rabbit serum containing the highest titer of anti-DARPP-32 activity was chosen for further characterization. Only a single band was detected when antiserum R2 was used to immunolabel nitrocellulose membranes to which tissue extracts prepared from bovine caudate nucleus or rat caudatoputamen and separated by SDS/PAGE had been transferred electrophoretically (data not shown). Moreover, antiserum R2 precipitated a single phosphoprotein from bovine caudate nucleus or rat caudatoputamen cytosol phosphorylated by the addition of 8-bromo-cAMP and  $\gamma\text{-}^{32}\text{P}$ -ATP (data not shown).

Immunized BALB/c mice were found to produce the highest

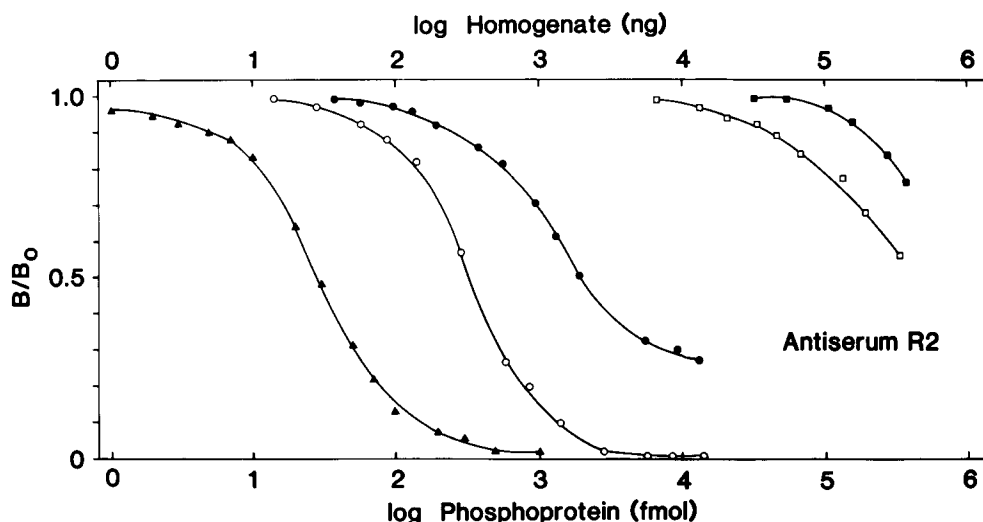
titer of anti-DARPP-32 activity and were used to produce monoclonal antibodies by the method of Köhler and Milstein (1975). Characterization of the anti-DARPP-32 monoclonal antibodies using immunolabeling of rat brain proteins separated by SDS/PAGE and transferred to nitrocellulose and immunoprecipitation of phosphorylated rat brain extracts was performed. Both techniques resulted in the identification of a single protein band using either monoclonal antibody C24-13a (Fig. 1) or any of the four other monoclonal antibodies (Ouimet et al., 1984; and data not shown). The amount of DARPP-32 detected was greater in caudatoputamen than in neocortex. Similar results were obtained with homogenates of bovine caudate nucleus and neocortex (data not shown). The immunolabeling of a single protein band in either rat or bovine brain homogenates and the immunoprecipitation of a single phosphoprotein from the mixture of cAMP-stimulated phosphoproteins present in crude cytosol preparations strongly suggest that both the antiserum and the monoclonal antibodies prepared against DARPP-32 are specific for DARPP-32. However, the possibility of immunologic reactivity with a protein not efficiently transferred to nitrocellulose or having an apparent molecular weight on SDS/PAGE identical to that of DARPP-32 cannot be ruled out.

### Radioimmunoassay of DARPP-32

Using antiserum R2, a detergent-based nonequilibrium RIA of DARPP-32 was developed based on the method described by Goetz et al. (1981) for the quantitation of synapsin I. The inclusion of a 10-fold weight excess of the nonionic detergent NP-40 over the ionic detergent SDS in the RIA permitted homogenization of tissue samples in 1% SDS, which reduced proteolysis during sample preparation and reduced background radioactivity without disruption of the antigen-antibody complex (Dimitriadis, 1979). The use of a competitive nonequilibrium assay involving sequential saturation conditions in which the antibody was preincubated with the samples for 12 hr before the  $^{125}\text{I}$ -labeled DARPP-32 was added increased the sensitivity of the assay and reduced overall incubation times.

The competition curves obtained using rabbit antiserum R2 to bovine DARPP-32 and  $^{125}\text{I}$ -labeled bovine DARPP-32 are shown in Figure 2. The range of sensitivity of the assay was from about 10 to 100 fmol of DARPP-32. Fifty percent displacement ( $B/B_0 = 0.5$ ) was obtained with about 29 fmol of DARPP-32. The phospho- and dephospho-forms of DARPP-32 were equipotent in the RIA (data not shown). Bovine caudate nucleus solubilized in 1% SDS produced a competition curve parallel to that of purified bovine DARPP-32 and resulted in essentially complete displacement of  $^{125}\text{I}$ -labeled bovine DARPP-32. However, rat caudatoputamen solubilized in 1% SDS did not produce a competition curve parallel to that of purified bovine DARPP-32, nor did it result in complete displacement of  $^{125}\text{I}$ -labeled bovine DARPP-32 (Fig. 2). Similar results showing lack of complete displacement were also obtained for mouse, guinea pig, and rabbit neostriatum homogenates (data not shown). The competition curves of neostriatum homogenates using antiserum R2 revealed heterogeneity between bovine and nonbovine DARPP-32. This was manifest in the absence of parallel competition curves, indicating heterogeneous antibody affinity, and in partial cross-reactivity (less than complete displacement), indicating heterogeneity in the specificity of the antiserum for different determinants on the immunogen (Berzofsky and Schechter, 1981).

The partial cross-reactivity of the nonbovine forms of DARPP-32 observed in the RIA using antiserum R2 made this RIA unsuitable for quantitation of DARPP-32 in species other than cow. True cross-reactivity is expected for certain homogeneous antibodies to a single determinant (Berzofsky and Schechter, 1981), a condition that applies to monoclonal antibodies. The monoclonal antibodies to bovine DARPP-32 were therefore



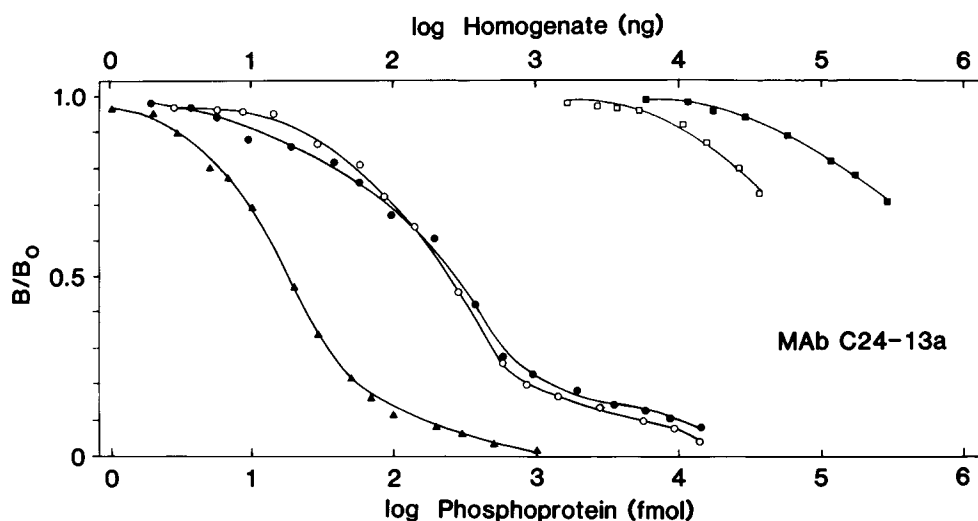
**Figure 2.** Competition curves using antiserum R2 for RIA of purified bovine DARPP-32 ( $\blacktriangle$ ), bovine caudate nucleus homogenate ( $\circ$ ), rat caudatoputamen homogenate ( $\bullet$ ), purified rabbit skeletal muscle protein phosphatase inhibitor-1 ( $\square$ ), and purified rabbit cerebellum G-substrate ( $\blacksquare$ ) with  $^{125}\text{I}$ -labeled bovine dephospho-DARPP-32. *Abscissa*, Log of total of DARPP-32, inhibitor-1, or G-substrate in assay (lower) or log of total homogenate protein from bovine caudate nucleus or rat caudatoputamen (upper); *ordinate*, ratio of  $^{125}\text{I}$ -labeled DARPP-32 bound in the sample ( $B$ ) to  $^{125}\text{I}$ -labeled DARPP-32 bound with no DARPP-32 added ( $B_0$ ), both corrected for nonspecific binding.

tested for true cross-reactivity to nonbovine DARPP-32 using the RIA technique. Of the five monoclonal antibodies prepared, antibody C24-13a resulted in the most sensitive RIA. As can be seen in Figure 3, the range of sensitivity of the RIA using antibody C24-13a was from about 5 to 80 fmol of DARPP-32. Fifty percent displacement ( $B/B_0 = 0.5$ ) was obtained with about 18 fmol of DARPP-32. The phospho- and dephospho-forms of DARPP-32 were equipotent in the RIA (data not shown). Bovine caudate nucleus solubilized in 1% SDS produced a competition curve parallel to that of the purified bovine DARPP-32 and resulted in complete displacement of  $^{125}\text{I}$ -labeled bovine DARPP-32. Comparison of the competition curve obtained using a homogenate of rat caudatoputamen (Fig. 3) with that of purified bovine DARPP-32 also revealed parallel curves with essentially complete displacement of  $^{125}\text{I}$ -labeled bovine DARPP-32. Homogenates of neostriatum from mouse, guinea pig, rabbit, rhesus monkey, canary, and turtle also produced parallel competition curves with essentially complete displacement of  $^{125}\text{I}$ -labeled bovine DARPP-32 (data not shown), comparable to the curves shown for rat or bovine neostriatum (Fig. 3), indicating true cross-reactivity of the homologous nonbovine forms of DARPP-32. The RIA employing monoclonal antibody C24-13a was therefore chosen for the quantitative determinations of DARPP-32 levels reported in this study. The RIA employing antiserum R2 was also used to analyze bovine tissue samples and to screen nonbovine species for the presence of

DARPP-32; the results from these assays were in good agreement with those obtained using monoclonal antibody C24-13a.

#### *Immunologic cross-reactivity of DARPP-32 with other phosphoproteins*

Previous studies have indicated many similarities between DARPP-32 and protein phosphatase inhibitor-1 (inhibitor-1), a potent inhibitor of protein phosphatase-1, and G-substrate, a Purkinje cell-specific phosphoprotein (Hemmings et al., 1984a, b, d; Williams et al., 1986). Inhibitor-1 and G-substrate were tested for immunologic cross-reactivity with DARPP-32 using the RIA (Figs. 2, 3). Limited displacement of  $^{125}\text{I}$ -labeled DARPP-32 was observed for both phosphoproteins in the RIA at very high concentrations, indicating some degree of immunological relatedness between these three phosphoproteins. Purified inhibitor-1 began to exhibit competition in the RIA at about 10 pmol with antiserum R2 and at about 5 pmol with monoclonal antibody C24-13a. Purified G-substrate also competed in the RIA but was less potent than inhibitor-1; G-substrate began to exhibit competition in the RIA at about 100 pmol with antiserum R2 and at about 20 pmol with monoclonal antibody C24-13a. The greater immunoreactivity of inhibitor-1 than of G-substrate in the RIA is consistent with the greater similarity in the known amino acid sequences of DARPP-32 and inhibitor-1 compared to G-substrate (Aitken et al., 1981, 1982; Hemmings et al., 1984d; Williams et al., 1986). The potency of either of



**Figure 3.** Competition curves using monoclonal antibody C24-13a for RIA of purified bovine DARPP-32 ( $\blacktriangle$ ), bovine caudate nucleus homogenate ( $\circ$ ), rat caudatoputamen homogenate ( $\bullet$ ), purified rabbit skeletal muscle protein phosphatase inhibitor-1 ( $\square$ ), and purified rabbit cerebellum G-substrate ( $\blacksquare$ ) with  $^{125}\text{I}$ -labeled bovine dephospho-DARPP-32. For explanation of *abscissa* and *ordinate*, see legend to Fig. 2.



**Table 1. Regional distribution of DARPP-32 in rat CNS**

Region	Immunoreactive DARPP-32 (pmol/mg total protein)
Substantia nigra	133.6 ± 11.0
Caudatoputamen	129.5 ± 9.4
Globus pallidus	112.3 ± 7.0
Olfactory tubercle	77.3 ± 14.6
Nucleus accumbens	60.3 ± 12.4
Thalamus	16.5 ± 3.2
Cerebellum	14.8 ± 2.6
Cerebral cortex	8.0 ± 3.2
Hippocampus	7.4 ± 2.1
Retina	6.3 ± 3.0
Frontal cortex	6.0 ± 1.0
Hypothalamus	5.0 ± 1.0
Amygdala	4.3 ± 1.2
Septum	3.4 ± 0.8
Pons/medulla	2.2 ± 0.6
Olfactory bulb	1.1 ± 0.6
Spinal cord	0.6 ± 0.4

Values represent the means ± SEM of at least three experiments, each employing two tissue concentrations assayed in triplicate, calculated using a molecular mass of 22,591 Da obtained by amino acid sequencing of bovine brain DARPP-32 (Williams et al., 1986).

these phosphoproteins in the DARPP-32 RIA was at least three orders of magnitude less than that of DARPP-32, indicating a high degree of specificity of the RIA for DARPP-32. Synapsin I, a neuronal phosphoprotein unrelated to DARPP-32 (Ueda and Greengard, 1977), did not exhibit any competition in the RIA at up to 1 nmol (data not shown).

The cross-reactivity of the antibodies to DARPP-32 with inhibitor-1 were further analyzed using the immunolabeling and immunoprecipitation techniques. Neither antiserum R2 nor monoclonal antibody C24-13a immunolabeled 5 µg of purified inhibitor-1, whereas they did immunolabel 0.010 µg of purified DARPP-32 (data not shown). Likewise, neither antibody preparation immunoprecipitated 10 µM purified phospho-inhibitor-1, while both immunoprecipitated 0.05 µM purified phospho-DARPP-32 (data not shown). These results further support the specificity of the antibodies to DARPP-32 in the presence of experimentally observable tissue concentrations of inhibitor-1, which is present at a concentration of 1.5 µM in rabbit skeletal muscle (Nimmo and Cohen, 1978).

#### Regional distribution of DARPP-32 in rat CNS

The distribution of DARPP-32 in 17 microdissected regions of rat CNS was studied using the RIA (Table 1). DARPP-32 was found to be highly enriched in the substantia nigra, caudatoputamen (neostriatum), and globus pallidus, which contained greater than 100 pmol of DARPP-32/mg of tissue protein. Intermediate DARPP-32 levels were measured in the olfactory tubercle and nucleus accumbens, which contained 77 and 60 pmol of DARPP-32/mg of tissue protein, respectively, while the other regions examined contained less than 17 pmol of DARPP-32/mg of tissue protein. The presence of DARPP-32 in each of the 17 CNS regions tested was confirmed by immunolabeling and by immunoprecipitation which resulted in the detection of a single immunoreactive protein with an  $M_r$  = 32,000 in each region.

#### Quantitation of DARPP-32 in the neostriatum of mammalian species of various orders

The amounts of DARPP-32 in neostriatal extracts prepared from six mammalian species representing four orders were mea-

**Table 2. Concentrations of DARPP-32 in the neostriatum of mammalian species of various orders**

Order	Immunoreactive DARPP-32	
	(pmol/mg total protein)	(% of total protein)
Rodentia		
Mouse ( <i>Mus musculus</i> )	102.5 ± 11.7	0.23
Rat ( <i>Rattus norvegicus</i> )	130.7 ± 10.6	0.29
Guinea pig ( <i>Cavia porcellis</i> )	110.7 ± 8.9	0.25
Lagomorpha		
Rabbit ( <i>Oryctolagus cuniculus</i> )	143.6 ± 9.6	0.32
Artiodactyla		
Cow ( <i>Bos taurus</i> )	97.4 ± 8.8 <sup>a</sup>	0.22
Primates		
Rhesus monkey ( <i>Macaca mulatta</i> )	95.8 ± 12.6	0.22

Values represent the means ± SEM of at least three experiments, each employing two tissue concentrations assayed in triplicate, calculated using a molecular mass of 22,591 Da obtained by amino acid sequencing of bovine brain DARPP-32 (Williams et al., 1986).

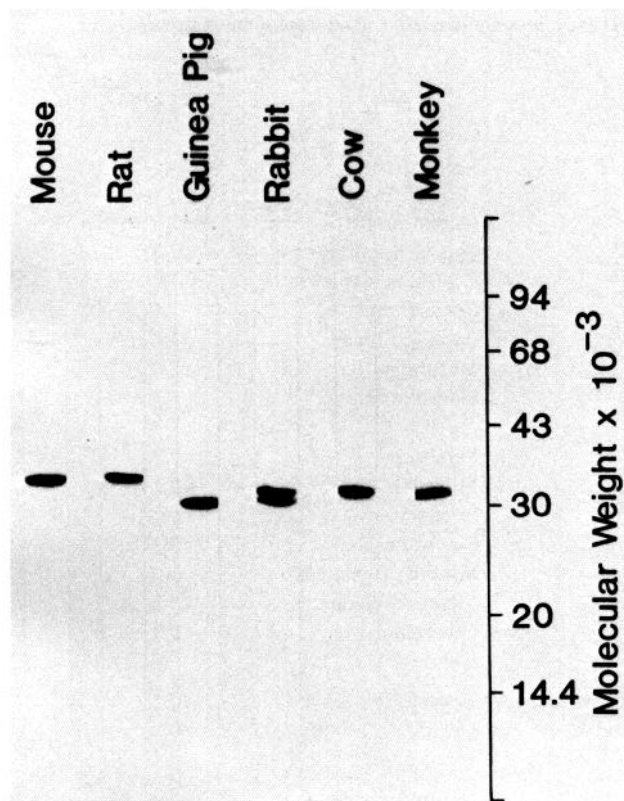
<sup>a</sup> Agrees with value determined by RIA using antiserum R2 (101.2 ± 6.3 pmol/mg total protein).

sured by use of the RIA (Table 2). DARPP-32 immunoreactivity was detected in the neostriatum of each of the six species at concentrations ranging from 96 to 144 pmol of DARPP-32/mg of total protein. DARPP-32 was highly concentrated in the neostriatum, constituting from 0.22 to 0.32% of the total neostriatal protein.

Immunoprecipitation of phosphorylated acid extracts allowed the identification of the phosphorylated acid-soluble proteins cross-reacting with the antibodies to DARPP-32 and supported the results obtained by RIA. The six immunoprecipitated phosphoproteins exhibited similar mobilities on SDS/PAGE, which corresponded to apparent  $M_r$  values of 30,000–34,000 (Fig. 4). The antibodies precipitated a doublet from the rabbit extract, both components of which yielded identical one-dimensional phosphopeptide maps after proteolysis with *S. aureus* V8 protease, and identical two-dimensional phosphopeptide maps after proteolysis with thermolysin (data not shown). Furthermore, the phosphoproteins immunoprecipitated from each of the five nonbovine species yielded one-dimensional phosphopeptide maps using *S. aureus* V8 protease and two-dimensional phosphopeptide maps using thermolysin that were similar to those previously published for bovine DARPP-32 (Hemmings et al., 1984b, c). Phosphothreonine was the only phosphorylated amino acid in each immunoprecipitated phosphoprotein (data not shown). The apparent  $M_r$  values determined by SDS/PAGE of the phosphoproteins immunoprecipitated from mouse, rat, guinea pig, rabbit, cow and rhesus monkey neostriatum were about 34,000, 34,000, 30,000, 32,000/30,000, 32,000, and 32,000, respectively (Fig. 4).

#### Quantitation of DARPP-32 in various peripheral tissues

The RIA for DARPP-32 was also used to analyze various peripheral non-nervous and nervous tissues (Table 3). No competition was observed when up to 25 µg of total protein from the peripheral non-nervous tissues lung, liver, heart, kidney, spleen, pancreas, skeletal muscle, or smooth muscle were assayed. These results indicate that DARPP-32, if present in these tissues, would comprise less than 0.2 pmol of DARPP-32/mg of total tissue protein. A very low level of DARPP-32 was detected in bovine parathyroid cells. These results were confirmed by immunolabeling and immunoprecipitation (data not



**Figure 4.** Immunoprecipitation of DARPP-32 from phosphorylated extracts prepared from various mammalian neostriata. Crude cytosol was prepared from samples of the neostriata of the mammalian species indicated, and acid extracts prepared and phosphorylated using the catalytic subunit of cAMP-dependent protein kinase and  $\gamma$ - $^{32}$ P-ATP. The phosphorylated proteins were immunoprecipitated with 2  $\mu$ l of monoclonal antibody C24-13a. The immunoprecipitated proteins were then subjected to SDS/PAGE (10% acrylamide) and analyzed by autoradiography.

shown). The fact that no immunoreactivity was observed in skeletal muscle by RIA, immunolabeling, or immunoprecipitation in spite of the presence of relatively high amounts (1.5  $\mu$ M) of inhibitor-1 in this tissue (Nimmo and Cohen, 1978) further attests to the specificity of the DARPP-32 immunoassay procedures.

Various peripheral nervous tissues were also analyzed for DARPP-32. Both bovine and rabbit superior cervical sympathetic ganglion contained DARPP-32. Although DARPP-32 was not detected in the anterior pituitary gland (adenohypophysis), low levels were measured in the posterior pituitary (neurohypophysis). Low levels of DARPP-32 were also measured in rat pineal gland, bovine adrenal medulla, isolated bovine adrenal chromaffin cells, and bovine choroid plexus. The identification of DARPP-32 in superior cervical sympathetic ganglion, isolated adrenal chromaffin and parathyroid cells, posterior pituitary, adrenal medulla, and choroid plexus by RIA was confirmed by immunolabeling and immunoprecipitation and by phosphopeptide mapping (data not shown).

#### Phylogenetic survey of DARPP-32 immunoreactivity

The RIA for DARPP-32 was used to carry out a survey of DARPP-32 immunoreactivity in the CNS of representative vertebrate and invertebrate species (Table 4), which included members of four classes of subphylum Vertebrata (bony fishes, amphibians, reptiles, and birds) and members of two invertebrate phyla—Arthropoda (*Homarus* and *Drosophila*) and Mollusca (*Aplysia*). No DARPP-32 immunoreactivity was detected when

**Table 3.** Distribution of DARPP-32 in various tissues

Tissue	Immunoreactive DARPP-32 (pmol/mg total protein)
Lung, rat	<0.2
Liver, rat	<0.2
Heart, rat	<0.2
Kidney, rat	<0.2
Spleen, rat	<0.2
Pancreas, rat	<0.2
Skeletal muscle, rat	<0.2
Skeletal muscle, rabbit	<0.2
Splenic artery, rabbit	<0.2
Mesenteric artery, rabbit	<0.2
Renal artery, rabbit	<0.2
Aorta, rabbit	<0.2
Parathyroid gland, cow	<0.2
Isolated parathyroid cells, cow	0.3 $\pm$ 0.1
Anterior pituitary, rat	<0.2
Posterior pituitary, rat	1.6 $\pm$ 0.4
Pineal gland, rat	6.0 $\pm$ 2.2
Superior cervical ganglion, rabbit	2.0 $\pm$ 0.3
Superior cervical ganglion, cow	3.8 $\pm$ 0.5
Adrenal medulla, cow	2.1 $\pm$ 0.4
Isolated adrenal chromaffin cells, cow	2.9 $\pm$ 0.6
Choroid plexus from lateral ventricle, cow	6.7 $\pm$ 2.1

Values represent the means  $\pm$  SEM of at least three experiments, each employing two tissue concentrations assayed in triplicate, calculated using a molecular mass of 22,591 Da obtained by amino acid sequencing of bovine brain DARPP-32 (Williams et al., 1986).

CNS samples containing up to 25  $\mu$ g of total protein from members of the two invertebrate phyla or from two of the vertebrate classes—the bony fishes (*Osteichthyes*) and the amphibians (*Amphibia*)—were analyzed. However, significant levels of DARPP-32 were observed in CNS samples from members of both the reptilian (*Reptilia*) and bird (*Aves*) classes. In the turtle, the highest amount of DARPP-32 immunoreactivity was measured in the basal forebrain; lower amounts were measured in the midbrain and dorsal neocortex. In the canary, DARPP-32 immunoreactivity was highly enriched in the paleostriatum; lower amounts were detected in the neostriatum and cerebellum.

The DARPP-32 immunoreactivity in the vertebrate species was further characterized by immunolabeling and immunoprecipitation. No immunolabeled proteins or immunoprecipitated phosphoproteins were detected in crude cytosol or acid extracts prepared from carp or frog CNS (Figs. 5, 6). However, both immunolabeling (Fig. 5) and immunoprecipitation of cAMP-stimulated phosphoproteins (Fig. 6) identified DARPP-32-immunoreactive proteins in turtle and canary brain extracts, with apparent  $M_r$  values of 44,000 and 32,500, respectively. The rank orders of the levels of the DARPP-32 identified in various turtle and canary brain regions by immunolabeling and immunoprecipitation agreed with those obtained using the RIA. One-dimensional phosphopeptide mapping using *S. aureus* V8 protease of the immunoprecipitated phosphoproteins obtained from turtle basal forebrain and canary paleostriatum yielded phosphopeptide maps similar to that of bovine DARPP-32 (data not shown).

#### Discussion

The RIA employing monoclonal antibody C24-13a was sensitive to DARPP-32 in the range of 5–80 fmol per assay well.

Table 4. Phylogenetic survey of DARPP-32 immunoreactivity using radioimmunoassay

Class	Region	Immunoreactive DARPP-32 (pmol/mg total protein)
Subphylum Vertebrata		
Amniotes		
<i>Aves</i> ( <i>Serinus canarius</i> , canary)	Paleostriatum	85.0 $\pm$ 10.4
	Caudal neostriatum	10.6 $\pm$ 4.7
	Rostral neostriatum	7.9 $\pm$ 2.5
	Cerebellum	2.4 $\pm$ 1.0
<i>Reptilia</i> ( <i>Pseudemys scripta</i> , turtle)	Basal forebrain	37.1 $\pm$ 5.4
	Midbrain	12.6 $\pm$ 3.6
	Dorsal neocortex	7.7 $\pm$ 2.2
Anamniotes <sup>a</sup>		
<i>Amphibia</i> ( <i>Rana pipiens</i> , frog)	Cerebrum	<0.2
	Midbrain	<0.2
	Cerebellum	<0.2
<i>Osteichthyes</i> ( <i>Cyprinus carpio</i> , carp)	Cerebrum	<0.2
	Midbrain/thalamus	<0.2
	Mesencephalon	<0.2
	Cerebellum	<0.2
Phylum Arthropoda <sup>a</sup>		
<i>Crustacea</i> ( <i>Homarus americanus</i> , lobster)	Nervous system	<0.2
<i>Insecta</i> ( <i>Drosophila melanogaster</i> , fly)	Head	<0.2
Phylum Mollusca <sup>a</sup>		
<i>Gastropoda</i> ( <i>Aplysia californica</i> , sea snail)	Nervous system	<0.2

Values represent the means  $\pm$  SEM of at least three experiments, each employing two tissue concentrations assayed in triplicate, calculated using a molecular mass of 22,591 Da obtained by amino acid sequencing of bovine brain DARPP-32 (Williams et al., 1986).

<sup>a</sup> Values were also <0.2 pmol/mg total protein when determined with RIA using antiserum R2.

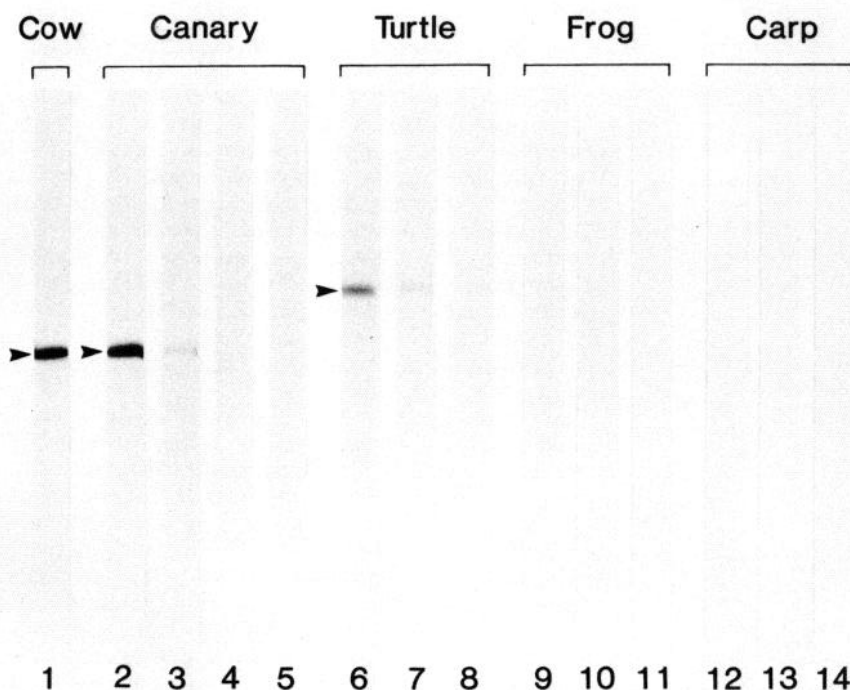
The sensitivity of the RIA was enhanced by using a nonequilibrium assay in which the antibody was preincubated with the samples prior to a short incubation with the labeled tracer (sequential saturation conditions), as has been demonstrated for the measurement of creatine kinase isoenzymes (Van Steirteghem et al., 1978). The antibodies did not distinguish between phospho- and dephospho-DARPP-32; thus, the RIA provides an estimate of the total immunoreactive DARPP-32 present, in contrast to the phosphorylation assay, which measures only the amount of dephospho-DARPP-32 (Walaas and Greengard, 1984). This suggests that monoclonal antibody C24-13a does not recognize the phosphorylation site or a determinant affected by phosphorylation of DARPP-32.

The RIA for DARPP-32 was used to survey the distribution of DARPP-32 immunoreactivity in microdissected regions of rat CNS. The highest concentrations of immunoreactive DARPP-32 were found in substantia nigra, caudatoputamen, and globus pallidus, and slightly lower concentrations were found in olfactory tubercle and nucleus accumbens. The concentrations of DARPP-32 in these five brain regions were approximately 10-fold higher than those in the other CNS regions examined. Four of the regions containing the highest levels of DARPP-32 (substantia nigra, caudatoputamen, nucleus accumbens, and olfactory tubercle) are also characterized by high dopamine-sensitive adenylate cyclase activities (Clement-Cormier et al., 1974; Horn et al., 1974; Keabian and Saavedra, 1976; Keabian et al., 1972; Krieger et al., 1977; Phillipson and Horn, 1976; Premont et al., 1976; Spano et al., 1976; for reviews, see Iversen, 1975, 1977; Miller and McDermed, 1979), by a high density of dopaminergic axon terminals or dendrites (Cheramy et al., 1981; Fuxe, 1965; Hökfelt et al., 1977; Lindvall and Björklund, 1974;

Ungerstedt, 1971; for reviews, see Björklund and Lindvall, 1984; Lindvall and Björklund, 1978; Moore and Bloom, 1978), by high dopamine concentrations (Brownstein et al., 1974; Versteeg et al., 1976), and by high levels of tyrosine hydroxylase activity (Bacopoulos and Bhatnagar, 1977), the rate-limiting enzyme in dopamine biosynthesis. The caudatoputamen, nucleus accumbens, and olfactory tubercle receive most of the terminals of the nigrostriatal and mesolimbic dopamine system neurons (Lindvall and Björklund, 1978), while the globus pallidus and substantia nigra are the main anatomical targets of these three dopamine-innervated brain regions.

Light- and electron-microscopic immunocytochemistry (Oui-met et al., 1983, 1984) and lesion studies (Walaas and Greengard, 1984) have been used to show that DARPP-32 is present in the cytoplasm of cell bodies, dendrites, axons, and axon terminals of the medium-sized spiny neurons of the caudatoputamen and nucleus accumbens and in the axons and axon terminals of the striatonigral and striatopallidal fibers, which originate from the medium-sized spiny neurons and terminate in the substantia nigra pars reticulata, globus pallidus, and ventral pallidum. The large amounts of DARPP-32 found in the substantia nigra and globus pallidus by RIA are probably due to its presence in the axons and axon terminals of striatonigral and striatopallidal neurons. In addition to constituting the terminal field of the dopaminergic striatal neurons, the globus pallidus also receives a modest dopaminergic innervation (Lindvall and Björklund, 1979). Similarly, dopamine is released in the substantia nigra pars reticulata, presumably from the dendrites of dopamine neurons (Cheramy et al., 1981). In summary, DARPP-32 is enriched in dopamine-innervated regions of the basal ganglia, probably in dopaminergic cells that contain





**Figure 5.** Phylogenetic survey of DARPP-32-immunoreactive proteins in the CNS of representatives of the five vertebrate classes by immunolabeling. Tissue samples were homogenized in SDS, subjected to SDS/PAGE (10% acrylamide), and electrophoretically transferred to nitrocellulose membranes as described in Materials and Methods. The nitrocellulose membranes were labeled with a 1:1000 dilution of antibody C24-13a followed by  $^{125}\text{I}$ -labeled goat anti-mouse Ig's, and analyzed by autoradiography. Lane 1 contains 75  $\mu\text{g}$  of total protein and lanes 2–14 contain 150  $\mu\text{g}$  of total protein each. Lane 1, Bovine caudate nucleus; lanes 2–5, canary paleostriatum, caudal neostriatum, rostral neostriatum, and cerebellum, respectively; lanes 6–8, turtle basal forebrain, midbrain, and dorsal neocortex, respectively; lanes 9–11, frog cerebrum, midbrain, and cerebellum, respectively; lanes 12–14, carp cerebrum, midbrain/thalamus, and cerebellum, respectively. The positions of the immunolabeled DARPP-32 are indicated by arrowheads.

D-1 dopamine receptors (Ouimet et al., 1983, 1984; Walaas and Greengard, 1984).

The distribution of DARPP-32 in other regions of the brain is also generally consistent with its localization in dopaminergic cells possessing D-1 dopamine receptors (for reviews, see Hemmings et al., 1986b; Walaas et al., 1986). There is now considerable evidence for the existence of a restricted dopamine input to the hippocampus (Ishikawa et al., 1982; Verney et al., 1985), where a dopamine-sensitive adenylate cyclase has also been demonstrated (Dolphin et al., 1979). The relatively low levels of DARPP-32 in some brain regions, such as the amygdala and hypothalamus, which are known to contain dense dopaminergic innervation and dopamine-sensitive adenylate cyclase activity (Clement-Cormier and Robison, 1977), may be explained by DARPP-32 being located in restricted populations of cells (Ouimet et al., 1984). The low concentration of DARPP-32 in frontal cortex appears to reflect a diffuse dopaminergic innervation primarily to the basal layers of this brain region, again with the concomitant localization of DARPP-32 to a subpopulation of neurons. The slightly higher levels of DARPP-32 in whole cerebral cortex may reflect the inclusion of several regions of the limbic cortex (cingulate, piriform, and entorhinal cortex) that contain DARPP-32 as determined by immunofluorescent labeling (Ouimet et al., 1984). These regions of the cerebral cortex also receive most of the cortical dopaminergic innervation and contain relatively high dopamine-sensitive adenylate cyclase activities (Tassin et al., 1978). Dopamine is present in vertebrate retina, where it appears to function as a neurotransmitter released by a subclass of intrinsic amacrine cells (Ehinger, 1977; Kramer, 1971), and dopamine-sensitive adenylate cyclase has been identified in homogenates of both rat and bovine retina (Brown and Makman, 1972). The septum, pons/medulla, olfactory bulb, and spinal cord, which contain low levels of DARPP-32 immunoreactivity, also contain low levels of dopamine (Holzbauer and Sharman, 1979) and, in some cases, identifiable dopaminergic axon terminals (Lindvall

and Björklund, 1978). The thalamus and cerebellum contain appreciable amounts of DARPP-32 but little dopamine (Versteeg et al., 1976) and apparently few dopaminergic axon terminals (Lindvall and Björklund, 1978).

Although the distribution of DARPP-32 in the CNS measured by RIA (this study) or by a phosphorylation assay (Walaas and Greengard, 1984) roughly parallels the distribution of dopamine-containing axon terminals, and more closely parallels the distribution of dopaminergic neurons possessing dopamine-sensitive adenylate cyclase (D-1 dopamine receptors coupled to adenylate cyclase), it is not quantitatively proportional to either the amount of dopaminergic innervation or of dopamine-sensitive adenylate cyclase activity in a particular region. There are several factors that may contribute to this disparity. First, in those dopamine-innervated regions that possess only D-2 dopamine receptors, DARPP-32 should not be present. Second, as a cytosolic protein (Walaas and Greengard, 1984), the amount of DARPP-32 in some brain regions may reflect the combined cell volume of the dopaminergic cells rather than the amount of dopamine input or dopamine-sensitive adenylate cyclase present. Third, apparent anomalies may exist when D-1 dopaminergic cells in one brain region, which contain DARPP-32 and receive a dopamine input, send processes, which contain DARPP-32, to another brain region that does not receive a dopamine input. Fourth, recent studies have demonstrated that the distribution of D-2 dopamine receptors within the brain may be greater than that known for dopaminergic innervation (Martres et al., 1985), a condition that may pertain to D-1 dopamine receptors as well. The monoaminergic neurotransmitters can probably diffuse a considerable distance through the brain (Reader et al., 1979), suggesting the possibility that in these cases DARPP-32 may identify certain neurons that are "physiologically" postsynaptic to dopamine, even in the absence of direct synaptic contact (Ouimet et al., 1984; Schmitt, 1984). Fifth, in some brain regions, a minor dopaminergic input may have escaped detection using the available methods. And finally,

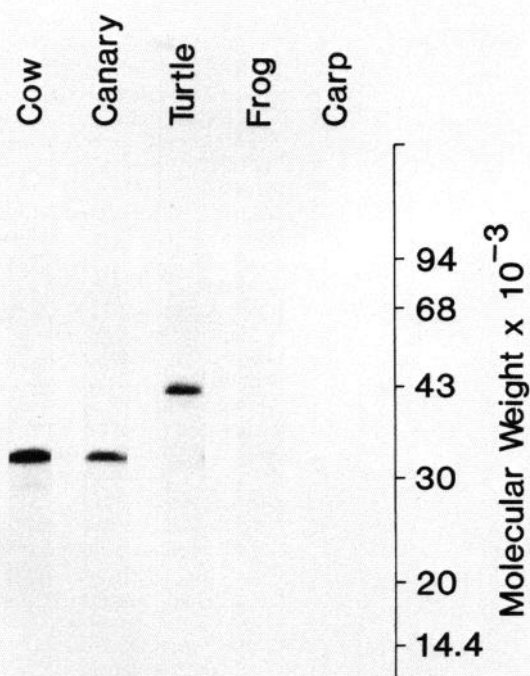


Figure 6. Identification by immunoprecipitation of phosphorylated DARPP-32-immunoreactive proteins in the CNS of representatives of the five vertebrate classes. Crude cytosol was prepared from samples of bovine caudate nucleus, canary paleostriatum, turtle basal forebrain, frog cerebrum, and carp cerebrum and subjected to endogenous phosphorylation with 8-bromo-cAMP and  $\gamma$ - $^{32}$ P-ATP followed by immunoprecipitation with 2  $\mu$ l of monoclonal antibody C24-13a. Immunoprecipitated proteins were subjected to SDS/PAGE (10% acrylamide) and analyzed by autoradiography. Samples of cytosol containing 100  $\mu$ g of total protein (cow and canary) or 200  $\mu$ g of total protein (turtle, frog, and carp) were used.

DARPP-32 may in some cases be able to act as an effector for neurotransmitters other than dopamine (e.g., norepinephrine) acting through cAMP, as suggested by the presence of DARPP-32 in the cerebellum.

The amounts of DARPP-32 determined by RIA in various regions of rat brain are higher than those determined by use of a phosphorylation assay (Walaas and Greengard, 1984), although the rank order of the levels is comparable. Possible explanations for the lower values obtained in the phosphorylation assay include: (1) interference by protein kinase inhibitors and/or inaccessibility of the protein kinase to DARPP-32 as a result of binding or occlusion, (2) the presence of phosphorylated DARPP-32 in the samples, and (3) endogenous protease or protein phosphatase activity. Our experience suggests that measurements made with the RIA more accurately reflect DARPP-32 levels.

Phosphoproteins immunologically related to bovine DARPP-32 were identified in mouse, rat, guinea pig, rabbit, and rhesus monkey brain, and were present in comparable amounts. DARPP-32 constituted from 0.22 to 0.32% of the total protein in the neostriatum of these six species representing four mammalian orders, an amount comparable to that of synapsin I, a well-characterized synaptic vesicle-associated phosphoprotein that appears to be involved in the regulation of neurotransmitter release (De Camilli et al., 1983; Huttner et al., 1983; Llinás et al., 1985) and that has also been shown to exist in several mammalian orders (Goelz et al., 1985). The apparent  $M_r$  values of the homologous forms of DARPP-32, as determined by SDS/PAGE, differed slightly.

Immunocytochemical studies of the cellular localization of DARPP-32 in rat caudoputamen have revealed that DARPP-32 is present in at least 60% of the medium-sized spiny neurons (Ouimet et al., 1983, 1984), which make up approximately 96% of the neurons in this nucleus (Groves, 1983). Assuming that these neurons make up about 50% of the volume of the caudoputamen, the estimated concentration of DARPP-32 within these neurons is on the order of 20–50  $\mu$ M. This concentration is about  $10\times$  higher than the apparent  $K_m$  values of DARPP-32 for phosphorylation by cAMP-dependent protein kinase (Hemmings et al., 1984c) and for dephosphorylation by protein phosphatase-2B (calcineurin; King et al., 1984), and  $10^4\times$  higher than the apparent  $K_i$  value (0.5–2.2 nM) of phospho-DARPP-32 for inhibition of protein phosphatase-1 *in vitro* (Hemmings et al., 1984a), suggesting that these reactions would be favorable kinetically *in vivo*.

In contrast to brain, most peripheral tissues examined contained less than 0.2 pmol DARPP-32/mg tissue protein as determined by RIA. Of the non-nervous tissues tested, only adrenal medulla, isolated bovine adrenal chromaffin and parathyroid cells, and choroid plexus contained detectable levels of DARPP-32 by RIA, immunolabeling and immunoprecipitation. Dopamine-sensitive adenylate cyclase has not been detected in bovine adrenal chromaffin cells (Serck-Hanssen et al., 1972; Gutman and Boonyaviroj, 1979). However, bovine parathyroid cells contain both D-1 dopamine receptors (Brown et al., 1977) and cAMP-dependent protein kinase (Brown and Thatcher, 1982), which may be involved in the regulation of DARPP-32 phosphorylation through cAMP. The presence of DARPP-32 in the choroid plexus may be related to the physiological effects of dopamine observed in this neuroectodermal secretory epithelium (Townsend et al., 1984), although a dopamine-sensitive adenylate cyclase has not been conclusively demonstrated (Nathanson, 1980). Recently, DARPP-32 has been demonstrated in another neuroectodermal secretory epithelium, the nonpigmented epithelial layer of the ciliary body (Stone et al., 1985, 1986).

DARPP-32 was not detected in kidney or in any of the peripheral vascular tissues tested. Renal vasculature and certain other vascular tissues are known to contain a cardiovascular dopamine receptor, known as the DA-1 dopamine receptor, which is coupled to the stimulation of adenylate cyclase and which is similar to the D-1 dopamine receptor of nervous and endocrine tissues in its pharmacological properties (for review, see Stoof and Kebabian, 1984). The absence of detectable DARPP-32 in kidney and peripheral vascular tissues may indicate a difference in the postreceptor mechanisms of D-1 and DA-1 dopamine receptors, or the amounts present in these tissues may be below the sensitivity of the RIA.

Studies of peripheral nervous tissues demonstrated that DARPP-32 was present in the posterior pituitary, which receives a dopamine input (Björklund et al., 1973) and possesses both D-1 and D-2 dopamine receptors. In contrast, DARPP-32 was not detectable in the anterior pituitary, which has only D-2 dopamine receptors (Kebabian and Calne, 1979); this provides an example of a dopamine-innervated tissue that possesses only D-2 dopamine receptors and does not contain DARPP-32.

DARPP-32 was demonstrated in both rabbit and bovine superior cervical sympathetic ganglion by RIA, immunolabeling, and immunoprecipitation. This ganglion contains dopamine in its small, intensely fluorescent cells (Björklund et al., 1970), and a dopamine-sensitive adenylate cyclase has been demonstrated also (Kebabian and Greengard, 1971), suggesting a role for DARPP-32 in mediating some of the effects of dopamine in this tissue.

The RIA was also used to carry out a phylogenetic survey of DARPP-32 distribution. Phosphoproteins antigenically related

to DARPP-32 were detected by RIA, immunolabeling and immunoprecipitation in specific regions of bird and reptile CNS. The apparent  $M_r$  of turtle DARPP-32 was significantly higher than that of any other species tested, presumably due to differences in its primary structure. Relatively strong DARPP-32 immunoreactivity was observed in canary paleostriatum and turtle basal forebrain. The results from canary (Burd et al., 1985; and G. Burd, H. C. Hemmings, Jr., J. Heinz, P. Greengard and F. Nottebohm, unpublished observations) and turtle (C. C. Ouimet, H. C. Hemmings, Jr., G. Burd and P. Greengard, unpublished observations) have been confirmed by immunocytochemical studies.

In both canary and turtle (as in rat), the brain regions that contain the highest levels of DARPP-32 (paleostriatum and basal forebrain, respectively) are regions that receive major catecholaminergic inputs and are thought to be homologous to mammalian neostriatum (Parent et al., 1984; Reiner et al., 1984). The avian paleostriatum, the acknowledged homolog of the mammalian neostriatum (Brauth et al., 1978), receives a major ascending catecholaminergic projection, probably dopaminergic. The turtle striatum, which is included in the basal forebrain dissection, also receives an ascending catecholaminergic input consisting primarily of dopamine (Juorio, 1973; Parent, 1979). The cellular localization of DARPP-32 in these species by immunocytochemical methods should yield valuable information concerning the phylogenetic development and comparative anatomy of D-1 dopaminergic neurons in the vertebrates, particularly within the basal ganglia.

No DARPP-32 immunoreactivity was detected by RIA, immunolabeling, or immunoprecipitation in CNS tissue from the two anamniote vertebrate classes, the amphibians and bony fishes, or in nervous tissue from three invertebrate classes, using either antiserum R2 or monoclonal antibody C24-13a. This could be due either to antigenic differences between the DARPP-32 of these species and bovine DARPP-32, the presence of levels of DARPP-32 below the sensitivity of the assay techniques, or the absence of a DARPP-32 homolog in these species. The presence of DARPP-32 in the amniote vertebrates (reptiles, birds, and mammals) and its apparent absence in anamniote vertebrates (bony fishes and amphibians) parallels the striking differences in the organization and relative development of the central catecholamine systems in these two vertebrate groups (see Parent et al., 1984). Thus, in the amniotes there is hypertrophy of the midbrain catecholamine neuronal population along with an increased dopaminergic projection to the striatum. The results of the study of the phylogenetic distribution of DARPP-32 in a limited number of species suggest that the phylogenetic development of DARPP-32 may correspond to that of the mesostriatal dopamine pathways. It will be important to test this hypothesis by the analysis of additional species from various vertebrate orders.

RIA provides a sensitive assay for the direct measurement of DARPP-32 levels and is free of many of the potential problems associated with quantitative measurements by the phosphorylation assay. The regional distribution of DARPP-32 in the mammalian CNS determined by RIA exhibits a remarkable correlation with the distribution of dopamine-containing axon terminals and dopamine-sensitive adenylate cyclase, although several possible exceptions involving less well characterized brain regions have been observed. Its distribution in peripheral tissues may also correspond to the distribution of dopaminergic neurons possessing D-1 dopamine receptors, although the amounts are considerably lower than those found in the CNS. The phylogenetic distribution of DARPP-32 appears to parallel the evolutionary development of the midbrain dopaminergic projection to the striatum. The determination of the normal distribution and concentration of DARPP-32 in various brain regions and peripheral tissues by immunochemical methods

provides the necessary basis for examining alterations in its concentration resulting from various pharmacological treatments and pathological processes.

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