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Dopaminergic neurons from embryonic mouse mesencephalon are enriched in culture through immunoreaction with monoclonal antibody to neural specific protein 4 and flow cytometry

(catecholaminergic neurons/tyrosine hydroxylase/fluorescence-activated cell sorting/membrane determinants/development)

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ABSTRACT **Dopaminergic neurons represent a rare neurotransmitter phenotype within the mammalian central nervous system. The mesencephalic dopaminergic neurons form the ascending dopaminergic pathways in mammals and are involved in motor and limbic functions. Here we report that about 30% of all developing mouse mesencephalic cells, including virtually all of the dopaminergic phenotype, express surface membrane determinant(s) recognized by a monoclonal antibody to neural specific protein 4 (NSP4). We have been able to isolate and culture neurons from the mesencephalon according to their expression of NSP4, using the anti-NSP4 immunoreaction in conjunction with fluorescence-activated cell sorting. Cultures of NSP4$^+$-sorted cells showed a significant enrichment in three morphologically distinct putative dopaminergic phenotypes when compared to unsorted mesencephalic cultures, whereas the cultures of NSP4$^-$-sorted cells were virtually devoid of dopaminergic neurons. This flow cytometric enrichment in dopaminergic neurons should provide the necessary cells for multidisciplinary study of dopaminergic phenotype differentiation.**

In a pilot study (1) we found that a monoclonal antibody to neural specific protein 4 (NSP4) (2), which is expressed on glia and neurons from different central nervous system (CNS) regions at different times in development (2, 3), bound in an apparently surface-reactive manner both to neurons cultured for several weeks from the embryonic mouse mesencephalon and to freshly dissociated embryonic mesencephalic cells. The mesencephalon contains, among other cellular phenotypes, dopaminergic neurons of the substantia nigra and the ventral tegmental area (4). Abnormality of dopaminergic transmission in humans contributes to Parkinson disease (5, 6). We have used fluorescence-activated cell sorting (FACS) in conjunction with NSP4 immunoreaction to isolate and culture cells from the embryonic mouse mesencephalon on the basis of their NSP4 expression. Here we show that NSP4$^+$-sorted cultures are enriched about 10-fold in three morphologically distinct dopaminergic phenotypes. Thus NSP4 is expressed on embryonic dopaminergic neurons, and its expression will permit us to enrich monolayer cultures in this relatively rare phenotype for multidisciplinary analysis of dopaminergic phenotype differentiation.

MATERIALS AND METHODS

Anti-NSP4 Antibody. The antibody is an IgM produced by a rat hybridoma and has been characterized previously (2).

Immunoblot Analysis of NSP4-Reactive Proteins. The methods used in preparing detergent extracts and immunodetection of the polypeptide chains after gel electrophoresis have been described elsewhere (7). The amount of protein loaded on top of each gel lane was 300 μg. The antibody reaction was carried out for 24 hr at 4°C and detected by reaction with ^{125}I-labeled rabbit anti-rat immunoglobulin (10 μCi/μg, Amersham; 1 Ci = 37 GBq) followed by autoradiography on x-ray film (Kodak) for 16 hr.

Cell Suspension and Tissue Culture. Cell suspensions were prepared as described (8, 9) using NIH albino Swiss mice at embryonic day (E) 13 from the mesencephalic region rich in dopaminergic neurons (10). In some experiments cell suspensions from E13–E15 spinal cord, cortex, striatum, and liver were used for FACS analysis. After dissociation, cell viability was about 90% as estimated by FACS analysis (see below) after incorporation of the dye ethidium bromide (EtdBr), which accumulates in dead cells (11). About 500,000 cells were plated in 35-mm Petri dishes (Falcon) or 20,000 in 1-cm^2 wells (Lab-Tek, Naperville, IL), either on poly(L-ornithine) (Sigma) (8) or cultured monolayers of cortical astrocytes (12). The cultures were kept at 37°C in 5% CO_2/95% air and saturated with water vapor.

Immunoreaction on Cells in Suspension. The cells in suspension (2×10^6/ml) were incubated for 1 hr at 22°C with undiluted culture medium from anti-NSP4-secreting hybridoma cells, washed with Dulbecco's phosphate-buffered saline (PBS) and then incubated for indirect immunofluorescence with fluorescein isothiocyanate-conjugated F(ab')$_2$ fragments of goat anti-rat IgG (both heavy and light chains) (Jackson ImmunoResearch, Avondale, PA) or IgM (Cappel Laboratories, Cochranville, PA) diluted 1:200 in PBS (anti-rat IgG and IgM gave comparable staining results). Cells were washed once in PBS, resuspended in PBS containing 3% fetal bovine serum (3×10^6 per ml), and filtered through a 62-μm-pore filter (Nitex) prior to FACS analysis and sorting. In control reactions the primary antibody was omitted.

FACS Analysis and Sorting. Between 20,000 and 25,000 events per sample were routinely analyzed by using a FACS 440 (Becton Dickinson) (11). For sorting, the FACS sheath buffer contained PBS with 1% FBS and the laser power was kept below 100 mW to reduce possible cell damage. The fluorescence window was set several channels above the background fluorescence associated with control cell suspensions. Live NSP4$^+$ and NSP4$^-$ cells in the relatively high region of forward-angle light scatter (FALS) were collected in separate tubes. Small aliquots of sorted cells were reanalyzed with the FACS to monitor their degree of homogeneity. Data are all expressed as dual-parameter (light scattering versus intensity of fluorescence) frequency distri-

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Abbreviations: CNS, central nervous system; E*n*, embryonic day *n*; TyrOHase, tyrosine hydroxylase; NSP4, neural specific protein 4; NCAM, neural cell adhesion molecule; NF, neurofilament; GFAP, glial fibrillary acidic protein; EtdBr, ethidium bromide; FACS, fluorescence-activated cell sorter (or sorting); FALS, forward angle light scatter.

butions in the form of three-dimensional "hidden-line" perspectives and contour plots (11).

Culture of FACS-Sorted Cells. The sorted cells were centrifuged, resuspended in culture medium, and plated in 1-cm^2 wells on feeder astrocytes, as above.

Labeling of Cells in Culture. In all cases surface immunoreaction was carried out on live cells. Unfixed cultures were labeled as above with anti-NSP4 or rabbit polyclonal antibodies to neural cell adhesion molecule (NCAM) (13), rinsed, and fixed in paraformaldehyde (13) prior to the reaction with fluorescent secondary antibodies. When stained for intracellular antigens, cells were permeablized after fixation with 0.1% Triton X-100. A mouse monoclonal antibody against the 200-kDa neurofilament (NF) subunit (1:1000, Labsystem Oy, Helsinki, Finland) or rabbit antiserum against glial fibrillary acidic protein (GFAP) (1:300, Accurate Chemicals, Westbury, NY) with fluorescein- or rhodamine-conjugated rat anti-mouse IgG (Boehringer Mannheim) or goat anti-rabbit IgG (Jackson ImmunoResearch) at dilutions 1:50 to 1:200 was used to identify neurons and astrocytes, respectively. Catecholaminergic neurons were visualized with a rabbit antiserum to tyrosine hydroxylase (TyrOHase; EC 1.14.16.2) (Eugene Tech Inc., Allendale, NJ) (1:1000) and fluorescent anti-rabbit IgG as above or by aldehyde-induced catecholamine fluorescence (14). Substitution of normal rat serum for anti-NSP4, normal mouse serum for anti-NF, or normal rabbit serum for anti-TyrOHase, anti-GFAP, or anti-NCAM antibodies (1:1000) resulted in no specific staining (background levels). In double-staining experiments the two secondary antibodies were labeled with different fluorochromes. Due to high cross-reactivity between rat and mouse anti-immunoglobulin, anti-NF and anti-NSP4 double labeling could not be done.

Fluorescence microscopy was performed on a Zeiss Photomicroscope III. Percent (±SD) of NSP4$^+$ cells in cultures was obtained by counting in 10 randomly chosen fields per plate (one field corresponded to 0.3 mm^2) of fluorescent cells and unstained process-bearing cell bodies (putative neurons) under combined or alternate phase-contrast and fluorescence optics; results from different experiments were pooled. In some experiments rhodamine-labeled TyrOHase$^+$ neurons were compared with fluorescein-labeled NF$^+$ cells and the total number of catecholaminergic neurons was determined. Given that catecholaminergic neurons are sparse and randomly distributed in these cultures, standard deviations were not calculated.

RESULTS

The anti-NSP4 antibody recognizes an antigenic determinant probably shared by a family of glycoproteins (2). Immunoblots of embryonic, postnatal, and adult mouse brain extracts (Fig. 1) showed that several proteins reacted with the anti-NSP4 antibody. In E13 and E16 tissues (lanes 1 and 2) three major bands of 140, 200, and 300 kDa (also seen as a doublet of 300/280 kDa) were identified, the 300-kDa band being the most prominent. In extracts from E13 and E16 mesencephalon the 300-kDa protein was the major band revealed (not shown). A less intense band of 170 kDa was present at E16, postnatally (lanes 3 and 4), and in the adult (lane 5). An apparently transient 120-kDa protein appeared postnatally. The 120-kDa polypeptide disappeared from adult extracts, while the other bands were preserved, with the addition of a faint band of about 52 kDa. None of these radioactive bands were detectable when an irrelevant monoclonal antibody of the same class as anti-NSP4 was used with iodinated secondary antibodies (lane 6). The anti-NSP4 did not react with postnatal day 7 muscle extracts (not shown).

These findings show that several molecular weight classes of proteins are recognized by the NSP4 antibody. They also revealed a developmental regulation of the expression of

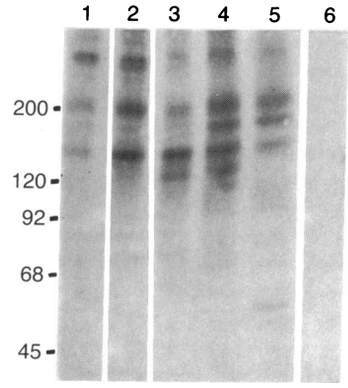

FIG. 1. Developmental expression of NSP4-reactive antigens. The figure illustrates autoradiographic immunodetection of detergent extracts from E13 (lane 1) and E15 (lane 2), postnatal days 1 (lane 3) and 5 (lane 4), and adult mouse brain (lane 5) that had reacted with anti-NSP4 antibody and ^{125}I-labeled anti-rat immunoglobulin after blotting (7). Lane 6 corresponds to adult brain extract that had reacted with MOPC, an irrelevant rat IgM monoclonal antibody used as a control, and ^{125}I-labeled anti-rat immunoglobulin. Molecular mass markers were myosin (200 kDa), β-galactosidase (120 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), and ovalbumin (45 kDa).

these molecules. Further experiments are required to establish whether the different bands represent different proteins or processing of the 300-kDa antigen. FACS analysis (Fig. 2) showed that the immunohistochemical reaction generated on E13 mesencephalic cell suspensions was complex, with a broad spectrum of NSP4$^+$ signals obtained in all of the analyses carried out thus far. The double-immunofluorescence signals (anti-NSP4 and anti-rat-immunoglobulins) were consistently more intense (Fig. 2 *A2* and *C2*) than nonspecific immunoreaction with secondary antibody alone (Fig. 2 *A1* and *C1*). Incubation of the cells with EtdBr revealed that about 10% of the intact cells were dead (Fig. 2 *B1* and *B2*). Comparison of the FALS values associated with the NSP4$^+$ elements, as well as dual-color FACS analysis of the NSP4$^+$ and EtdBr$^+$ signals together demonstrated that the NSP4 signals were distributed on either vital cells or subcellular debris. Thus some of the NSP4 immunoreaction occurs, as expected, on the surface of intact vital cells. Reanalysis of NSP4$^+$ cells showed that the majority of the cells were not apparently damaged by the labeling and sorting procedure (not shown). By FACS analyses 32 ± 5% of the vital cells were NSP4$^+$ (n = 8). About 15–25% NSP4$^+$ cells were also found in dissociated cell suspensions from other areas of E13–E15 CNS (spinal cord, striatum, cortex), but no positive cells could be detected in liver cells obtained from the same embryos (data not shown).

Vital mesencephalic cells were sorted by flow cytometry according to the intensity of their NSP4 immunoreaction. NSP4$^-$ cells were identified as those expressing low fluorescent signals, similar in intensity to levels associated with the nonspecific secondary immunoreaction (Fig. 2*C1*), while NSP4$^+$ cells were characterized by fluorescence intensity significantly greater than the secondary immunoreaction signal distribution (Fig. 2*C2*). For comparison, cells that were not sorted were also cultured (unsorted cultures). The sorted cells adhered much less effectively to the substrate normally used for these cultures (polylysine or polyornithine). However, they would attach and survive for several weeks if plated on a 3-week-old monolayer of cortical astrocytes. The rate of cell survival after plating was difficult to evaluate. On average, in 10- to 15-day-old cultures, about 9500 ± 4000 (n = 5) cells were NF$^+$, thus accounting for at least a 50–60% survival. In older cultures high cell loss occurred randomly.

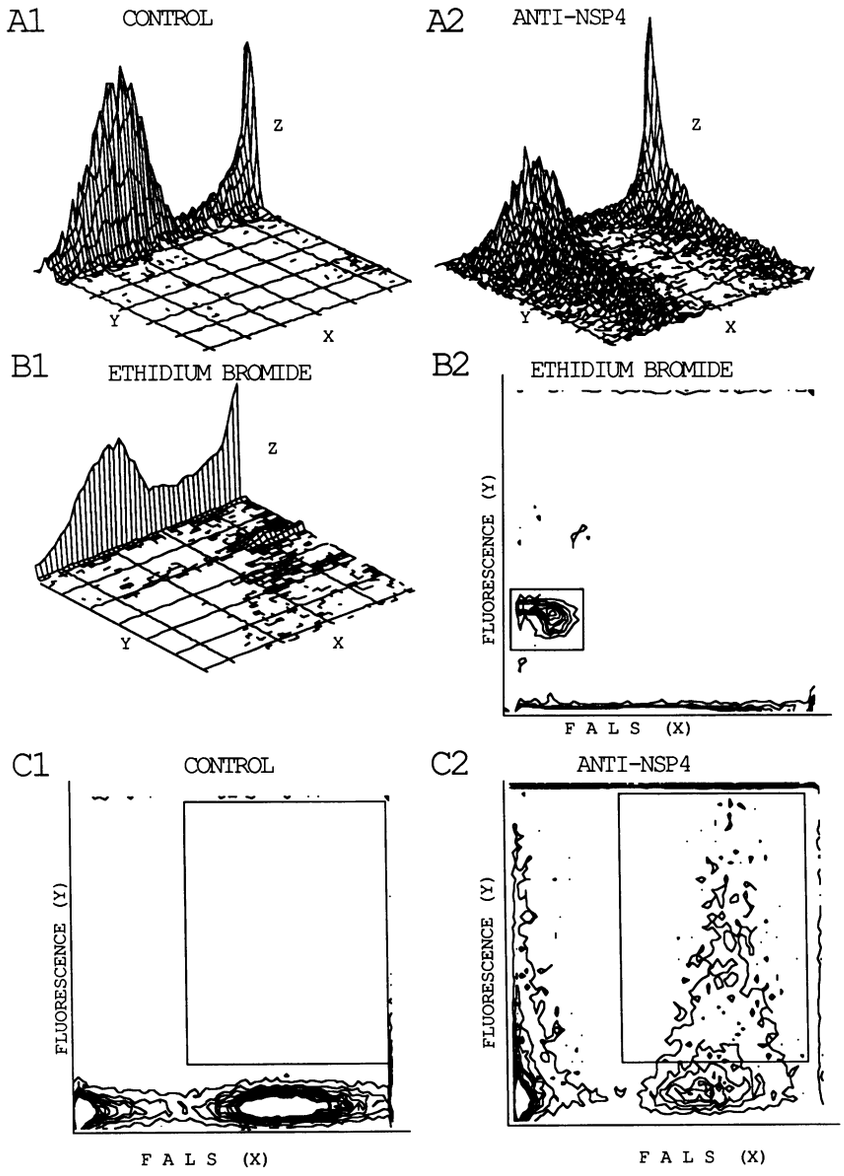

FIG. 2. Anti-NSP4 stains vital embryonic mesencephalic cells. Representative FACS analyses of the NSP4 immunoreaction (*A* and *C*) and nonvital EtdBr stain (*B*) of E13 mouse mesencephalic cell suspensions are displayed in "hidden-line" three-dimensional perspective (*A* and *B1*) and contour plots (*B2* and *C*). The perspectives show frequency (*Z*) distributions of 25,000 events according to FALS (*X*) and fluorescence (*Y*) for the control immunoreaction with fluorescein-coupled secondary antibody alone (*A1* and *C1*), the double immunoreaction with primary anti-NSP4 followed by fluorescein-coupled secondary antibody (*A2* and *C2*), or EtdBr staining (*B1* and *B2*). The perspectives are outlined with grids to allow clearer comparison of the signal distribution. The most intense fluorescence signals accumulating in the last fluorescence channel have been deleted in the perspectives to permit unobstructed views of the signal distribution accounting for more than 95% of the data. Although the axes have not been numbered to simplify the data presentation, the perspectives and contours in *A* and *C* can be compared, since each was acquired and accumulated at the same gain settings on the amplifier. The gain setting in *B* was lower due to the high intensity of EtdBr fluorescence. FACS analysis shows that almost all of the control immunoreaction signals accumulate close to the origin (*A1* and *C1*), while the NSP4 signals are abundantly distributed throughout the fluorescence axis (*A2* and *C2*). EtdBr stains less than 10% of the elements and those that do stain scatter relatively little light (*B1* and *B2*). Thus, those elements that scatter more light and make up the prominent peaks and contours are intact vital cells. The window used to sort NSP4$^+$ cells according to their fluorescence and FALS values is shown superimposed on the contour plots (*C*).

In the cultures the NSP4 labeling was confined to neuron-like cells, decorating perikarya and neurites but not the background layer of glial cells (not shown). In unsorted cultures we found about 20 ± 4% (n = 12) NSP4$^+$ neurons, whereas after NSP4$^+$ sorting, the large majority of neuron-like cells were labeled (86 ± 4% in 16 2-week-old NSP4$^+$-sorted cultures). Cells sorted according to the lack of NSP4 expression remained NSP4$^-$ for several weeks *in vitro* in two experiments, while in three others between 2% and 10% of the neurons became NSP4$^+$ at 2 weeks in culture (not shown). Thus, most of the NSP4 antigen expression on the surface of mesencephalic neurons appears to occur by E13. In sorted and unsorted cultures from E13 mesencephalon the NSP4 expression seemed confined exclusively to neurons. We never detected NSP4 immunoreaction on flat, GFAP$^+$, type-1-like astrocytes (10). Double labeling competition experiments with anti-NSP4 and anti-NCAM on 1- to 2-week-old unsorted cultures (not shown) confirmed that neurons labeled with anti-NSP4 (about 25%) were always also immunolabeled with anti-NCAM, independently of the order of incubation with the antibodies and confirmed that almost all the neurons expressed NCAM. These results suggest that there is little competition between the two antibodies for neurons.

Cultured neurons expressing the catecholaminergic phenotype were identified by using either an anti-TyrOHase immunoreaction or the fluorescent histochemical reaction for catecholamines. Catecholaminergic neurons were sparse in the unsorted cultures (Fig. 3 *A1* and *B1*) and extremely rare within the NSP4$^-$-sorted population (Table 1), whereas they were more abundant, often clustered, in the NSP4$^+$-sorted cultures (Fig. 3 *A2*, *A3*, *B2*, and *B3*).

As shown in Table 1, in five separate experiments there were about 100 TyrOHase$^+$ neurons per cm^2 in NSP4$^+$-sorted cultures, while NSP4$^-$-sorted cultures contained less than 1 catecholaminergic cell per cm^2. Unsorted cultures had about 10 catecholaminergic cells per cm^2. There was interexperiment variability in the absolute numbers of catecholaminergic neurons differentiating in culture. When this was taken into account by computing the ratio of TyrOHase$^+$ neurons in NSP4$^+$ sorted cultures to TyrOHase$^+$ cells in unsorted cultures, there were about 15 times as many TyrOHase$^+$ neurons in the NSP4$^+$-sorted cultures as in the unsorted monolayers. The immunocytochemical and histochemical reactivities of these neurons indicated that they are of the catecholaminergic phenotype, most likely dopaminergic. Examination of the TyrOHase$^+$ neurons in NSP4$^+$-sorted cultures revealed three distinct morphologies corresponding to three different dopaminergic phenotypes (Fig. 4). The more

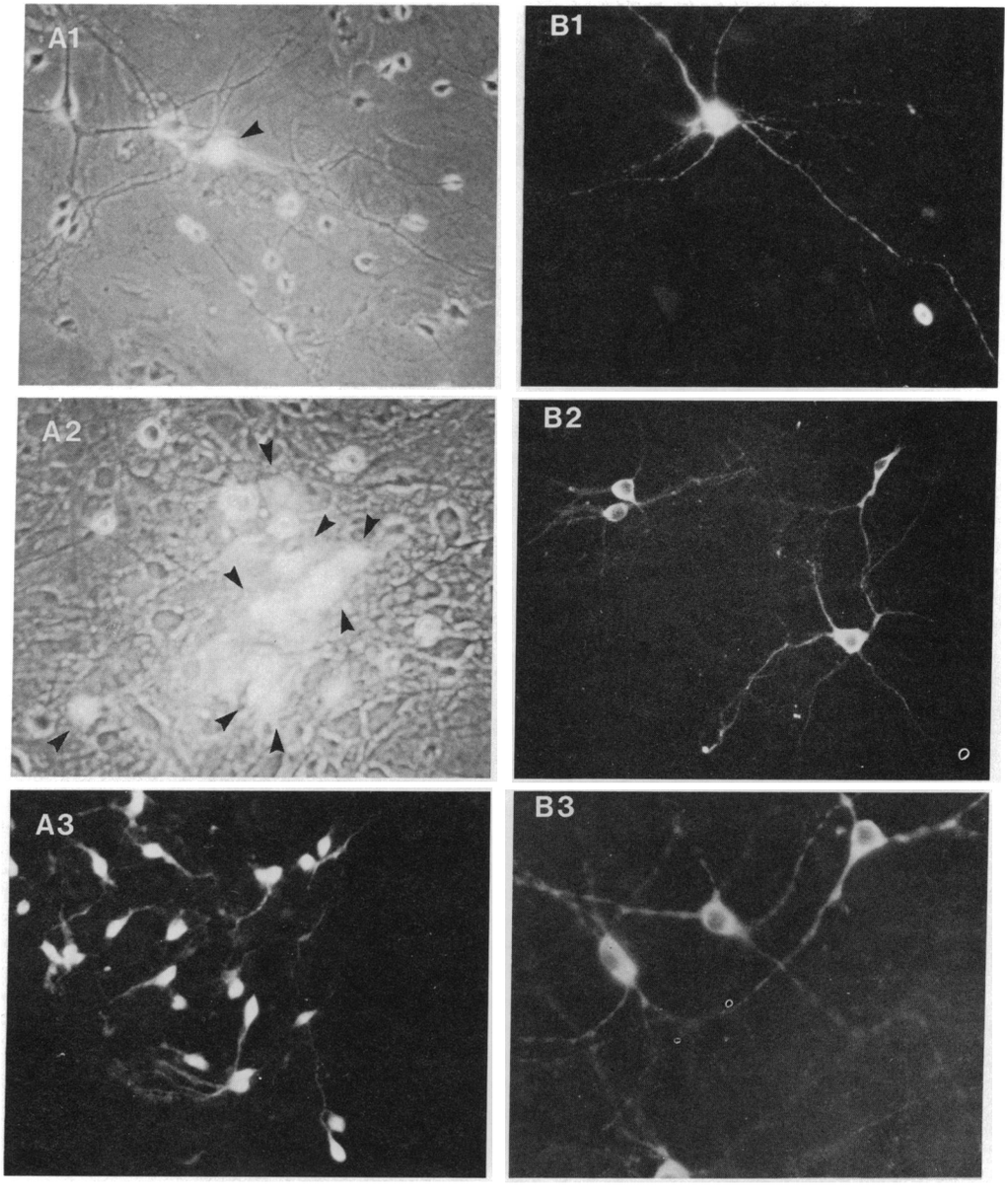

FIG. 3. Catecholaminergic neurons are enriched in NSP4$^+$-sorted cultures. The figure illustrates formaldehyde-induced catecholamine fluorescence (*A*) and anti-TyrOHase immunofluorescence (*B*) in different 10- (*A1*, *A3*, *B1*, and *B2*) to 15-day-old (*A2* and *B3*) cultures of unsorted (*A1* and *B1*) and NSP4$^+$-sorted (*A2*, *A3*, *B2*, and *B3*) mesencephalic cells. *A1* and *A2* show combined phase contrast and catecholamine fluorescence (arrowheads). Catecholaminergic neurons are rare in heterogeneous unsorted cultures (*A1* and *B1*), but they appear highly enriched in NSP4$^+$-sorted cultures, both by catecholamine fluorescence (*A2* and *A3*) and TyrOHase immunostaining (*B2* and *B3*). (*A1* and *A3*, ×180; *A2*, *B1*, and *B2*, ×270; *B3*, ×360.)

common type (about 50%) was ovoid (10–15 μm diameter) and bipolar with two processes that bifurcated at variable distances from the soma (Fig. 4*A2*). The next most common type (about 40%) was pyramidal (15–20 μm diameter) and multipolar (Fig. 4*B2*). The least common (about 10%) was irregularly shaped (25–30 μm diameter) with several long processes projecting from one side and numerous short rapidly bifurcating processes emanating from the opposite side of the cell (Fig. 4*C2*).

Table 1. Catecholaminergic neurons are enriched in NSP4$^+$-sorted cultures

Exp.	FACS-sorted NSP4$^-$	FACS-sorted NSP4$^+$	Unsorted
1	1 (0–2; n = 6)	74 (65–85; n = 12)	4 (1–7; n = 28)
2	1 (0–2; n = 6)	111 (93–141; n = 6)	23 (18–33; n = 6)
3	Not detected	83 (68–104; n = 5)	4 (2–6; n = 22)
4	Not detected	90 (77–133; n = 6)	12 (6–29; n = 9)
5	Not detected	156 (159–173; n = 6)	10 (8–12; n = 6)

Cells from the mouse embryonic ventral mesencephalon (E13) were dissociated and either plated in culture (Unsorted) or stained with anti-NSP4 and then sorted with a FACS (FACS-sorted) according to their positive (NSP4$^+$) or negative (NSP4$^-$) labeling. After 10–15 days of culture the number of catecholaminergic neurons was determined by counting the TyrOHase$^+$ neurons in a 1-cm^2 well. Results are expressed as the mean number of TyrOHase$^+$ neurons in each experiment. In parentheses are indicated minimal and maximal TyrOHase$^+$ neurons per cm^2 found in each experiment and the number of wells examined, n.

DISCUSSION

We have used immunocytochemical and flow cytometric techniques to study the expression of anti-NSP4 antigens in embryonic CNS tissue. We have found by immunoblotting that the NSP4 antibody recognizes a variety of epitopes preserved in detergent extracts of brain tissues during the course of CNS development in mice and that their expression is developmentally regulated.

In cultures from perinatal rat optic nerve, anti-NSP4 antibody labeled 0-2A progenitor cells, thought to be the precursors for type-2 fibrous astrocytes and for oligodendrocytes, but not type-1 flat astrocytes (3). In early cultures of postnatal mouse cerebellum (2 weeks *in vitro*) anti-NSP4 marked only neuronal elements, but later it was also expressed on presumptive type-2 astrocytes (2). Evidently

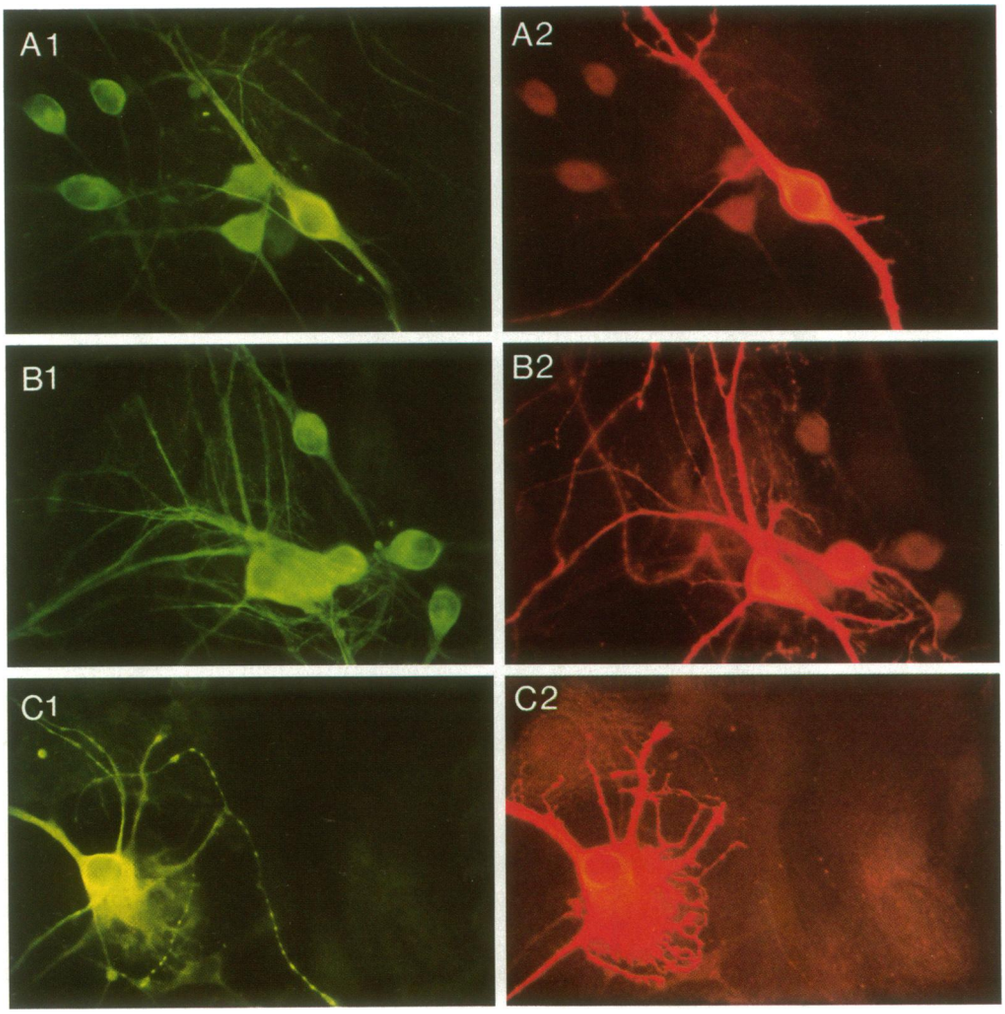

FIG. 4. Three morphological phenotypes of TyrOHase$^+$ neurons are present in NSP4$^+$-sorted cultures. (*A1*, *B1*, and *C1*) Fluoresceinated anti-NF immunoreaction in 18-day-old NSP4$^+$-sorted cultures (green). (*A2*, *B2*, and *C2*) Rhodamine-labeled anti-TyrOHase antibodies were used to identify catecholaminergic neurons in the same fields (red). The three morphologically distinct dopaminergic phenotypes sorted with anti-NSP4 antibodies are shown in *A2*, *B2*, and *C2*: ovoid and bipolar, pyramidal and multipolar, and stellate and multipolar, respectively. In *A2* and *B2* green fluorescence of some NF$^+$ TyrOHase$^-$ neurons passes through the rhodamine filter. (×500.)

NSP4 antigens are expressed by different cellular phenotypes at different times in development. In cultures derived from E13 mesencephalon, anti-NSP4 never stained GFAP$^+$ astrocytes as expected since only the NSP4$^-$ GFAP$^+$ flat astrocytes seem to be present in these cultures, while the NSP4$^+$ glial cells found in optic nerve and cerebellar cultures (0-2A stem cells, fibrous astrocytes, and oligodendrocytes), which appear quite late in embryogenesis, are absent. Although NSP4 immunoreactivity is not specific for a single neuronal phenotype, it appears that most mesencephalic dopaminergic neurons at this time in development expressed NSP4 on their surface, thus explaining their relative enrichment in the NSP4$^+$-sorted plates and their virtual absence from the NSP4$^-$-sorted cultures. NSP4 was also expressed by 15–25% of the cells dissociated from other embryonic CNS regions. Further experiments are required to ascertain whether the NSP4 antigenic determinant is common to different proteins, each specific for different neuronal phenotypes.

Vital labeling and sorting of cells by surface immunoreaction has been used recently to isolate and culture bipotential embryonic precursor cells from the adrenal medulla that develop into chromaffin cells or differentiate along a neuronal pathway (15). Our results show that CNS neurons, including putative dopaminergic neurons dissociated from the embryonic mouse mesencephalon, can be enriched in monolayer culture using a strategy involving surface immunoreaction and FACS. At least three morphologically distinct dopaminergic phenotypes were sorted. In time, the enrichment of dopaminergic neurons in cultures may make it possible to elucidate details regarding the differentiation of the three dopaminergic phenotypes and to demonstrate how their cellular properties function in establishing dopaminergic circuits. This enrichment strategy could also form the basis for experiments involving transplantation of dopaminergic cells to restore function in parkinsonian patients.

1. di Porzio, U., Rougon, G., Mazzetta, J. S., Kell, W. M. & Barker, J. L. (1985) *Soc. Neurosci. Abstr.* **11**, 978.
2. Rougon, G., Hirsch, M. R., Hirn, M., Guenet, J. L. & Goridis, C. (1983) *Neuroscience* **10**, 511–520.
3. ffrench-Constant, C. & Raff, M. C. (1986) *Nature (London)* **323**, 335–338.
4. Glowinski, J. (1979) in *The Neurosciences: Fourth Study Program*, eds. Schmitt, F. O. & Worden, F. G. (MIT Press, Cambridge, MA), pp. 1069–1083.
5. Price, K. S. & Farley, I. J. (1978) *Adv. Biochem. Psychopharmacol.* **19**, 293–300.
6. Joyce, J. N. (1983) *Neurosci. Biobehav. Rev.* **7**, 227–256.
7. Rougon, G., Deagostini-Bazin, H., Hirn, M. & Goridis, C. (1982) *EMBO J.* **1**, 1239–1244.
8. Prochiantz, A., di Porzio, U., Kato, A., Berger, B. & Glowinski, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5387–5391.
9. di Porzio, U., Daguet, M. C., Glowinski, J. & Prochiantz, A. (1980) *Nature (London)* **288**, 370–373.
10. di Porzio, U., Murphy, D. B. & Barker, J. L. (1986) *Soc. Neurosci. Abstr.* **12**, 866.
11. St. John, P. A., Kell, W. M., Mazzetta, J. S., Lange, G. D. & Barker J. L. (1986) *J. Neurosci.* **6**, 1492–1512.
12. Landis, D. M. D. & Weinstein, L. A. (1983) *Brain Res.* **276**, 31–41.
13. van den Pol, A. N., di Porzio, U. & Rutishauser, U. (1986) *J. Cell Biol.* **102**, 2281–2294.
14. Furness, J. B., Costa, M. & Wilson, A. J. (1977) *Histochemistry* **52**, 159–170.
15. Anderson, D. J. & Axel, R. (1986) *Cell* **47**, 1079–1090.