Temporal regulation of ephrin/Eph signalling is required for the spatial patterning of the mammalian striatum

Lara Passante¹, Nicolas Gaspard¹, Mélanie Degraeve¹, Jonas Frisén², Klas Kullander³, Viviane De Maertelaer¹ and Pierre Vanderhaeghen¹,*

Brain structures, whether mature or developing, display a wide diversity of pattern and shape, such as layers, nuclei or segments. The striatum in the mammalian forebrain displays a unique mosaic organization (subdivided into two morphologically and functionally defined neuronal compartments: the matrix and the striosomes) that underlies important functional features of the basal ganglia. Matrix and striosome neurons are generated sequentially during embryonic development, and segregate from each other to form a mosaic of distinct compartments. However, the molecular mechanisms that underlie this time-dependent process of neuronal segregation remain largely unknown. Using a novel organotypic assay, we identified ephrin/Eph family members as guidance cues that regulate matrix/striosome compartmentalization. We found that EphA4 and its ephrin ligands displayed specific temporal patterns of expression and function that play a significant role in the spatial segregation of matrix and striosome neurons. Analysis of the striatal patterning in ephrin A5/EphA4 mutant mice further revealed the requirement of EphA4 signalling for the proper sorting of matrix and striosome neuronal populations in vivo. These data constitute the first identification of genes involved in striatal compartmentalization, and reveal a novel mechanism by which the temporal control of guidance cues enables neuronal segregation, and thereby the generation of complex cellular patterns in the brain.

KEY WORDS: Forebrain, Neuronal migration, Ephrin, Striatum, Guidance

INTRODUCTION

The striatum is a major structure in the mammalian forebrain, playing a prominent role in the control of movement and emotions. It displays a unique mosaic organization, made of two major compartments that can be identified neuroanatomically and neurochemically: the matrix and the striosomes (Gerfen, 1992; Graybiel and Ragsdale, 1978; Johnston et al., 1990). The matrix compartment contains the majority (80-85%) of the striatal medium spiny (MS) projection neurons and is organized around the striosomes, which contain the rest of the MS neurons and appear as patches of neurons scattered in the striatum.

The matrix and the striosomes can be distinguished on the basis of various neurochemical markers selectively enriched in one of the compartments, such as calbindin in the matrix and μ-opioid receptor in the striosomes (Gerfen et al., 1987; Herkenham and Pert, 1981; Liu and Graybiel, 1992; Nastuk and Graybiel, 1985). Importantly, the striatal compartmentalization is also related to the organization of cortical inputs to the striatum, with matrix and striosome neurons receiving preferential inputs from distinct cortical layers and areas (Gerfen, 1989; Gerfen, 1992; Kincaid and Wilson, 1996). These anatomical differences are tightly linked to functional differences that have started to be unravelled recently. Neurons from striosome and matrix compartments display differential activity during natural behaviour or following psychomotor stimulant treatments (Brown et al., 2002; Canales and Graybiel, 2000). Similarly, the specific loss of neurons from either compartment has been correlated with distinct clinical features in Huntington’s disease (Tippett et al., 2007), while selective impairment of the function of either neuronal compartment can have different effects on motor function and behaviour (Tappe and Kuner, 2006).

The development of striatal compartments is a highly orchestrated process. Striatal projection neurons are generated in the lateral ganglionic eminence (LGE) in the ventral forebrain (Marin and Rubenstein, 2003; Wilson and Houart, 2004; Wilson and Rubenstein, 2000), but interestingly the commitment to one specific striatal compartment is linked to the developmental stage at which embryonic neurons are generated (Song and Harlan, 1994; van der Kooy and Fishell, 1987). Early-generated neurons are destined to the striosomal compartment, whereas neurons exiting the cell cycle at mid and late embryogenesis are committed to the matrix compartment (Mason et al., 2005; van der Kooy and Fishell, 1987; Yun et al., 2002). This birthdate-based spatial confinement is reminiscent of the layered organisation found in the cerebral cortex, where neurons populate non-overlapping layers depending on their birthdate (Bayer and Altman, 1991). However, in the case of the striatum, neurons that are generated sequentially first migrate to the same domains of the striatal mantle (the striatal primordium), where they intermix at embryonic stages, before segregating during early postnatal periods, forming a mosaic pattern rather than distinct layers (Krushel et al., 1995; Lanca et al., 1986) (Fig. 1A). In vitro assays showed that the striosome neurons display homophilic adhesive properties, providing a first hint about potential underlying cellular mechanisms (Krushel et al., 1995; Krushel and van der Kooy, 1993). Although earlier in vivo studies tended to minimize the role played by extrinsic factors such as the projections from the cortex and substantia nigra (Snyder-Keller, 1991; van der Kooy and Fishell, 1992), recent in vitro studies have suggested that the early-generated striosomal neurons may cluster around corticostratial fibres (Snyder-Keller et al., 2001; Snyder-Keller, 2004) and that striatal compartmentalization may thus rely on such extrinsic cues.
Striatal development thus constitutes an original model of pattern
generation through cell sorting, the mechanisms of which may be
quite different from those operating during hindbrain segmentation,
for example (Poliaakov et al., 2004). Although some of the genes
implicated in the specification of striatal MS neurons have been
identified (Arlotta et al., 2008; Mason et al., 2005; Yun et al., 2002),
the molecular cues involved in striatal neuron sorting remain
completely unknown. Cadherins have been found previously to be
expressed differentially between the murine striatal compartments
(Redies et al., 2002), as are some Eph receptors, which are
selectively enriched in the matrix compartment (Janis et al., 1999);
however, the functional involvement of these molecules in striatal
patterning has not been explored. Ephrins and Eph receptors have
been involved in the generation of distinct developmental
compartments, such as the segmentation of the hindbrain into
distinct rhombomeres, making them attractive candidates for the
segregation of striatal compartments (Barrios et al., 2003; Klein,
2004; Pasquale, 2004; Poliaakov et al., 2004; Swartz et al., 2001).

Using in vivo analyses of mutant mice and a novel organotypic
assay that recapitulates striatal development in vitro, we have
identified ephrin/Eph family members as guidance cues that control
matrix/striosome compartmentalization. These data constitute the
first identification of genes involved in the formation of the mosaic
pattern of the striatum, supporting a model whereby the temporal
control of membrane-bound cues is tightly linked to the spatial
organization of this structure.

MATERIALS AND METHODS

Transgenic mice, breeding and genotyping

Timed-pregnant mice were obtained from Harlan and from local colonies of
mutant and wild-type mice. The plug date was defined as embryonic day
E0.5, and the day of birth as P0. Ephrin A5, EphA4 and ephrin A5/EphA4
double knockout mutants have been described previously (Friessen et al.,
1998; Kullander et al., 2001; Dufour et al., 2003).

In situ RNA hybridization

In situ hybridization probes have been described previously (Dufour et al.,
2003; Vanderhaeghen et al., 2000). In situ hybridization using digoxigenin-
labeled RNA probes was performed as described (Vanderhaeghen et al.,
2000). All hybridization results obtained with antisense probes were
compared with control sense probes. Pictures of the in situ RNA
hybridization were acquired with Axioplan2 Zeiss microscope and a Spot
RT camera, and converted in false colours and overlayed using Adobe
Photoshop software.

Organotypic overlay assay

Vibratome coronal slices (250 μm) were isolated from transgenic embryos
ubiquitously expressing GFP at E12 or E15 (Okabe et al., 1997). The lateral
ganglionic eminence (LGE) was dissected out in ice-cold L15 and
mechanically dissociated. Up to 500 × 10^3 cells were laid down on top of
postnatal striatal vibratome slices (P0-P2) and cultured within cell culture
inserts (1 μm pore size PET membranes; Becton Dickinson), as previously
described (Pellex and Ghosh, 2002). Organotypic co-cultures were
performed using an air-interface protocol and were maintained in a 5% CO2
humidified incubator for 20 hours in vitro. Ephrin/Eph inhibitors (EphA3-
Fc, EphB2-Fc) and control reagent (Fc) were purchased from R&D Systems.
GFP and DARPP32 were detected by immunofluorescence as previously
described (Dufour et al., 2003), and imaged using a Bio-Rad MRC1024 or
Zeiss LSM510 confocal microscope.

BrdU incorporation and immunofluorescence

For BrdU labelling, timed-pregnant female mice were injected
intraperitoneally, with four pulses (50 mg kg^-1 body weight) every 2 hours,
of 5-bromo-2’-deoxyuridine (Sigma) dissolved in physiological sterile
solution, at E16 and E17. Newborns were sacrificed 2 days after birth, fixed
by perfusion with PFA 4%, followed by overnight immersion in the same
fixative. The forebrain was vibratome sectioned at 50 μm. Sections were
then processed for double-immunofluorescence against BrdU (mouse
antibody, 1/1000, Becton Dickinson) and DARPP32 (rabbit antibody, 1/500,
Chemicon).

Quantification methods of matrix/striosome distribution in vitro
and in vivo

To determine the matrix/striosome (M/S) values in organotypic assays, the
area of each single DARPP32-positive striosome was determined in
Photoshop using the Lasso tool, and GFP-positive pixels (representing the
GFP+ cells) were quantified by selection with the Magic Wand tool in each
DARPP32-positive striosome, giving a first value of GFP+ cell density in the
striosome (S). Next, the same selected area corresponding to the
striosome was moved into three different adjacent matrix regions outside the
striosome, where GFP-positive pixels were quantified similarly. A mean
value for the pixels counted in the three matrix regions outside the
striosome was then calculated (as M, the mean density of GFP+ cells in the
adjacent matrix) and divided by the number of pixels counted in the adjacent
striosome (S) to obtain a matrix/striosome value that reflects the ratio of
GFP+ cell densities between each striosome and adjacent matrix area. Thus,
the M/S value reflects a cell density and is independent of the size of the area
where cells are counted, and an M/S value of 1 is obtained if cells are
distributed in a uniform fashion across striosome and matrix compartments.
This procedure was applied to all the striosomes and to the corresponding
adjacent matrix areas of all the striatal sections to obtain a mean M/S value
for each condition.

The distribution of BrdU cells in matrix versus striosome compartments
in wild-type and ephrin A5/EphA4 mutants were quantified on all visible
striosomes of brain sections (n=5 sections for each animal) using similarly
determined M/S values, by quantifying the distribution of BrdU-positive
cells within DARPP32-positive striosomes (S) and neighbouring
DARPP32-negative matrix domains (M) (owing to the single cell resolution
of the BrdU staining, BrdU-positive cells were counted manually).

M/S means were compared using classical Student’s t-test with Welch’s
correction to account for unequal variances. The hypothesis that the mean
value of M/S could be equal to 1 was tested with compatibility Student’s t-
test.

RESULTS

A novel organotypic assay to study striatal compartmentalization

To address the issue of the cellular and molecular mechanisms of
striatal compartmentalization, we set up a novel organotypic assay
to study in vitro the sorting of striatal neurons. In particular we
sought to recapitulate the temporal pattern observed in vivo, where
neurons born at a different time end up in distinct compartments.

This assay consisted of a heterochronic co-culture where striatal
neurons generated at different embryonic ages were confronted with
and allowed to sort out between nascent matrix and striosome
compartments. First, cells were dissociated from the LGE (where all
striatal projection neurons are generated) of ubiquitously GFP-
expressing embryos (Okabe et al., 1997) at embryonic stages E12 or
E15-16, to obtain a population enriched for either the striosome or
matrix neurons, respectively (Fig. 1B). The dissociated cell
suspensions were then plated onto organotypic slices of the striatum
at early postnatal stages (P0-P2), the stage at which the two
compartments just start to emerge clearly in vivo (Fig. 1A). The
culture was stopped after 20 hours in vitro, and the slices were
processed to allow examination of the distribution of the GFP+
neurons throughout the striatum, in comparison with the pattern of
DARPP32, which marks selectively the striosomes at early stages of
striatal development (Anderson et al., 1997) (Fig. 1B).

The distribution of the GFP+ cells in each compartment was
quantified by comparing the density of GFP+ cells that settled in
each DARPP32-positive striosome compartment (S) with the
density that settled in the adjacent DARPP32-negative matrix compartments (M). The relative distribution of the GFP+ cells in each compartment was then expressed as the ratio between M and S cellular densities as an M/S value (Fig. 1B; see also Materials and methods). Thus, M/S values of 1 indicate that the cells are distributed in a random fashion across striosome and matrix compartments, whereas M/S values less than 1 indicate a preferential distribution in the striosome compartment and, conversely, M/S values greater than 1 indicate a preferential distribution in the matrix compartment (Fig. 1O).

When plating E15-LGE derived cells, we found that the GFP+ cells were not distributed uniformly over the striatal surface (Fig. 1C), but rather a preference for the DARPP32-negative matrix compartment (Fig. 1C-H) with a mean M/S value greater than 1 (1.65±0.106, P<0.001, Student’s t-test). By contrast, when the same experiment was performed with E12-LGE derived cells, they were found to settle preferentially in the DARPP32-positive striosomal compartment (Fig. 1I-N), with a mean M/S value less than 1 (0.58±0.061, P<0.001, Student’s t-test), which is very different from the M/S value observed for the E15-derived cells (P<0.001, Student’s t-test, Fig. 1O). As a control cell population for compartment sorting, we used physiologically irrelevant E14 dorsal thalamic cells in the same striatal overlay assays. Quantification showed that they were randomly distributed with a mean M/S value not significantly different from 1 (1.05±0,067, P=0.403, Student’s t-test).

These results indicate that presumptive matrix- and striosome-destined neurons (isolated at E15 and E12, respectively) can respond differentially to local cues present in the early postnatal striatal slice, and thereby can segregate into the appropriate compartment. This quantitative assay thus recapitulates a major feature of striatal patterning: its time dependence. It enables us to reveal the differences in responsiveness of striatal progenitors to local cues (depending on their age), and also that early striatal cells preferentially set on striosome compartments while later striatal cells preferentially set on the matrix compartment.

These results prompted us to use this system to test for the involvement of candidate molecules in matrix/striosome patterning. To this end, we first tried to identify which chemoaffinity molecules might be differentially expressed between striatal compartments and could control the sorting of the two striatal populations. In view of our results, we looked for guidance cues that might show: (1) a
Differential temporal and spatial patterns of expression of ephrin/Eph genes in the developing striatum

Given their prominent role in compartmentalization in the hindbrain, ephrin/Eph genes constituted logical candidates in our search for matrix/striosome-sorting factors. The EphA4 receptor has previously been shown to be expressed in the matrix compartment at postnatal stages [P6 (Janis et al., 1999)], although its expression at time points relevant to matrix/striosome establishment was not explored, nor was the expression of candidate ligands for EphA4.

To explore the involvement of EphA4 in matrix/striosome formation, we performed in situ hybridization for this receptor, as well as for putative ligands known to be expressed in the developing forebrain (ephrin A2, ephrin A3, ephrin A5, ephrin B1, ephrin B2 and ephrin B3), focusing on their expression at embryonic and early postnatal stages, covering the whole period of genesis, migration and sorting of the two striatal populations. This analysis revealed striking temporal patterns of expression of EphA4 and its ligands ephrin A5 and ephrin B2 (Fig. 2), and ephrin B3 (similar to ephrin B2, data not shown).

By E12.5, when the genesis and the migration of the striosome-destined neurons have started, EphA4 was detected only in the ventricular zone and not in the striatum per se (Fig. 2A), whereas expression of ephrin A5 and ephrin B2 was already detected both in the ventricular zone (VZ) and the striatal mantle (Fig. 2B, arrows). By E14.5, when most of the striosomal neurons have migrated in the striatal mantle, expression of ephrin A5 and ephrin B2 was detected throughout the striatal mantle. At this time, most of EphA4 receptor expression still remained mostly confined to the ventricular zone, with some expression found in the mantle (Fig. 2D-F, arrows). By E16.5, when many matrix neurons have migrated in the striatal mantle, expression of EphA4 could be detected throughout the striatal mantle, similar to ephrin A5 and ephrin B2 (Fig. 2G-K). These data indicate that EphA4 and its ligands ephrin B2 and ephrin A5 display a differential temporal pattern of expression, suggesting that ligands are preferentially expressed in early-generated striatal neurons, and that EphA4 is preferentially expressed in later-generated neurons.

At early postnatal stages (P0-P2), when matrix/striosome patterns first appear visible, the EphA4 receptor showed a matrix-like distribution all over the striatum (Fig. 2J,M), with small and evenly distributed patches devoid of staining that become more and more distinct from P0 to P2. E16.5 exhibited a differential expression profile, with a progressively lower expression level and a more restricted patch distribution, reminiscent of the striosomal compartment (Fig. 2K,N), whereas ephrin B2 was expressed at progressively weaker levels (Fig. 2L,O).

To compare more precisely the distinct patterns of expression of ephrin A5 and EphA4 with matrix and striosome compartments at postnatal ages, we performed, on alternate striatal sections, immunohistochemistry for DARPP32, an early marker for the striosomal compartment (Anderson et al., 1997), and compared it with the patterns of the ligand-receptor pair.

EphA4 receptor distribution across the striatum was found to complement the DARPP32-positive striosomes, from postnatal stage P2 (Fig. 3A-C, arrowheads) to P4 (Fig. 3G-I, arrowheads), implying that the receptor belongs mainly to the matrix compartment, although also to some striosomal cells at a lower level. At P0, EphA4 expression already exhibited a matrix-like distribution, but failed to strictly complement the DARPP32 staining, consistent with the notion that the process of compartmentalization between the two striatal populations is not completed at that stage (data not shown).

Next, we compared the EphA4 staining with the ephrin A5 distribution on alternate sections, and found a partial complementarity between the two that was dependent on the striatal
altogether, these results suggest a complex and highly dynamic spatiotemporal pattern, where ephrin A5/B2 levels of expression are highest in the early striatal population and EphA4 levels are highest in later-generated population at embryonic ages. At early postnatal stages, EphA4 is expressed at high levels and is mostly restricted to the matrix compartment, whereas ephrin A5 retains a partially complementary pattern and ephrin B2 becomes gradually downregulated. This distinctive temporal and spatial pattern of distribution in the developing striatum is compatible with an involvement of EphA4-ephrin signalling in the control of matrix/striosome compartmentalization. Specifically, it leads to the hypothesis that the differential, partially complementary, expression of EphA4 and ephrin ligands by the two striatal populations could trigger a bi-directional repulsive guidance response that would then cause the sorting of the matrix and striosome neurons and, as a consequence, the segregation of the two striatal populations into distinct compartments.

**Disruption of ephrin-Eph signalling partially alters the matrix/striosome neuronal sorting in vitro**

To test directly our hypothesis on the involvement of ephrin/Eph signalling in striatal compartmentalization, we first used the organotypic overlay assays described above, in combination with specific soluble inhibitors of ephrin/Eph signalling added to the culture media (Dufour et al., 2003). Given the unique functional promiscuity of EphA4 (it can bind and activate most ligands of the ephrin A and ephrin B subfamilies (Flanagan and Vanderhaeghen, 1998)), we used EphA3-Fc and EphB2-Fc inhibitors in combination, to provide efficient inhibition of both ephrin A and ephrin B signalling. Control conditions consisted of addition of Fc proteins only.

When performing these experiments with cells derived from E16 LGE cells (presumptive matrix neurons), pre-incubation of the receiving striatal slices with EphA3/B2-Fc resulted in a significant decrease of the preference of these cells for any specific striatal compartment (Fig. 4A-F,M), as assessed by a decreased M/S value (1.15±0.105), which was not different from 1 (P=0.151, Student’s t-test), and different from the mean M/S value obtained under the control condition (incubation with control Fc reagents; P=0.001, unpaired t-test with Welch’s correction). Similarly, when performing these experiments with cells derived from E12 LGE cells (presumptive striosome neurons), pre-incubation of the receiving striatal slices with EphA3/B2-Fc resulted in a significant decrease of the preference of these cells for any striatal compartment (Fig. 4G-L,M), with a mean M/S value (0.99±0.117) not different from 1 (P=0.945, Student’s t-test), and different from the mean M/S value obtained under the control conditions (incubation with control Fc reagents, P<0.0047, unpaired t-test with Welch’s correction).

Interestingly, when EphA3-Fc or EphB2-Fc were used alone, they did not affect the matrix/striosome preference of LGE embryonic cells (data not shown), thus providing evidence that a combination of ephrin A and ephrin B signalling is required for proper striatal neuron sorting in vitro.

These data indicate that acute inhibition of ephrin/Eph interactions normally present in the striatum results in a partial loss of the appropriate sorting of matrix and striosome neuronal populations, consistent with the hypothesis that ephrin/Eph signalling is indeed involved in this process, at least in the context of the organotypic assay.
consequence, the correct formation of striatal compartments. To
the neuronal segregation during striatal development and, as a
result, either ephrin ligands or EphA4 receptor, or both, would impair
patterning of striatal compartments in vivo.

EphA4 signalling is required for the normal
patterning of striosomal neurons in vitro.

When mouse E16 LGE-derived cells, enriched for the matrix neurons, are overlaid on postnatal striatal slices preincubated with control Fc reagents (A-C) they display a preference for the DARPP32-negative matrix compartment (arrows). This preference is partially lost when slices are preincubated with soluble ephrin inhibitors EphA3-Fc/EphB2-Fc (D-F), revealing more GFP+ cells in DARPP32-positive compartments (arrowheads). (G-L) When E12-LGE derived cells, enriched for the striosome neurons are overlaid on postnatal striatal slice preincubated with control Fc reagents (G-I), they display a preference for the DARPP32-positive striosome compartments (arrowheads). This preference is partially lost when slices are preincubated with soluble ephrin inhibitors (J-L), revealing more GFP+ cells in DARPP32-negative compartments (arrows). (M) Quantification of the relative densities of GFP+ cells in matrix (M) and striosome (S) compartments. Treatment with ephrin soluble inhibitors results in a decrease of matrix preference for E16 cells, and conversely a decreased striosome preference for E12 cells (*P=0.001, **P=0.0047). Scale bar: 100 μm.

EphA4 signalling is required for the normal patterning of striatal compartments in vivo.

According to the expression patterns and in vitro analysis, the loss of either ephrin ligands or EphA4 receptor, or both, would impair the neuronal segregation during striatal development and, as a consequence, the correct formation of striatal compartments. To further test this hypothesis, we analyzed ephrin A5 knockout mice, EphA4 receptor knockout mice (Frisen et al., 1998; Kullander et al., 2001) and ephrin A5/EphB4 double knockout (DKO) mice, given their known interactions in other systems in vivo (Dufour et al., 2003; Eberhart et al., 2004; Marquardt et al., 2005; Swartz et al., 2001).

To determine whether the compartment formation was affected in the mutant mice, we again took advantage of the temporal pattern of matrix/striosome formation. We treated embryos with BrdU pulses at embryonic days E16 and E17 to label a majority of endogenous matrix neurons, then sacrificed the newborn pups at P2 and performed a double immunofluorescence against BrdU and DARPP32, in order to visualize the matrix neurons and the striosomal compartments, respectively.

The relative distribution of the presumptive matrix neurons between the two striatal compartments was assessed by comparing the density of BrdU+ cells in matrix and striosomal compartments. The data were quantified as in the in vitro assay, by determining the cell density within each compartment, and expressed as an M/S value (as for in vitro assays), which would be equal to 1 if the matrix neurons were homogeneously distributed, and greater than 1 if BrdU+ neurons were preferentially located in the matrix compartment. This value, thus, reflects the degree of segregation between the two striatal populations. In addition, the DARPP32 staining enabled the qualitative determination of the spatial distribution of striosomal neurons across the striatum.

Control animals showed a distribution of BrdU+ neurons that was typical of the matrix neurons across the striatum: a dense matrix of BrdU+ cells (Fig. 5A) with interspersed zones almost devoid of BrdU+ cells (Fig. 5A, arrowheads and insert), corresponding to the DARPP32+ striosomes (Fig. 5B, arrowheads and insert), from which the BrdU+ cells were largely excluded (Fig. 5C, arrowheads and insert). The mean M/S value was significantly greater than 1 (1.3±0.069, P=0.001, Student’s t-test, Fig. 6), indicating that, as expected in control animals, the BrdU+ neurons were significantly enriched in the matrix compartment. Thus, this labelling method appeared to be a sensitive and specific method to determine the level of matrix/striosome segregation in vivo, which we next applied to the analysis of ephrin A5, EphA4 and compound ephrin A5/EphA4 mutants.

When analysed and compared with control animals, the single ephrin A5 knockout mice (n=5) displayed a similar distribution of BrdU+ matrix neurons across the striatum, with a preferential distribution of BrdU neurons in the matrix compartment (Fig. 5D-F, Fig. 6). In addition, examination of the DARPP32 pattern did not reveal any obvious difference compared with control animals, suggesting that all compartments are overall properly formed in this mutant. Consistent with this qualitative analysis, the M/S value was significantly greater than 1 (1.37±0.083, P<0.001; Student’s t-test, Fig. 6).

By contrast, a similar analysis performed on EphA4 receptor knockout mice revealed several striking defects in matrix/striosome organization. First, the BrdU+ cells appeared to be distributed more uniformly across the striatum (Fig. 5G), with much less distinct zones devoid of BrdU+ cells (Fig. 5G, inset). In addition, the DARPP32 staining appeared much more diffuse (Fig. 5H, arrowheads and insert) than in the control animals, suggesting an alteration in the distribution of presumptive striosomal neurons. Finally, BrdU+ cells were found to be distributed at ectopic locations within the remaining striosomes (Fig. 5I, arrowheads and insert). Consistent with these observations, the M/S value was not different from 1 in these mutants (0.99±0.055, P=0.798 Student’s t-test, Fig. 6), indicating that the matrix neurons...
were distributed much more uniformly between the two compartments. Given the known interactions between ephrin A5 and EphA4, and the likelihood that other redundant ephrin/Eph genes are involved in the system, we next turned our analysis to ephrin A5/EphA4 DKO, reasoning that, as in other systems (Dufour et al., 2003), this may reveal further the involvement of these genes in striatal development. The analysis of DKO mice (n=6) revealed several qualitative and quantitative changes in the striatal patterning. First, the BrdU+ cells were more uniformly distributed over the striatal mantle (Fig. 5J); second, the DARPP32+ cells were less densely packed into well-defined striosomes (Fig. 5K); and finally, ectopic BrdU+ matrix cells were found abundantly in the striosomal compartment (Fig. 5L, arrowhead and inset). These qualitative defects were further quantified by determining the M/S values in DKO mice, which revealed an M/S value that was not different from 1 (0.96±0.045, P=0.421, Student’s t-test, Fig. 6). Although the analysis of the DKO thus clearly confirmed the defects observed in the single EphA4 KOs, it failed to reveal a quantitative interaction between ephrin A5 and EphA4 genes, suggesting compensatory mechanisms by the other ligands (including ephrin Bs) acting in combination with ephrin A5 in this context. Importantly, the absolute density of BrdU neurons throughout both striatal compartments was similar in all mutants analysed (data not shown), indicating that the changes observed in EphA4 and ephrin A5/EphA4 mutants was not due to a change in the absolute number of striatal neurons, related, for example, to changes in proliferative or apoptotic patterns (Depaep et al., 2005; Holmberg et al., 2005).

These data thus indicate that EphA4 signalling is a major player in vivo for the normal segregation between the two striatal populations and, as a consequence, for the correct formation of the striatal compartments during development.

**DISCUSSION**

The functions of the mammalian striatum rely on its mosaic compartmentalized structure, including the matrix and striosome neurons (Gerfen, 1992; Johnston et al., 1990). Here, we report that ephrin/Eph family members act as guidance factors that regulate the compartmentalization of the striatum, both in vitro and in vivo. Our data shed new light on the molecular and cellular mechanisms that control the cytoarchitecture of this important forebrain structure. In addition they reveal a novel developmental mechanism by which the temporal control of guidance cues can control the spatial patterning of brain nuclei.

**Ephrin/Eph and other guidance cues controlling matrix/striosome sorting: a combination of repulsion and adhesion**

Although the development of the unique cytoarchitecture of the striatum must rely on differential cell guidance and adhesion, the underlying mechanisms have remained essentially unknown. Our data identify the first molecular cues involved in striatal compartmentalization, and point to a cellular mechanism involving bidirectional repulsion, according to the following scenario (Fig. 7B).
At early stages of striatal neurogenesis, ephrin A5/B2+ neurons start to be generated and progressively migrate to populate uniformly the striatum. Later on, the expression profile of the newly generated striatal neurons progressively shifts to the EphA4 receptor, and these late-generated EphA4+ neurons mix with the resident ephrin A5/B2+ neurons. Following cell-cell interactions, ephrin/Eph signalling will result in mutual segregation of the two cell populations, which will cause the EphA4+ neurons to adopt a matrix distribution around the clustered ephrin A5/B2+ neurons.

This model thus strongly implies the involvement of bi-directional signalling, mediated not only by Eph receptors (forward signalling) but also by ephrin ligands (reverse signalling), in accordance with our in vitro and in vivo data, where disruption of ephrin/Eph signalling also results in the mislocalization of matrix and striosome neurons.

Although, in principle, such a model could fully account for the segregation of matrix/striosome neurons, it is very likely that other cues act in concert, in particular to enable the preferential homoadhesion between striosome neurons, as suggested by previous in vitro studies (Krushel and van der Kooy, 1993; Krushel, 1989). In this context, an attractive set of cues would be cadherins, which have previously been shown to be expressed in the developing striatum (Redies et al., 2002), and control the compartmentalization of neuronal populations within the spinal cord (Price et al., 2002). In addition, other Eph receptors, in concert with EphA4, could also be involved in homophilic cellular adhesion processes (Holmberg et al., 2000). Such a scenario would be reminiscent of other complex systems involving ephrins, such as the retinotectal system, hindbrain patterning or motoneuron guidance, where ligands and receptors control differentially the guidance of distinct neuronal populations through bi-directional signalling (Eberhart et al., 2004; Flanagan, 2006; McLaughlin and O’Leary, 2005; Pasquale, 2005; Pasquale, 2008; Poliakov et al., 2004).

**Generating mosaic patterns of cytoarchitecture: the importance of partially overlapping expression of repulsive cues**

The patterns of expression of EphA4 and its ephrin ligands in the striatum may appear to be only partially consistent with a simple model of cell repulsion. Indeed, their temporal and spatial patterns are not strictly complementary, contrary to other systems of ephrin/Eph-dependent cell segregation [such as hindbrain rhombomere formation (Fig. 7A) (Wilkinson, 2001)]. Interestingly, the striatal architecture constitutes a unique model of organization, very distinct from the strictly segmented pattern observed for hindbrain rhombomeres. Instead, they form compartments that are intermingled with each other to form a mosaic pattern, and the formation of such patterns may require the use of partially complementary labels, as recently suggested by theoretical modelling of ephrin/Eph patterning mechanisms (Honda and Mochizuki, 2002). According to these models, if two cellular populations express reciprocally a ligand and its receptor, following a strict mutually exclusive expression pattern, they will segregate into two adjacent distinct compartments, similar to hindbrain rhombomeres, for example (Fig. 7A). Most interestingly, however, if the two populations co-express the ligand and the receptor but differ in the relative levels of ligand/receptor in a complementary fashion, then a very different pattern can be achieved, resembling a mosaic pattern strikingly similar to a matrix/striosome organization (Fig. 7B).

Such a mixed distribution of guidance cues is highly reminiscent of the expression pattern observed for striatal ephrin/Eph genes, where the two striatal populations express, in a partially
complementary pattern, ephrin ligands and the EphA4 receptor. Thus, the fact that each striatal population expresses ligands and receptors, but at different relative levels, might contribute to the formation of a mosaic matrix-striosome organization, as opposed to a strictly segmented structure such as the hindbrain, where cell populations express repulsive cues in a mutually exclusive pattern (Fig. 7A,B).

**Intrinsic and extrinsic mechanisms of striatal compartmentalization**

The cellular mechanisms orchestrating the compartmentalization of the striatum remain almost completely unknown. In particular, it has remained unclear whether the two neuronal populations segregate from each other through direct interactions, or through the influence of extrinsic factors, such as the nigrostriatal and corticostriatal projections. Earlier in vivo experiments tended to minimize the role of the nigrostriatal afferences in the segregation of the striatal populations (van der Kooy and Fishell, 1992; Snyder-Keller, 1991), whereas, more recently, organotypic assays involving co-cultures of the striatum with cortex or substantia nigra suggested that the afferents from this structure had a prominent influence on the emergence of striatal patterning (Snyder-Keller, 2004; Snyder-Keller, 2001).

Here, we provide direct in vitro and in vivo evidence that intrinsic cues expressed within the striatum contribute importantly to compartment formation, by a mechanism relying on contact-dependent cell sorting. Indeed, the organotypic assays that we used included striatal tissue only, in absence of nigral or cortical afferents, and yet they allow a faithful recapitulation of the cell sorting that occurs normally in vivo, achieving an M/S density score strikingly similar to the one measured in vivo.

In addition, the pattern of ephrin/Eph expression also suggests that EphA4 and ephrin ligands act, at least in part, locally in the striatum to contribute to the sorting of the two main striatal neuron populations. However, although the phenotype observed in ephrin A5/EphA4 DKO s and EphA4 KOs provides clear indication for a key role of this receptor in striatal patterning, the contribution of ephrin A5 appears to be more limited. Indeed, the absence of any striatal phenotype in the ephrin A5 KO mice strongly suggests that other ligands are involved in the EphA4-mediated neuronal sorting, including ephrin B2/B3. This is also consistent with the expression data, where striatal ephrin A5 expression is only partially complementary to EphA4 expression, with preferential expression in a subset of the striosomes. The requirement for combined signalling involving ephrin A5 and ephrin B proteins is also consistent with the in vitro data, where only the combination of EphA3/B2-Fc inhibitors was able to inhibit efficiently the matrix/striosome sorting.

Finally, other ligands or receptors enriched in the striosomes may be brought by the afferent innervations, such as the nigrostriatal projections, as such extrinsic cues would not be detected at the transcript level in the striatum. Consistent with this hypothesis, earlier studies revealed that dopaminergic neuron innervation coming from the substantia nigra leads to increased striatal expression of the EphB1 receptor (Halladay et al., 2000). Similarly, it is clear that, as in any other system, there must be genes and mechanisms other than ephrin/Eph genes involved in this process, which would include other types of cues, acting locally or from the afferents from the cortex and substantia nigra. In any case, the identification of the role of ephrin/Eph signalling in matrix/striosome formation provides an important framework for identifying these other cues and the pathways involved.

**Temporal patterning of guidance cues and spatial patterning**

The matrix/striosome compartmentalization relies highly on temporal patterning, whereby neurons generated at different time-points end up in different spatial compartments. This is highly reminiscent of the formation of distinct layers within the cerebral cortex, each of which consists of distinct populations generated at different time points (Bayer and Altman, 1991). The process by which neurons born at a different time end up in different cortical layers remains unclear, although the reelin signalling pathway has emerged as an important player in this process (Tissir and Goffinet, 2003). Our data provide evidence for a model of spatial segregation that relies on the temporal control of neuronal guidance cues: it will be interesting to test whether a similar mechanism may underlie the establishment of other patterned brain structures.

**Implication of matrix/striosome disruption for striatal connectivity and function**

Several mouse mutants have been reported to display alterations in the specification of matrix or striosome neurons, including *Mash1* (Ascl1), *Notch1* and Ctip2 (*Bcl11b*) mutant mice, where early striatal populations are reduced in size or lost (Arlotta et al., 2008; Casarosa et al., 1999; Mason et al., 2005), and *Dlx1*/*2* and *Ebf1* mutants, where the matrix population is mostly affected (Anderson et al., 1997; Garel et al., 1999). However, these mutants display no defect in striatal compartmentalization per se, so that EphA4 mutants constitute the first model of selective disruption of the cytoarchitecture of the striatum. Matrix/striosome organization has been shown to be important for several aspects of striatal function, but the exact relationships between striatal function and cytoarchitecture remain poorly known. Ephrin A5/EphA4 mutants constitute the first example of genetic disruption of the cytoarchitecture of the striatum, and thereby constitute a unique model with which to test how the disruption of matrix/striosome compartments might be associated with abnormalities in connectivity and function of the basal ganglia, and how these may be related to abnormal behavioural traits.

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