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Basal ganglia precursors found in aggregates following embryonic transplantation adopt a striatal phenotype in heterotopic locations

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SUMMARY

Transplantation of immature CNS-derived cells into the developing brain is a powerful approach to investigate the factors that regulate neuronal position and phenotype. CNS progenitor cells dissociated from the embryonic striatum and implanted into the brain of embryos of the same species generate cells that reaggregate to form easily recognizable structures that we previously called clusters and cells that disperse and integrate as single cells into the host brain. We sought to determine if the neurons in the clusters differentiate according to their final location or acquire a striatal phenotype in heterotopic positions. We transplanted dissociated cells from the E14 rat medial and lateral ganglionic eminences, either combined or in isolation, into the E16 embryonic rat brain. At all time points, we found clusters of BrdU- and DiI-labelled donor cells located in the forebrain and hindbrain, without any apparent preference for striatum. Immunocytochemical analyses revealed that cells in the clusters expressed DARPP-32 and ARPP-21, two antigens typically co-expressed in striatal medium-sized spiny neurons. In agreement with observations previously noted by several groups, isolated cells integrated into heterologous host areas do not express basal ganglia phenotypes. These data imply that immature striatal neuronal progenitors exert a community effect on each other that is permissive and/or instructive for development of a striatal phenotype in heterotopic locations.

Key words: Neuroblast, Heterotopic location, Cluster, Striatum, CNS

INTRODUCTION

During forebrain development, a relatively homogeneous neuroepithelium becomes regionalized and within each region, a multitude of differentiated phenotypes arise from seemingly identical progenitor cells. A major issue in developmental neurobiology is the delineation of the factors regulating neuronal position and phenotype, including intrinsic fate and environment. A powerful strategy to study mechanisms of central nervous system development is based on intracerebral grafting procedures in which progenitor cells are implanted into the immature nervous system (McConnell, 1985; Cattaneo et al., 1994; Brustle et al., 1995; Campbell et al., 1995a,b; Fishell, 1995; Barbe, 1996). The transplanted cells may be placed into either homotopic or heterotopic positions, and the age of the donor and recipient can also be altered. The grafted cells are thereby provided with environments that are varied, but highly permissive for cell proliferation, migration and differentiation. Based on the ability to identify the grafted cells, their fate choice and the influence of the new environment can be assessed.

A well-established experimental model with clinical implications is the grafting of cells derived from the ganglionic eminence(s) and the assessment of their survival, integration and phenotypic choices (Graybiel et al., 1989). During forebrain regionalization, the basal ganglia develop from the medial and lateral ganglionic eminences (MGE and LGE), although there is evidence that the LGE makes the major contribution to the caudate-putamen (striatum) (Deacon et al., 1994; Nakao et al., 1994b). The LGE, however, does not contribute only to the striatum and both ganglionic eminences may also contribute to the globus pallidus (GP), amygdala and cortex (Marchand and Lajoie, 1986).

Medium-sized spiny neurons (MSNs) constitute greater than 95% of the striatal neurons and GABA is their primary neurotransmitter. MSNs are born between E14 and P2 in the rat, and between E13 and P1 in the mouse (Smart and Sturrock, 1979; Sturrock, 1980). MSNs are characterized by the postmitotic, postmigrational expression of DARPP-32 and ARPP-21 (Ouimet et al., 1984, 1989) (Foster et al., 1987, 1988; Gustafson et al., 1992). In many studies, DARPP-32 is the only marker used to identify striatal medium-sized spiny neurons (e.g. Deacon et al., 1994; Nakao et al., 1994a; Campbell et al.,

1995a,b). DARPP-32 is expressed in the vast majority of the MSNs, but is also expressed, to a lesser extent, in many other regions, including the septum, choroid plexus and layers II, III and VI of the cortex (Ouimet et al., 1984). Co-expression of DARPP-32 and ARPP-21 is exclusive of the basal ganglia with perhaps a small number of cortical neurons expressing these two antigens. Cells expressing DARPP-32 or ARPP-21 are not found in the brainstem (Ouimet et al., 1984, 1989).

Transplants of striatal E14 mouse or E15 rat neuroblasts into the adult striatum result in a percentage of DARPP-32+ cells, which reaches a maximum of 90% of the volume of the surviving transplant after two months (Campbell et al., 1995a). Grafts of MGE into adult striatum have been shown to yield implants that are 25% DARPP-32-positive (Olsson et al., 1995). In these studies, embryonic cells are transplanted directly into adult striatum and are exposed only to a mature, striatal environment. These data therefore indicate that the potential of the dividing transplanted cells to become DARPP-32-positive is present and that the adult in vivo environment (striatum or substantia nigra) is providing the necessary differentiation factors. However in vitro studies showed that LGE and MGE cells maintain their regional identity as determined by expression of homeobox genes (Robel et al., 1995), but fewer than 2% of these cells are DARPP-32 immunopositive in defined media (Nakao et al., 1994a,b, 1996; Ivkovic et al., 1997). These data suggest a requirement for an environmental factor(s) for terminal differentiation.

The in utero grafting procedure was devised to investigate the ability of the embryonic environment to influence the fate of immature cells taken from different regions of the developing brain. Using this procedure, several groups have reported that rodent embryonic striatal cells found in isolation in heterotopic regions of the embryonic brain accommodate their fate to the new host environment (Brustle et al., 1995; Campbell et al., 1995b; Fishell, 1995; Magrassi et al., 1996a). These authors differed on whether or not these precursors preferentially integrate into the host striatum. A single study looked at the phenotype of LGE and MGE cells injected directly into heterotopic locations and focused on the cells that were phenotypically respecified (Olsson, 1997). Ectopic expression of a striatal phenotype, even in clusters of engrafted cells, has never been reported.

In our previous short-term analysis, we found that, after mechanical dissociation and transplantation into the fetal brain, a great portion of the donor neuronal progenitor cells derived from the E14.5 rat ganglionic eminences reaggregate into multicellular clusters (Cattaneo et al., 1994; Magrassi et al., 1996a). In our current study, we used our paradigm of rat-to-rat in utero transplants to examine whether precursors obtained from the embryonic ganglionic eminences can maintain their developmental program following a challenge with a heterologous embryonic environment and subsequent clustering. Results from some of the experiments were previously presented in a preliminary form (Soc. Neurosci. Abstract 1996, 392.7).

MATERIALS AND METHODS

Cell dissociation and labeling

Donor cells were prepared as previously described (Cattaneo et al., 1994). Briefly, striatal precursor cells were obtained from the striatal primordia (medial and/or lateral ganglionic eminence) of embryos of timed-pregnant Sprague-Dawley rats of 14 days postcoitum (E14). Three 5′-bromodeoxyuridine (BrdU 100 mg/kg) injections to the pregnant mother were given every 6 hours, starting 18 hours before embryo collection. A single-cell suspension was obtained by gentle mechanical dissociation of the pooled eminences. After dissociation, the cells were resuspended in Dulbecco's Minimal Essential Medium containing 40 µg/ml of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probe, Eugene, OR) (Honig and Hume, 1986; Paramore et al., 1992), incubated for 30 minutes and then extensively washed in fresh medium by three rounds of centrifugation and resuspension. Cell viability as estimated by trypan blue exclusion was normally over 95%. Following these procedures 100% of the donor cells were labelled by DiI and 30% of them were double-labelled with BrdU. These BrdU-labelled cells represented those that were in the S-phase of the mitotic cycle during about 1 hour after each intraperitoneal injection. In this paper, we analyzed the fate of the cells that were dividing at the time of the BrdU injections and that retained sufficient BrdU to be detected (i.e. those cells that, after transplantation, have gone through a limited number of cell divisions).

Transplantation

In utero surgical manipulations on rat fetuses were performed according to a previously described technique (Cattaneo et al., 1994; Conti et al., 1997). Anesthesia was obtained by ketamine HCl 50 mg/kg supplemented by Diazepam 2.5 mg/kg. Timed-pregnant E16 Sprague-Dawley rats were used in all experiments. The volume of the transplants was approximately 1 µl containing $2\text{-}4{\times}10^4$ cells. Cells were grafted into the CNS at different targets (developing cerebellum, brain hemispheres, colliculi and ventricles) by injecting them with a glass microelectrode with a tip of approximate internal diameter of 25-30 µm. The initial location of the transplant was ascertained immediately by visual inspection made possible by adding trypan blue (0.05%) to the donor cell suspension. Leakage of cells into the lateral ventricle during the injection into the deep region of the hemisphere always occurs.

Histology, immunocytochemistry and cell counts

Hosts were examined up to 12 weeks postnatal. Animals were fixed by transcardial perfusion with 4% paraformaldehyde dissolved in PBS (pH 7.4). Serial sections were obtained either by cryostat (10 µm) or vibratome (150 µm) cutting. Every tenth section was inspected for evidence of DiI-labelled cells and, if positive, the slice was reacted with a monoclonal antibody against BrdU (Becton Dickinson 1:100 dilution) after protease digestion (pepsin 0.05%, 15 minutes at 37°C) and DNA denaturation as previously described (Cattaneo et al., 1994). Mouse monoclonal antibodies against DARPP-32 and ARPP-21 were used as described (Ouimet et al., 1984, 1989) and revealed by the ABC method (Vector Laboratories) and diaminobenzidine. When double staining was performed on the same section, the brown precipitate obtained by the oxidation of DAB was turned to a grey by the addition of $NiSO_4$ and $ZnCl_2$ in the reaction mixture. Human serum obtained from a patient affected by the stiff man syndrome was used to reveal GAD (Baekkeskov et al., 1990) followed by fluoresceinated goat anti-human antibodies (Sigma). Monoclonal antibodies against TH and GFAP were used according to the manufacturer's instructions (Sigma).

Volume of the clusters was estimated using the Cavalieri estimator (Sterio, 1984) starting from a random systematic sampling of all 10 µm cryostate sections containing the cluster. Cluster profiles for area calculation and cell counting were drawn according to DiI staining. The number of cells contained in the cluster and the number of BrdU-labelled nuclei inside the cluster were estimated according to the dissector method (Gundersen and Jensen, 1987; Oorschot, 1994).

RESULTS

Striatal precursors form stable clusters throughout the brain without obvious preference for developmentally related regions

The donor nature of the cells in the clusters was unequivocally determined by the presence of DiI, overlapping with the BrdU-

positive signal, on serial consecutive sections. This is illustrated in Fig. 1A,B in a cluster from a 1-week-old animal. BrdU-immunoreactive cells (Fig. 1B) constitute approximately 40% of the cells in the area of this cluster as determined by a nuclei count.

We studied clusters for long periods following grafting to determine if the structures are stable and the cells remain viable. Fig. 1C,D show an intraparenchymal cluster 12 weeks after birth. The architecture of the cluster is virtually identical to that in Fig. 1A,B. Hoechst staining does not reveal pyknotic nuclei. Not uncommonly, we found a network of blood vessels surrounding the clusters (see black arrows in Fig. 4A). Of note, intraparenchymal clusters were consistently surrounded by isolated DiI-positive cells (arrows in Fig. 1C) within the host structures, but outside the limits of the aggregate. In our previous report (Cattaneo et al., 1994) using identical transplant methods, only 2-5% of the identifiable donor cells were found outside the clusters. These few isolated BrdU- and DiI-positive cells outside the clusters remain visible at the longer time points (Figs 1, 7).

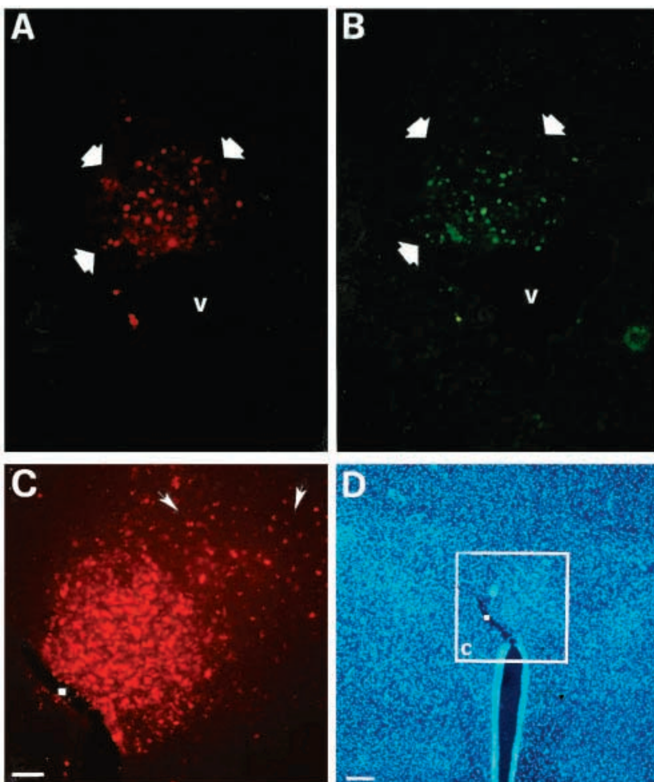

Fig. 1. Examples of clusters obtained after grafting cells dissociated from the striatal primordia of E14 rat embryos into the brains of 16-day-old rat embryos (E16). The cells were labeled with BrdU and DiI (see methods). (A) Section from a 1-week-old transplanted animal. DiI-labelled cells forming a cluster close to the cerebral acqueduct (v) are shown. (B) The same cluster as in A after BrdU immunocytochemistry. About 30 % of the cells in the cluster are labeled. Intensity of labelling is not uniform indicating either limited division of the cells after transplantation or shorter duration in the S-phase during exposure of the cells to BrdU in vivo. (A,B) The arrows help to outline the border of the cluster. (C,D) 50 µm vibratome section from the cerebral hemisphere of a 12-week-old host. (C) The outlines of the cluster are visualized by the typical fluorescence of DiI-labelled cells. Isolated DiI-labelled cells are also seen in the surrounding brain (arrows). (D) The box indicates the same cluster shown in C after staining of the nuclei by Hoechst. The white dot indicates a tear in the section that can be used as a landmark. Transplanted cells inside the clusters were more densely packed than in the surrounding parenchyma, but the shape of their nucleus, their dimension and chromatin texture were similar. Scale bars: A,B,D, 50 µm; C, 100 µm.

We define a cluster as a cellular aggregate with six or more cells along its largest diameter. This definition is consistent with the minimal aggregate of cells that we found ectopically expressing striatal markers (see below, Fig. 6) and required observation of the volumetric display of the cell mass in serial sections. An example of the spatial organization of these clusters is shown in Fig. 2. The tridimensional outline of this and other clusters is that of an irregular ovoid often oriented parallel to the fiber tracts and nuclei of the host region where they are located. In this figure, the reconstruction of a large cluster spanning the distance from the mesencephalon to the inferior pons is shown 4 weeks after transplant. We calculated that this particular cluster was composed of 7.5×10^4 cells. The number of cells in this unusually large cluster is approximately twice the number of cells originally transplanted. Since not all labeled transplanted cells are in this cluster, these data imply that there were cells that underwent more than one division following transplantation and that the progeny of these cells will have diluted the DiI and BrdU labels.

Using the Cavalieri estimator (Sterio, 1984; Gundersen and

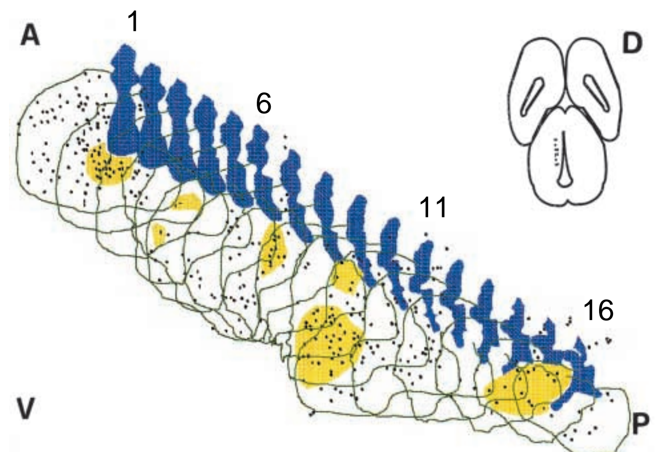

Fig. 2. Serial reconstruction of a large cluster (4 weeks after transplant) located in close proximity to the third ventricle and spanning the distance between the ventral mesencephalon and pons. The drawing on the upper right is a schematic of the position of the cluster shown in the reconstruction. The outline of the CSF spaces (end of the third ventricle, aqueduct and ostium of the fourth ventricle) is in blue. Numbers refer to the sections used in the reconstruction ordered in an anteroposterior direction. Letters are for orientation A, anterior; D, dorsal; V, ventral; P, posterior. The thin continuous lines (green) outline the extension of the cluster as determined by the presence of the DiI-labelled cells. To avoid overcrowding of the reconstruction, BrdU-labelled cells (represented by single black dots) were drawn only for the numbered sections. Notice the presence of BrdU-labelled cells outside the limit of the cluster (e.g. in section 16). The yellow colour maps the DARPP-32-positive regions in the cluster. DARPP-32 staining was not uniform in the cluster and at least two non-overlapping regions were present.

Table 1. Volumes and ratios of BrdU-labelled to unlabelled cells

Cluster	Vol.	BrdU	Total	Ratio
A	1.8×10^6	794	3626	0.22
B	3.5×10^6	2020	8240	0.24
C	8.5×10^5	480	2060	0.23

(A) Cluster in the mesencephalon (periacqueductal area). (B) Cluster in the diencephalon. (C) cluster in superior colliculus.

All animals were killed four weeks post transplantation. The numbers of labeled and unlabeled cells were estimated using the disector method (Sterio 1994; Oorschot, 1994). All nuclei inside the cluster were counted. Vol., volume calculated according to the Cavalieri estimator and expressed in μm^3. BrdU, estimated number of BrdU-positive nuclei inside the cluster. Total, labeled and unlabeled nuclei inside the cluster. Ratio, ratio between BrdU and total nuclei inside the cluster.

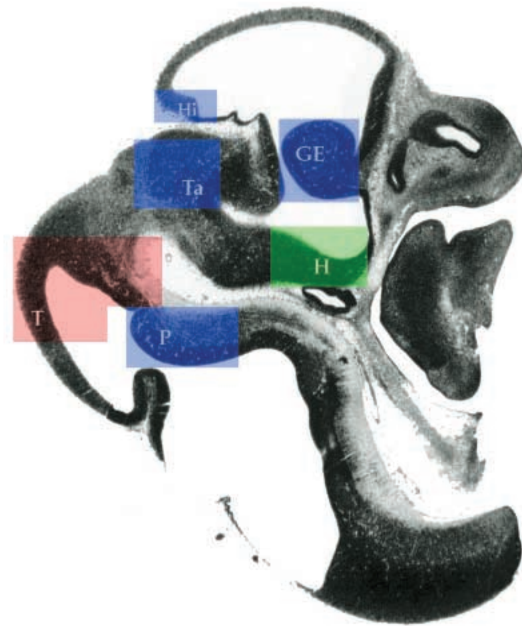

Fig. 3. Schematic representaton of sites of integration of clusters of donor cells. Sagittal section of a rat embryo brain at E16. The areas outlined by the colored boxes represent the sites where intraparenchimal clusters were found. Colour represents frequency class (red, high frequency; green, medium frequency; blue, low frequency). In 10 analyzed transplanted animals, clusters were located in H; in 15 cases, clusters were found in T and 5 in Ta, P or GE. 6 additional clusters were found in the ventricles. The data were obtained by pooling the observations on all hosts at all survival times. Clusters were located throughout the brain, without any apparent bias for developmentally related regions. GE, ganglionic eminence; Hi, hippocampus; Ta, thalamus; H, hypothalamus; P, ventral mesencephalon and pons; T, tectum.

Jensen, 1987), 78% of the clusters had an estimated volume between 10^5 and 5×10^6 μm^3, 13% smaller than 10^5 μm^3 and 9% larger than 5×10^6 μm^3. The cluster reconstructed in Fig. 2 is an example of a large cluster with an estimated volume of $4.3\times10^7 \mu m^3$. In contrast to the large variation in size of the clusters, the ratio of BrdU-labelled cells versus unlabeled cells is remarkably constant among clusters (Table 1). Clusters at shorter survival times (postnatal day 0) have a slightly higher BrdU cell percentage than clusters at long survival times (0.27±0.05) but this difference does not reach statistical significance.

Cluster formation was observed after both intraparenchymal and intraventricular injection irrespective of the origin of the cells (LGE alone or LGE plus MGE) or the injection target. Donor cells were found in all regions of the brain, including the hindbrain and the ventricular system. There was no obvious preference for the basal ganglia. In particular, cells derived exclusively from the lateral ganglionic eminence were not preferentially integrated into the host striatum. A detailed description of the position of the clusters is outlined in Fig. 3. In the majority of the host animals, there was only a single cluster. There were, however, two or three clusters in 19% of the grafted fetuses and, in these individual animals, clusters were widely dispersed. 27 out of 30 observed clusters derived from the LGE were located outside the striatum. Six additional animals demonstrated LGE-derived clusters that developed inside the ventricles but retained contact with the choroid plexus (Fig. 4B) or ependyma (not shown), which appeared to provide the initial vascular support to the cluster. No sign of degeneration in the clusters, as determined by cavitation or gliosis, was visible up to 3 months after birth of the host.

Clusters are not separated from the surrounding structures by a astrogliotic barrier

To determine whether the integrity of the clusters is dependent on a reactive barrier, we investigated whether or not a gliotic ring formed around the clusters. This was accomplished both by analyzing serial sections stained with nuclear dyes (Hoechst, toluidine blue) for morphological or histological identification of glial cells and by GFAP immunohistochemistry. We looked at both intraparenchymal and intraventricular clusters up to 2 months after surgery. In Fig. 4A, an intrathalamic cluster from a 2-month-old animal is identified by BrdU staining (open arrows). Double-labelling with GFAP reveals the absence of a gliotic barrier (arrowheads). In fact, this analysis showed no indication of the formation of a glial barrier in any of the locations of the clusters. Within the limits of the cluster, BrdU-positive donor cells are GFAP-negative. Fig. 4B is a toluidine-blue-stained intraventricular cluster attached to the choroid plexus (arrows delineate the limits of the cluster). Fig. 4C shows the electron microscopic analysis of the same cluster in B. Inside the cluster, there are no signs of degenerating cells and there is a continuum between cells in the cluster and those of the host without any intervening layer of reactive tissue. Analysis of the clusters by Hoechst staining (Fig. 1D) also did not reveal any enrichment of small fusiform nuclei suggestive of a glial reaction around the clusters.

Clusters derived from the ganglionic eminence co-express the distinctive striatal antigens DARPP-32 and ARPP-21 independent of their positions in the neuraxis

We utilized expression of DARPP-32 and ARPP-21 to confirm phenotypic differentiation into striatal neurons. Clusters of cells derived from either the combined medial and lateral ganglionic eminences or the lateral ganglionic eminence alone contained well-differentiated groups of DARPP-32 neurons (Figs 5, 6).

Regions of DARPP-32- and ARPP-21-immunopositive cells were detected in clusters beginning on postnatal day 8 (P8) and the immunostaining reached maximal intensity at P18 (not shown), consistent with development of endogenous mRNA and protein (Foster et al., 1987, 1988; Gustafson et al., 1992). In a large pontomesencephalic cluster 4 weeks after transplant, no distinction in nuclear morphology is observed between donor and host cells (Fig. 5B). DARPP-32 and ARPP-21 immunostaining is limited to cells contained within the cluster (Fig. 5C,D). In this cluster, a distinct area of DARPP-32 immunopositive cells (arrows) was present. In six clusters in which DARPP-32 was quantitated, greater than 65% of the volume was DARPP-32 positive. To confirm the striatal phenotype of these cells, we show that the same region of the cluster is immunopositive for ARPP-21 (Fig. 5D). As expected, the surrounding tissue is immunonegative for DARPP-32 and ARPP-21. Clusters were also immunopositive for glutamic acid decarboxylase, the biosynthetic enzyme of the primary medium-size spiny neuron neurotransmitter, but negative for tyrosine hydroxylase (not shown).

Fig. 6A,B show areas of DARPP-32 immunopositivity within two separate clusters in a single host animal 4 weeks after birth. The clusters are located in the ventral mesencephalon, a region that normally only contains DARPP-32-positive projection fibers. As delineated by BrdU and DiI staining (not shown), the clusters are not connected to each other. Fig. 6A shows one cluster stained with DARPP-32 through its maximal diameter, whereas Fig. 6B shows a section through the posterior limit of the second cluster. The identification of DARPP-32 immunopositive cells within both clusters suggests that the donor cells that ultimately will express the same phenotype do not all aggregate together prior to integration into the host tissue. Fig. 6C shows a section through a small subcortical cluster double-labelled with BrdU and DARPP-32. This double-labelling is further evidence of the donor origin of the cells and also indicates that differentiation occurs after transplantation. An additional small cluster composed of seven cells along its largest diameter in a single dimension is shown in Fig. 6D. In this small cluster located in the hindbrain adjacent to the fourth ventricle, all seven cells are DARPP-32 positive. Thus, even the cells that are clearly exposed to non-striatal influences express the DARPP-32 gene when aggregated as a cluster.

Transplanted cells that have integrated into the host tissue but are not part of a cluster are never ARPP-21 or DARPP-32-positive (Fig. 7). As an example, pictures of different microscopic fields taken from the same section double-stained with antibodies against DARPP-32 and BrdU are shown. Despite the strong DARPP-32 immunopositivity of the striatal fibers projecting to the substantia nigra (C) and of the graft-derived cells contained in the cluster (B), no staining is visible in the donor-derived BrdU-labelled cells (A) dispersed in the molecular layer of the hippocampus, 4 weeks after transplant.

DISCUSSION

Transplantation is a powerful tool for the study of fate determination in the central nervous system. Since transplantation is also potentially an important therapeutic

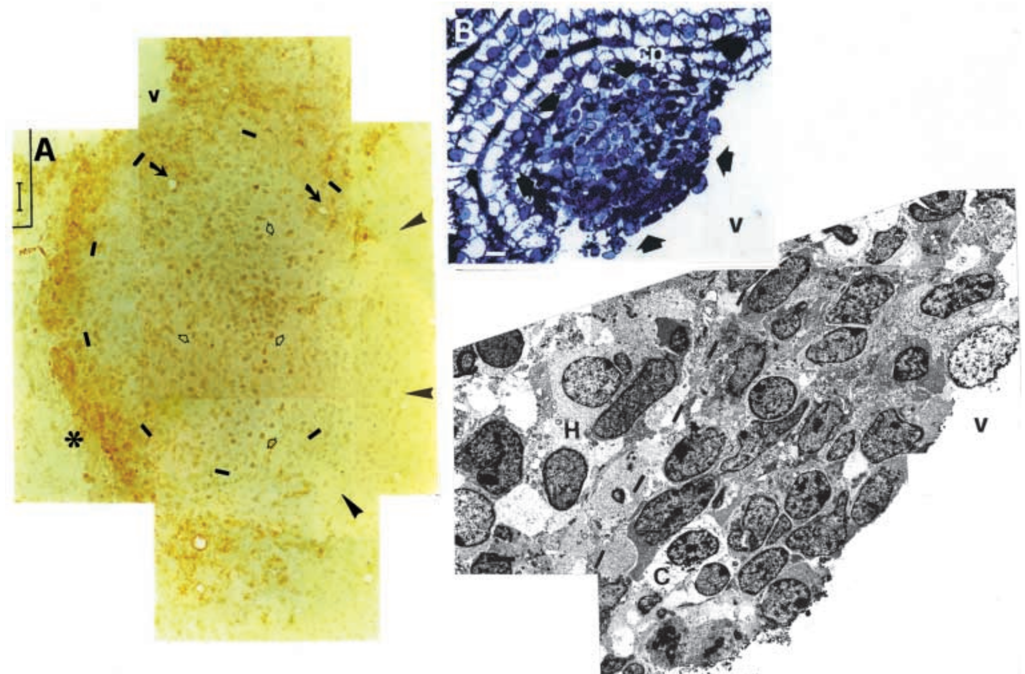

Fig. 4. (A) A photographic montage of adjacent fields in the same cryostat section showing a cluster in the thalamus. The cluster is located close to the wall of the third ventricle in a host animal 8 weeks after transplant (v, ventricle). The broken line delineates the limit of the cluster as indicated by BrdU and DiI labelling on an adjacent section. The section was stained with an antibody against GFAP, and subsequently BrdU-positive cells were detected immunocytochemically (some indicated by open arrows). Cells within the cluster are largely GFAP-negative. The cluster is not surrounded by a wall of reactive astrocytes and the border of the cluster forms a continuum with the host tissue (arrowheads). The coronal section was slightly skewed in the sagittal plane and on the left it is touching tangentially the inner surface of the third ventricle (asterisk). This also causes an artificially stronger background in the subependimal layer. Arrows show the blood vessels. Scale bar, 90 µm. (B) Light and (C) electron micrograph of a cluster completely contained within the right lateral ventricle from a 1-week-old host. Both light and electron microscopic studies show the continuity of the two tissues without any intervening reactive tissue layer between the grafted cells and the choroid plexus (cp). No degenerating cells are present either in the cluster or the sourrounding tissue. (B) Toluidine-blue-stained 1.5 µm plastic section of a small cluster (arrows) adherent to the choroid plexus. (C) Montage of electron microscopic plates showing the border between the cluster and the choroid plexus (broken line). The cells in the cluster appear healthy without signs of apoptosis (e.g. chromatin condensation, increased electron density of the cytoplasm). C, cluster; H, host. Scale bars: B, 12 µm; C, 5 µm.

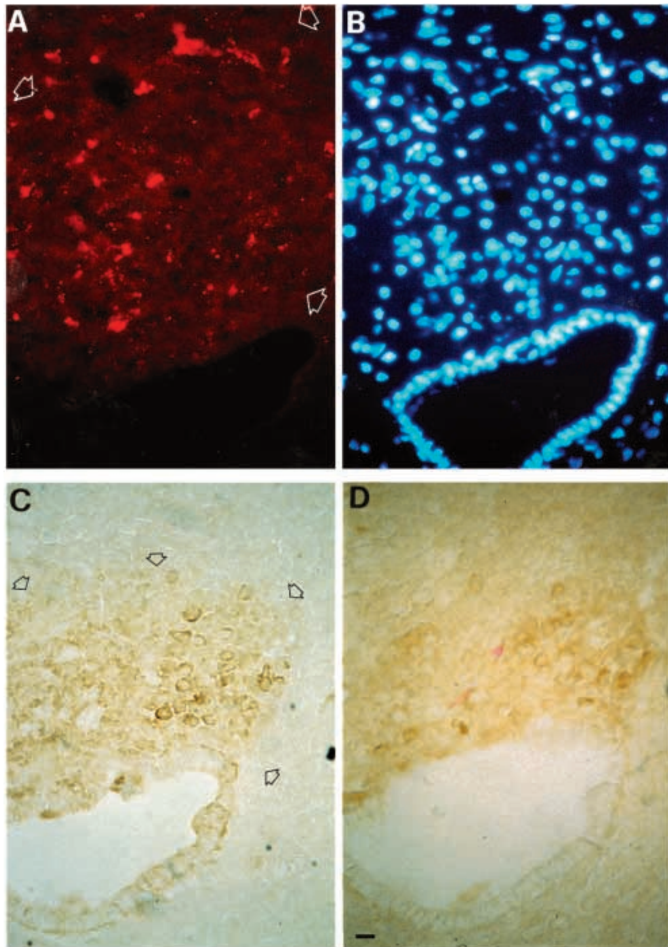

Fig. 5. DARPP-32-/ARPP-21-positive cluster in the pontomesencephalon in a 4-week-old host animal. (A,B) Same cryostate section (15 μm) obtained after: (A) fluorescent excitation to show DiI-labelled cells (the border of the cluster is delineated by the open arrows), and (B) fluorescent excitation to show DNA staining by Hoechst. (C) Light micrograph of a section adjacent to A after immunohistochemical staining with the anti-DARPP-32 mAb; (D) Adjacent section stained with an antibody against ARPP-21. Examples of cells within the cluster that are positive both for DARPP-32 and ARPP-21 are shown (arrows). No DARPP-32- or ARPP-21-labelled cell bodies are seen in the surrounding host tissue. Scale bar, 12 μm.

approach, knowledge of the ultimate phenotypic choice of the transplanted cells is critical. Analysis and comparison of results from individual studies, however, requires accounting for all variables. These include, but are not limited to, donor and host species and age, anatomic origin of donor cells, host location of transplant, and methods of identification of grafted cells and assignation of phenotype. Finally, conclusions regarding fate can obviously be drawn only from those grafted cells that survive and can be identified. In all experimental paradigms, it is possible that specific cell types, e.g. stem cells and/or terminally differentiated neurons, do not survive in the host and/or can not be identified.

Our major conclusions are as follows. First, E14 rat neuroepithelial precursors derived from the ganglionic eminences and transplanted into E16 rat embryos tend to segregate from the surrounding host cells and form aggregates. These clusters are easily recognized by their compact tridimensional structure and by the presence of both BrdU- and DiI-labelled cells. Second, the site of integration of a cluster is not restricted to the striatum and shows no predilection for homotypic sites. Third, within clusters, many cells differentiate according to their origin and not according to their final integration site. Fourth, BrdU- or DiI-positive single cells that integrate in isolation do not develop a striatal phenotype (i.e. they are negative for DARPP-32 and ARPP-21).

About 30% of engrafted cells were double-labelled with DiI and BrdU, indicating that, at the time of transplant, many donor cells were still dividing. Some of the labelled cells may have ended their division within a few hours of one of the in vivo injections of BrdU. However, according to previous cell-cycle kinetics studies (Bayer, 1984; van der Kooy and Fishell, 1987; Bhide, 1996), most of the medium spiny neurons in the rat striatum are generated after E14. Furthermore, even those cells that have completed their final mitosis before transplantation are

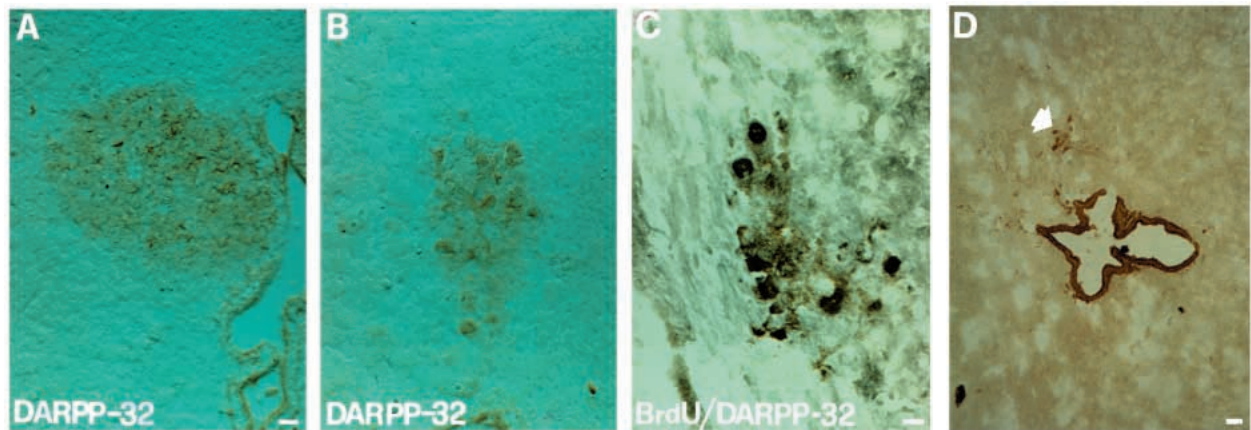

Fig. 6. (A,B) Two separate areas of DARPP-32 immunoreactivity within two separate clusters are shown in a single host animal 4 weeks after birth. The clusters are located in the ventral mesencephalon but not connected to each other. Scale bar, 12 μm. (C) Section through the periphery of a small subcortical cluster double-labelled with BrdU and DARPP-32. Scale bar, 8 μm. (D) Section through a 7-cell cluster located in the hindbrain adjacent to the fourth ventricle. The brown-stained cells indicated by the white arrow are DARPP-32 positive. Scale bar, 24 μm.

unlikely to have migrated to the lateral striatum and terminally differentiate prior to the time of harvesting. This suggests that the vast majority of the BrdU-labelled cells in the suspension used for the transplant were still undifferentiated. Moreover, BrdU (Gratzner, 1982; Miller and Nowakowsky, 1988) and lipophilic dyes like DiI (Renfranz et al., 1991) or PKH26 (Olsson et al., 1997) are lost by dilution following cell division. Transplanted cells with a high proliferative capacity, i.e. stem cells or astrocytes, will not be detected. Therefore, double-labelled cells that were identified in the host must have been close to their final mitosis at the time of transplant. Other studies (Fishell, 1995; Campbell et al., 1995b; Brustle et al., 1995; Magrassi et al., 1996a,b; Olsson et al., 1997) have focussed on the fate of cells that actively divide in the host fetal brain and integrate in isolation. Our analysis of cells integrated in isolation revealed that these BrdU- or DiI-labelled cells accounted for only a small percentage of the transplanted donor cells. Of note, this was already true at short survival times, i.e. 24 hours, when the majority of the donor cells were found in clusters (Cattaneo, 1994). Furthermore, some clusters are even located on the lumenal side of the ependyma, inside the ventricles. The absence of mechanical constraints within the ventricles and the lack of surrounding fibrotic tissue at the sites of parenchymal clusters, indicates that cluster formation in the embryo is not due to the inability of the grafted cells to move out from the transplantation site into the host tissue.

Our different results regarding the number of cells found in isolation compared to those of others (Fishell, 1995; Campbell et al., 1995b; Brustle, 1995; Olsson et al., 1997) may result from dilution of the label by the dispersed cells, species differences and to the use of a different transplantation protocol, including the method of dissociation and the number of transplanted cells. We used a single species and our population of donor cells was subjected only to mechanical dissociation. By not employing DNase treatment or proteolytic enzymes, membranes were preserved intact. These two peculiarities of our study allowed us to analyze the fate of donor cells with unmodified membrane properties exposed to heterotopic locations within the same species. Thus, the same-species embryonic host environment supports migration, integration, survival and segregation of engrafted cells.

It has been suggested that selective adhesion represents a conserved mechanism to restrict cell mixing, resulting in defined regions during development (Gotz et al., 1996). These authors demonstrated that selective adhesion is age dependent, and that cortical and GE cells segregate from each other at early (E14) stages only. In our

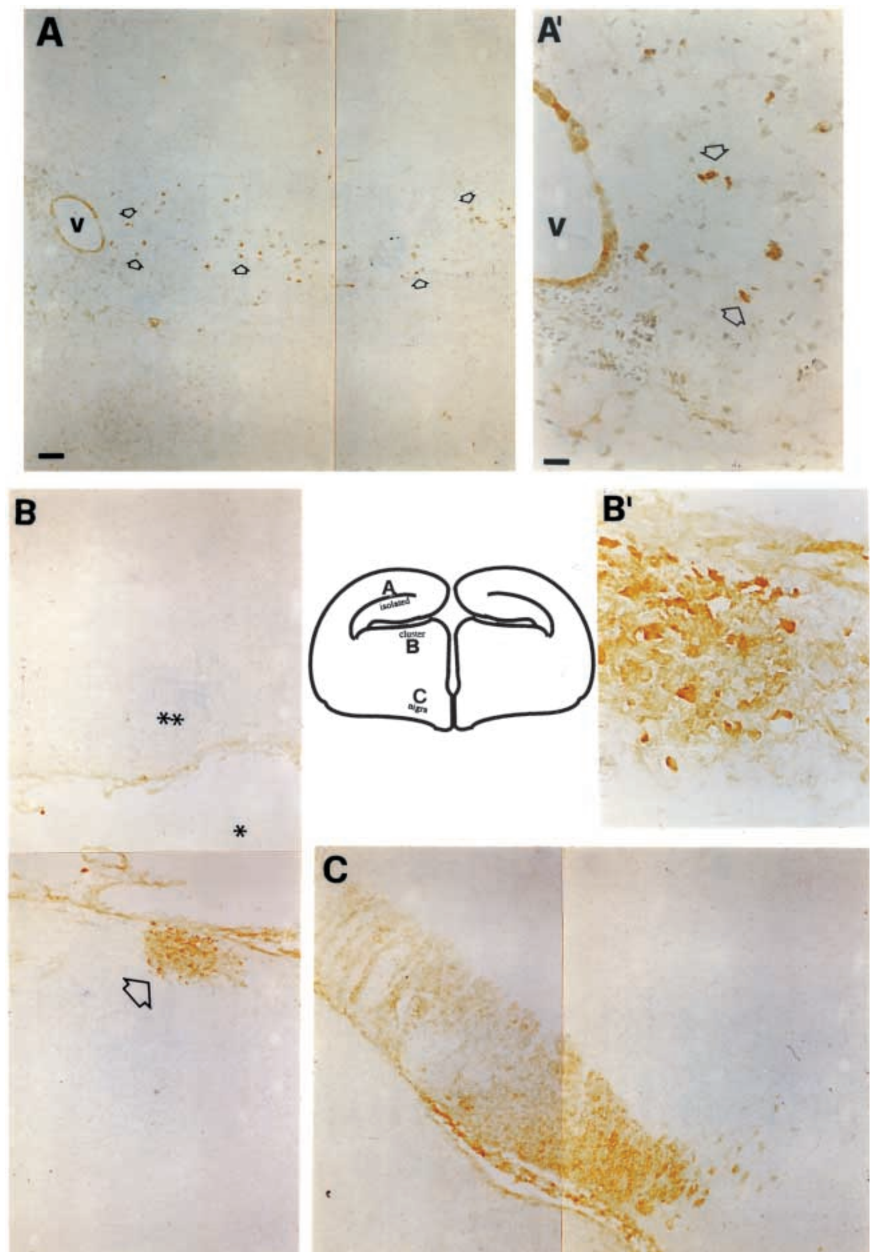

Fig. 7. Different fields taken from the same section of a 4-week-old host animal brain double-stained with antibodies against DARPP-32 and BrdU. A schematic drawing of the section is presented in the middle. (A) Molecular layer of the hippocampus. V, blood vessels. Open arrows point to some of the BrdU-labelled nuclei (in brown) of the donor-derived dispersed cells. (A′) Enlargement of the same field centered on the cells close to the vessel, showing that the cytoplasm of isolated BrdU-positive nuclei is DARPP-32 negative. Positivity to the BrdU is revealed by the brown precipitate in the nuclei. (B) The arrow points to a cluster of DARPP-32-positive cells in the superior colliculus. Double asterisks are in the hippocampus. Single asterisk is in the quadrigeminal cistern. (B′) Higher magnification of the region of the cluster. The DARPP-32-positive cytoplasm of the cells is visible. (C) The heavily labelled plexus of striatal efferents in the substantia nigra is shown as a positive control for the DARPP-32 staining. No DARPP-32-positive cells or fibers were visible in this section outside the cluster in the superior colliculus and the substantia nigra. Scale bars: A-C, 80 μm; A′,B′, 12 μm.

study, the final destinations of the transplanted cells do not display spatial predisposition and derivatives of ganglionic eminence cells were found throughout the telencephalon and even in the hindbrain. This is consistent with the results of Brustle et al. (1995), who transplanted E13.5-E14 mouse ganglionic eminence cells into E15 rat forebrain ventricle. These data, however, contrast with those of Campbell et al. (1995b), who transplanted E14 mouse lateral or medial ganglionic eminence cells into E16 or E18 rat ventricle and found that LGE cells showed a distinct preference to integrate into the host striatum. These differences may be due to the older ages of the hosts in the latter study in addition to species differences. Our data are thus consistent with data by Gotz et al. (1996) and suggest that, among the various possibilities, cell adhesion does not drive position in the neuraxis, but may drive phenotypic determination.

Assignation of a phenotype to transplanted cells is also fraught with difficulties. Ganglionic eminence cells at the time of transplant already express region-specific homeobox genes while in the mitotic stage (Porteus et al., 1994), but must migrate to their final location in vivo prior to expressing DARPP-32 and ARPP-21. The use of both DiI and BrdU associated with the characteristic disposition of the cells in clusters unequivocally identified transplanted cells. Co-expression of DARPP-32 and ARPP-21 likewise identifies differentiated cells derived from the ganglionic eminences (Ouimet et al., 1984, 1989). As noted above, although DARPP-32 is the most widely used antigenic marker of striatal medium spiny neurons, it is in fact endogenously expressed in many other regions, and therefore we used both markers.

In agreement with the hypothesis that cluster formation may influence phenotypic determination, we found that the majority of donor cells present in clusters develop an identity correlated to their site of origin (i.e. striatum). Clusters of engrafted cells identified by BrdU and DiI labelling on the same or adjacent sections, and located outside the host striatum, contained groups of DARPP-32+/ARPP-21+ cells. Our data show that, once clustered, many of the donor cells continue along their developmental pathway and develop a striatal phenotype regardless of the conflicting microenvironment to which they are exposed after transplantation and before clustering. These data further indicate that even cells still in the mitotic cycle at the time of transplant are able to differentiate into a striatal phenotype when organized in clusters. Similar data were obtained in other systems using neural precursors dissociated from the developing and adult olfactory mucosa (Magrassi and Graziadei, 1996).

We suggest that the mechanisms involved in cluster formation and differentiation (Cattaneo et al., 1994; Magrassi and Graziadei, 1996) may be compared to the cell-community effect, which was first described by Gurdon (1988) and Kato and Gurdon (1993) in *Xenopus laevis* embryos. Single muscle precursor cells from the late gastrula stage, comparable to transplanting phenotypically committed vertebrate postmitotic cells, can differentiate into mature muscle cells even in an ectopic position. However, remarkably, single muscle precursors from the earlier, mid-gastrula stage do not differentiate into muscle when surrounded by nonmuscle cells, but they can do so when surrounded by cells from the same region. Gurdon (1988) proposed that a certain number of muscle precursor cells is necessary to accumulate a high concentration of diffusible factors. In our striatal transplantation paradigm, specific cell surface molecules may also be necessary for the clustering mechanisms. Proper differentiation according to the tissue of origin can then proceed through a community effect mechanism.

This hypothesis would predict that ganglionic eminence cells that integrate in isolation, and differentiate without the community influence of similarly derived cells, may be unable to establish a striatal phenotype. This would be true despite the fact that cells that disperse into the host brain and cells that reaggregate into clusters are initially subjected to similar influences by the host environment. Previous studies of the fate of cells derived from the ganglionic eminences after mouse-to-rat in utero transplantation that integrate in isolation (Fishell, 1995; Brustle et al., 1995; Campbell et al., 1995b) demonstrated that these cells differentiate according to their site of integration. Olsson et al. (1997) further analyzed the short-term fate of mouse E13.5 (equivalent to E14.5-E15 rat) ganglionic eminence cells derived from the germinal zone and purposely transplanted, using ultrasound guidance, into heterotopic locations. In this mouse-to-mouse paradigm, these authors also found that isolated cells differentiate with a phenotype appropriate for the site of integration, without having been through the migratory step. In another system, Magrassi and Graziadei (1996) showed that transplanted olfactory cells that migrate and integrate as single cells into the host central nervous system differentiate into neurons and glia according to the local environment, while cells that aggregate into clusters differentiate into an olfactory epithelium. We also found that cells integrated in isolation were never positive for DARPP-32 or ARPP-21. In fact, careful review of all immunostained sections failed to detect ectopic expression of DARPP-32- or ARPP-21-positive cells in isolation. These data support the hypothesis that differentiation of the transplanted cells into a mature striatal phenotype requires being surrounded by similarly derived cells. These results are also consistent with our previous ontogeny and in vitro studies, which suggested the requirement for extracellular stimuli in postnatal maturation of the striatum (Gustafson et al., 1992; Ivkovic et al., 1997).

In summary, we conclude that dividing neuronal progenitor cells from E14 rat ganglionic eminence differentiate according to their origin after embryonic transplantation, integration and clustering into heterotopic ventricular and parenchymal sites in E16 rat. The finding that cells that adopt a striatal phenotype are always found in clusters strongly indicates that striatal differentiation requires contact-mediated or diffusible signals that act on a local basis on ganglionic eminence-derived precursors.

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Note added in proof

After this manuscript was submitted, Olsson et al. (1998) described the preferential integration of striatal donor cells before or after trypsin treatment.

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