The COUP-TF nuclear receptors regulate cell migration in the mammalian basal forebrain

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Summary

Cells migrate via diverse pathways and in different modes to reach their final destinations during development. Tangential migration has been shown to contribute significantly to the generation of neuronal diversity in the mammalian telencephalon. GABAergic interneurons are the best-characterized neurons that migrate tangentially, from the ventral telencephalon, dorsally into the cortex. However, the molecular mechanisms and nature of these migratory pathways are only just beginning to be unravelled. In this study we have first identified a novel dorsal-to-ventral migratory route, in which cells migrate from the interganglionic sulcus, located in the basal telencephalon between the lateral and medial ganglionic eminences, towards the pre-optic area and anterior hypothalamus in the diencephalon. Next, with the help of transplantations and gain-of-function studies in organotypic cultures, we have shown that COUP-TFI and COUP-TFII are expressed in distinct and non-overlapping migratory routes. Ectopic expression of COUP-TFs induces an increased rate of cell migration and cell dispersal, suggesting roles in cellular adhesion and migration processes. Moreover, cells follow a distinct migratory path, dorsal versus ventral, which is dependent on the expression of COUP-TFI or COUP-TFII, suggesting an intrinsic role of COUP-TFs in guiding migrating neurons towards their target regions. Therefore, we propose that COUP-TFs are directly involved in tangential cell migration in the developing brain, through the regulation of short- and long-range guidance cues.

Key words: COUP-TFs, Cell migration, Forebrain, Mouse

Introduction

It is well recognized that both radial and tangential migration contribute significantly to neuronal diversity within the telencephalon (reviewed by Marin and Rubenstein, 2003). Projection neurons originate in the ventricular zone of the cortical anlage (pallium) and migrate radially to form the developing cortex, while GABAergic interneurons originate in restricted regions of the ventricular zone of the subcortical telencephalon (subpallium) and migrate tangentially to reach their final destination, in defined telencephalic regions. Within the subpallium, the medial ganglionic eminence (MGE) appears to be the major source of cortical interneurons (Anderson et al., 2001; Jimenez et al., 2002; Marin et al., 2000; Wichterle et al., 2001). Furthermore, it has been shown in vivo that neurons that derive from the lateral ganglionic eminence (LGE) and the caudal ganglionic eminence (CGE) contribute to the generation of projection neurons in the striatum and GABAergic interneurons in the cortex, respectively (Nery et al., 2002; Wichterle et al., 2001). Although there is an overlap in the structures populated by neurons originating from three ganglionic eminences (GEs), the nuclei derived from the progeny of each eminence are distinct and can vary in their neurochemical profiles (Nery et al., 2002). As a further source of cellular diversity, the anterior entopeduncular area, which is located between the MGE and the hypothalamus, contributes to the generation of oligodendrocytes in the cortex and hippocampus (He et al., 2001). Therefore, interneurons and oligodendrocytes appear to follow a mainly ventral-to-dorsal migratory route before reaching their target tissues, and it is not known whether these cells can also migrate in a dorsal-to-ventral direction. The identification of other migratory pathways, such as the gangliothalamic body connecting the GEs with the thalamus (Letinic and Kostovic, 1997; Rakic and Sidman, 1969), and the rostral migratory stream between the LGE and the olfactory bulb (Altman, 1969; Lois and Alvarez-Buylla, 1994; Luskin, 1993), makes it plausible that the GEs generate neurons directed to other embryonic brain regions besides the dorsal telencephalon.

As suggested by various studies, directional migration can be achieved by short-range guidance cues and by long-range diffusible gradients (Tessier-Lavigne and Goodman, 1996). Contact guidance relies on a permissive environment, on which gradients of diffusible guidance molecules can be superimposed, to achieve directional migration. However, the distribution and molecular nature of short- and long-range molecules, and the substratum that interneurons use in their migration towards the cortex, are poorly understood. Motogenic factors, such as hepatocyte growth factor and the
neurotrophin molecules BDNF and NT4, have been shown to influence the numbers of cells migrating away from the subpallium (Polleux et al., 2002; Powell et al., 2001). Guidance molecules with repulsive and attractive activities are likely to be involved in guiding migrating cells from the subpallium to the pallium, and within the subpallium (Marin et al., 2003; Wichterle et al., 2003). For the substratum, one report has shown that blocking the function of the TAG1 adhesion molecule results in a marked reduction in GABAergic neurons in the cortex (Denaxa et al., 2001). However, in Tagl mutant mice, there is no major alteration in the tangential migration of interneurons (Marin and Rubenstein, 2003), and treatment of cortical slices with phosphoinositide-specific phospholipase C (PI-PLC), an enzyme that cleaves GPI-anchored proteins, such as TAG1, does not alter interneuron migration (Tanaka et al., 2003). Therefore, nothing is known about the nature of short-range guidance contact cues involved in tangential migration.

The nuclear orphan receptor COUP-TFI favours migration of neurons through an integrin-dependent mechanism in cell cultures (Adam et al., 2000). Overexpression of COUP-TFI in retinoic-acid-treated neuronal aggregates coated with different cell substrata, such as polylysine, laminin or fibronectin, results in a higher rate of migration compared with controls, whereas overexpression of a mutated form of COUP-TFI has no effects on cell migration. Moreover, increased levels of vitronectin, an extracellular matrix (ECM) molecule involved in neurite extension, motor neuron differentiation and possibly neuronal migration, are detected both in aggregate and monolayer cell cultures after overexpression of COUP-TFI. This, and the data that show that COUP-TFI stimulates transcription from the vitronectin promoter in vitro, strongly suggests a role of COUP-TFI in the regulation of ECM synthesis during the process of neuronal migration. Although COUP-TFI has been shown to be involved in cortical regionalization and axon pathfinding in the developing telencephalon (Zhou et al., 1999; Zhou et al., 2001), its role in promoting cell migration in vivo has not been established so far.

Here, we show that COUP-TFI and COUP-TFII are expressed in migrating cells in specific pathways in the basal telencephalon of mouse embryos at a stage when interneuron migration is robust. Moreover, expression of COUP-TFI in the cortex co-localizes with the GABAergic marker calbindin, suggesting that a subpopulation of COUP-TFI-positive cells in the cortex are GABAergic interneurons. With the help of transplantation and tracing techniques in organotypic slice cultures, we first show the presence of a dorsal-to-ventral migratory pathway that originates in the region of the interganglionic sulcus. We then demonstrate that COUP-TFI is expressed in the basal telencephalon in neurons not only migrating dorsally towards the cortex, but also ventrally towards the pre-optic area (POa) and the hypothalamus. Finally, by ectopic expression of COUP-TFI and COUP-TFII in the GEs, we show an increased rate of migrating cells. While COUP-TFI appears to control dorsal and ventral migration in the basal telencephalon, COUP-TFII is more specific in modulating tangential migration into the intermediate zone of the cortex, suggesting distinct functions of COUP-TFIs in regulating cell migration in the developing forebrain.

Materials and methods

**Generation of polyclonal antibodies of COUP-TFI and COUP-TFII**

To eliminate any possibility of crossreactions between the antibodies against COUP-TFI and COUP-TFII, and to identify the less conserved, more exposed and immune-reactive regions of the proteins, we performed an in-silico analysis using Blastp, PredAccServer (Protein Hydropathic Profile) and Antigenic (following Kolasker-Tangaonkar algorithm) software. GST-fusion proteins were produced using the pGEX cloning vector, in which the N-termini of COUP-TFI and COUP-TFII, comprising the first 203 amino acids, were cloned into the EcoRI-SphiI (for COUP-TFI) and the MscI-SphiI (for COUP-TFII) restriction sites of pGEX. The fusion proteins were grown in BL21 cells, and purified by affinity chromatography on glutathione-activated columns before being injected into rabbits. The immunization protocol consisted of five rounds of injections before the final bleeding, and it was carried out by Primm srl, Milan, Italy. The specificities of the antibodies were tested by blocking them with their respective immunogens, and using them in control frozen sections. No signals were detected (data not shown).

**Slice culture experiments**

Organotypic slice cultures of embryonic mouse telencephalon were prepared as previously described (Anderson et al., 1997). Brain slices 250 μm thick were used for the grafting experiments (see below), for tracing with the fluorescent carbocyanide dye 1,1′-dioctadecyl 3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes), and for electroporation and lipofectamine injections (see below).

**Grafting experiments**

For the grafting experiments, the MGE, the LGE, or the region at the MGE/LGE border (MLGE) were removed from the donor brain slices derived from a green fluorescent protein (GFP)-expressing transgenic embryo (Hadjantonakis et al., 1998) and transferred to the same position in a host wild-type brain slice. The chimaeric slices were examined 1 hour after the transplant under a stereo microscope with a GFP filter, to confirm that the transplants were successfully and correctly integrated into the host; slices where the grafts did not integrate, or integrated in an incorrect location, were not used for this study. The medium was changed every day, and after about 60 hours the slices were fixed in 4% paraformaldehyde.

**Electroporation and lipofectamine-mediated injection**

For the fate map, a GFP-expressing vector was electroporated into the GEs of embryonic day (E) 13.5 brain slices using the same method and conditions as described previously (Stuhmer et al., 2002). For the gain-of-function approach, we used the PolyFect Transfection Kit (Qiagen, Hilden, Germany), containing 250 ng/μl of the expression vectors (CMV-GFP or CMV-COUP-TFs-IRES-GFP). Full-length mouse COUP-TFI and COUP-TFII (kindly donated by M. Tsai, Baylor College, USA) were cloned into the PIRES-hrGFP-2a expression vector (Stratagene, California, USA).

**Immunohistochemistry and immunofluorescence**

The brains were sectioned and treated for immunostaining according to standard procedures. Combined immunohistochemistry and in-situ hybridization on cryosections were carried out as described previously (Hirsch et al., 1998). The times of incubation with the primary
antibodies ranged from 90 minutes (anti-COUP-TFs, anti-reelin) to overnight. The following antibodies were used: rabbit α-COUP-TFI (1:500), rabbit α-COUP-TFII (1:1000), mouse α-reelin (clone G10, 1:500; kind gift of A. Goffinet), mouse α-calbindin (clone CB-955, Sigma, 1:500), mouse α-GABA (clone GB-69, Sigma, 1:1000) and mouse δ-β-tubulin type III (clone SDL.3D10, Sigma, 1:400). Sections were alternatively incubated in a biotinylated secondary antibody (Vector, 1:200) and processed by the ABC histochemical method (Vector) or with a fluorescent secondary antibody (Molecular probes 1:400; Alexafluor 488 α-rabbit, Alexafluor 594 α-rabbit, Alexafluor 594 α-mouse) for 1 hour at room temperature. The immunohistochemistry-treated sections were dried, dehydrated and mounted on a coverslip with Permount (Fisher); the immunofluorescence-treated sections were mounted with Vectashield (Vector). The GFP was detected by its endogenous fluorescence.

Cell counting
In the grafting experiments, the cells were counted on images obtained from 6 µm sections of the slices that were acquired with a CCD camera attached to a conventional microscope. To count the cells, a standardized box (a 40x enlargement of the slice) was used; for each transplant, three sections were evaluated, and for each section, the cell's cell in two different regions were counted. In the gain-of-function experiments, an image of the whole slice was obtained by confocal microscopy, after reconstruction of all of the confocal planes. The GFP-positive cells that migrated towards the cortex or towards the POa were counted on this reconstructed image, and normalized for the total number of GFP-positive cells [e.g. cortex/cortex + basal ganglia], or POa/(POa + basal ganglia)]. The data from control and overexpressing cells within the same slice were compared, and paired Student’s t-tests were used for the analysis.

Results
COUP-TFI and COUP-TFII are expressed in distinct domains in the developing telencephalon
To analyse the expression of COUP-TFs at a cellular level in the developing telencephalon, we generated polyclonal antibodies against COUP-TFI and COUP-TFII. Because of the high sequence homology between COUP-TFI and COUP-TFII and the other members of the steroid/thyroid superfamily, we chose the N-termini of the proteins, as these show the highest sequence divergence (see Materials and methods for more details). Western blotting and immunohistochemistry demonstrated no crossreactivities between the two members of the family (data not shown).

To study the expression profile of both COUP-TFs during the period of neural migration, we performed immunohistochemistry on E13.5 mouse forebrains, when tangential migration is well pronounced. At this stage, COUP-TFI expression was localized to all layers of the neocortex, to the piriform cortex, to the ventricular and subventricular zones of the LGE, to the ventricular zone of the MGE, to the POa, and in scattered cells in various regions of the basal telencephalon (Fig. 1A). By contrast, COUP-TFII expression was mainly restricted to the marginal zone of the neocortex and to the piriform cortex and POa of the basal telencephalon. Scattered COUP-TFII-positive cells were also identified in the LGE, at the cortico-striatal boundary, and in the intermediate zone of the neocortex (arrowheads in Fig. 1A′). Expression of COUP-TFI and COUP-TFII was also seen in the interganglionic region where the MGE and LGE are fused, but before the appearance of the CGE (asterisk in Fig. 1A, A′). For convenience, we will define this area as the ‘MLGE border’

Fig. 1. COUP-TFs are expressed in migrating cells. Adjacent coronal sections of an E13.5 mouse brain at a medio-caudal level immunostained with anti-COUP-TFI (A) and COUP-TFII antibodies (A′). The asterisks in A and A′ indicate expression in the interganglionic region (the ‘MLGE’). The insets show an enlargement of this region after double labelling of COUP-TFs (COUP-TFI mRNA/COUP-TFII antibody in A; COUP-TFI antibody/COUP-TFII mRNA in A′). The arrow in A indicates the expression boundary of COUP-TFI at the level of the medial cortex, whereas in the same region COUP-TFII is expressed in the putative cortical hem region (ch in A′). An arrow in A′ indicates expression in the marginal zone of the ncx, and the arrowheads show expression at the cortico-striatal boundary and in the intermediate zone of the ncx. (B-B′) Two different enlargements of the regions indicated in A and A′. Most COUP-TF-positive cells show a polarized soma (arrows in B-B′′′), indicative of migrating cells. (C-C′) Expression of COUP-TFs in the POa region. Note that COUP-TFI is mostly restricted in the ventricular zone and in two streams of migrating cells, as indicated by the arrows. High expression of COUP-TFI is mainly localized in the mantle zone and in a dorsally migrating stream (arrow in C′). ch, cortical hem region; cp, choroid plexus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; mz, mantle zone; ncx, neocortex; pcr, piriform cortex; POa, pre-optic area; VZ, ventricular zone.
henceforth. Double labelling for COUP-TFI and COUP-TFII and COUP-TFI and COUP-TFII highlighted an increased expression of COUP-TFs in this region (insets in Fig. 1A,A').

Higher magnifications of the region between the MLGE border and POa showed that the scattered COUP-TF-positive cells were grouped into streams of cells with polarized bodies, indicative of migrating neurons (Fig. 1B-B'''). Although both proteins were localized in the same area, double detection of COUP-TFI and COUP-TFII indicated that expression in these populations overlapped only partially (data not shown). In the POa region, COUP-TFs presented complementary expression (Fig. 1C,C'). While COUP-TFI was expressed at high levels in the ventricular zone and in streams of cells oriented dorsally and ventrally (arrows in Fig. 1C), COUP-TFII was mainly expressed in the mantle zone and in cells dorsal to the POa region (arrow in Fig. 1C'). An additional complementary expression was evident at the level of the caudomedial wall of the telencephalon. While cortical COUP-TFI expression stopped abruptly at the level of the hem region (Fig. 1A), COUP-TFI was specifically expressed in this region and in the choroid plexus (Fig. 1A').

In summary, COUP-TFI and COUP-TFII showed distinct and mostly non-overlapping expression profiles in the developing telencephalon at E13.5. Furthermore, COUP-TFs appeared to be expressed in migrating cells at the stages in which several tangential migratory pathways within the developing telencephalon are well pronounced.

**COUP-TFs are detected in specific and non-overlapping cellular populations in the developing cortex**

To characterize the types of cells in which COUP-TFs are expressed, we performed a series of double immunofluorescence studies for both COUP-TFs and cell-type-specific markers (Fig. 2).

The expression data described previously suggested that COUP-TFs are expressed in migrating neurons. To test this hypothesis, we used an antibody against calbindin that labels a subpopulation of GABAergic interneurons. At E13.5, the majority of calbindin-positive cells co-labelled with COUP-TFI-positive cells in the neocortex and piriform cortex (Fig. 2A,B), but not with COUP-TFII-positive cells (Fig. 2D,E). Similar results were obtained with an anti-GABA antibody (data not shown). Thus, in the marginal zone of the cortex, GABAergic neurons are positive for COUP-TFI and negative for COUP-TFII. To assess whether COUP-TFII-positive cells in the marginal zone were rather Cajal-Retzius cells, we used an antibody against reelin. All COUP-TFII-positive cells co-expressed reelin (Fig. 2F), whereas no COUP-TFI-positive cells were positive for reelin in the cortex (Fig. 2C). At E13.5 and E15.5 co-localization of COUP-TFI and reelin was also detected in the caudomedial wall of the telencephalic vesicles (Fig. 2G,H), which has been recently described as one of the sources of Cajal-Retzius cells (Takiguchi-Hayashi et al., 2004).

This is not the case for COUP-TFI, in which no co-expression of COUP-TFI and reelin has ever been detected in this region (Fig. 2I).

Taken together, these data strongly suggest that in the cortex COUP-TFI is expressed in GABAergic interneurons, whereas COUP-TFI is expressed in Cajal-Retzius cells.

**COUP-TFI, and not COUP-TFII, is expressed in neurons migrating tangentially from the basal telencephalon to the cortex**

We have previously shown that some COUP-TFI-positive cells in the cortex are GABAergic interneurons. It is now well established that the GEs are the major source of most neocortical interneurons (reviewed by Corbin et al., 2001; Marin and Rubenstein, 2001). Therefore, to assess whether COUP-TFI-positive cells located in the basal telencephalon are interneurons migrating to the cortex, a series of grafts from the GEs of E13.5 GFP-expressing transgenic brain slices were homotopically transplanted into wild-type E13.5 brains (n=27)
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Fig. 3. COUP-TFI-positive cells in the MZ of the cortex originate from the basal telencephalon. (A) A grafting experiment performed at E13.5, in which dorsally migrating cells are analysed. The box delineates the area shown in C-H. (B) Example of an MLGE transplant that shows GFP-positive cells migrating dorsally into the ctx, laterally into the pcx, and ventrally into the POa/hypo. (C-H) Double immunofluorescence for GFP (green) and COUP-TFs (red) shows that GFP-positive cells originating from the graft are double positive for COUP-TFI in the MZ (arrowheads in C-E) and in the ventricle-oriented cells deriving from the MZ (arrows in C-E). Arrows in insets show labelling of cells in the intermediate zone (IZ). (F-H) Double immunofluorescence of GFP (green) and COUP-TFII (red) shows no co-localization of these two proteins. ctx, cortex; MZ, marginal zone; pcx, piriform cortex; POa/hypo, pre-opic area/anterior hypothalamus.

Fig. 4. Dorsal-to-ventral migration within the basal telencephalon. (A) A grafting experiment performed at E13.5, in which ventrally migrating cells are analysed. The box delineates the area shown in C-E”’. (B) Example of a graft in which GFP-positive cells have reached the POa. Arrows in C show co-localization of COUP-TFI (red) with GFP (green), whereas no co-labelling with COUP-TFII (red) has been identified (D) within the migratory cells. (E-E’’) Arrows in (E’’) indicate that migrating cells are neurons by double labelling of GFP (green) with TuJ1 (red). (E’’) is a merge of panels (E) and (E’). (F) Double immunofluorescence of COUP-TFI (green) and GABA (red) shows that some COUP-TFI-positive cells (arrows) are GABAergic in the ventral migratory stream. (G) By electroporation of a GFP-expressing construct into the MLGE region, GFP-positive cells migrate ventrally, and these cells express COUP-TFI (arrows in H). Similarly, by inserting a DiI crystal into the MLGE region, fluorescent cells are detected ventrally, in the POa/hypothalamic region (I). These cells co-express COUP-TFI (J). The arrow indicates a leading process. Note that the diffuse red signal in J is the result of the DiI signal after fixation.
expression of COUP-TFI and COUP-TFII was subsequently monitored in adjacent sections of the same slices. An example of an MLGE graft is shown in Fig. 3B, which demonstrates the fate of fluorescent cells after 60 hours of incubation, at which point GFP-positive cells were distributed in the mantle layer of the basal telencephalon and in various layers of the cortex. High magnification of the lateral and medial cortex showed that GFP-positive cells were present in the marginal and intermediate zones (Fig. 3C,E). Interestingly, some GFP-positive cells were also present in deeper layers, with a radial orientation towards the surface of the cortex (arrows in Fig. 3C-E). These cells were previously reported to migrate from the marginal zone to the ventricular zone before moving radially, to take up their positions in the cortical anlage (Nadarajah et al., 2002). After double labelling with anti-COUP-TFI, 75% of the GFP-positive cells (n=250) were also positive for COUP-TFI, including the ventricle-directed cells (Fig. 3E). Adjacent sections of the same grafted brain slices showed no co-localization of COUP-TFI and GFP (Fig. 3H). This is in agreement with the observation that COUP-TFI-positive cells in the cortex are non-GABAergic, and therefore do not originate from the basal telencephalon. Thus, our data confirm that COUP-TFI in the cortex is expressed in GABAergic interneurons originating from the subpallium, whereas COUP-TFII expression has a pallial origin.

**COUP-TFI is expressed in neurons migrating tangentially towards ventral regions of the telencephalon**

In the transplantation experiments described above, we observed that when the graft region included the MLGE region, a significant number of neurons also migrated towards the piriform cortex and towards more ventral regions, such as the POa and the anterior hypothalamus (Fig. 3B). In some grafts, GFP-positive cells were also identified in the POa (Fig. 4B), suggesting that cells deriving from the interganglionic region migrate in various directions: dorsally, laterally and ventrally. By double labelling GFP-positive cells with anti-COUP-TFI and anti-COUP-TFII antibodies, we found double-positive cells for COUP-TFI, but not for COUP-TFII, in this ventral migratory path (Fig. 4C,D). This suggests that a subpopulation of COUP-TFI-positive cells located in the POa/anterior hypothalamus might originate from the GEs. Furthermore, some of these migrating cells were neurons, as demonstrated by the double staining of GFP with the neuronal marker Tuj1 (Fig. 4E-E′). Finally, a subpopulation of COUP-TFI-positive cells in the ventral stream was positive for GABA (Fig. 4F), suggesting that some ventrally migrating cells are GABAergic interneurons.

To validate the origin of this ventral stream, we used two further experimental approaches. We first electroporated a GFP expression vector into the MLGE region of E13.5 brain slices and followed the fate of the electroporated cells after 60 hours of incubation (Fig. 4G). GFP-positive cells located ventrally with respect to the injected side showed a leading process and co-labelled with COUP-TFI, but not with COUP-TFII (Fig. 4H, and data not shown). Similarly, upon placing a crystal of DiI in the MLGE region (Fig. 4I), fluorescent cells were subsequently identified in more ventral regions, and these cells were positive for COUP-TFI (Fig. 4J). The morphology of the DiI/COUP-TFI-positive cells fits well with the morphology of tangentially migrating cells observed by other authors (Nadarajah and Parnavelas, 2002). Indeed, the nuclei of these cells were oriented in the sense of the migration and had a process lagging behind the soma (arrow in Fig. 4J).
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Finally, to assess whether ventrally directed migration also originates from more caudal levels, we grafted the CGE of a GFP E13.5 mouse brain to a wild-type brain of the same stage (n=5) (Fig. 5A). After 60 hours of incubation, GFP-positive cells migrated out of the graft into two main streams: a medial stream directed towards the third ventricle, and a lateral stream directed laterally and ventrally into the hypothalamic region (Fig. 5B). Both streams contained single neurons with short and polarized leading processes indicative of migrating cells (Fig. 5C-F). Adjacent sections of the same brain slices showed co-labelling of GFP with COUP-TFI (Fig. 5C,D), whereas no COUP-TFII-positive cells were co-localized with GFP-positive cells (Fig. 5E,F).

Taken together, these data show that cells located in the basal telencephalon migrate tangentially not only dorsally into the developing neocortex, as previously described, but also ventrally into the basal diencephalon, including the pre-optic and hypothalamic regions. Furthermore, our results show that COUP-TFI, but not COUP-TFII, is expressed in these populations of migrating neurons, suggesting a functional role of COUP-TFI in the patterning of tangential migration.

Ectopic expression of COUP-TFI in the basal telencephalon promotes cells to migrate in both dorsal and ventral directions

At this stage, we have demonstrated that a subpopulation of COUP-TFI-positive cells in the basal telencephalon are cells that are in the process of migrating; however, this does not demonstrate that COUP-TFI is directly involved in migration. To address this issue, we set up a functional assay in E13.5 brain slice cultures, in which we ectopically expressed COUP-TFI in the GEs at different dorso-ventral and antero-posterior locations. The migrating cells were followed after 60 hours of incubation. On one side, and as a control, we injected a construct expressing GFP under the control of a CMV promoter, while on the contralateral side, we injected a construct expressing COUP-TFI followed by an IRES-GFP sequence. In this way, we could follow the fate of the injected cells by following GFP fluorescence in the same slices (Fig. 6A). The histogram in Fig. 6B shows the average value of GFP/GFP<sub>tot</sub> ratio (black columns) or the GFP-COUP-TFI/GFP<sub>tot</sub> ratio (white columns) of all of the injected slices, calculated independently in the cortex or ventrally in the POa (see also Materials and methods). Bars indicate standard errors of the mean. In the COUP-TFI injected side, 72±4% of the GFP-positive cells migrate into the cortex, versus 24±4% of the GFP-positive cells in the control side. For the POa ectopic expression of COUP-TFI, 46±3% of the GFP-positive cells were induced to migrate, versus 17±3% in the control situation. Significant P-values are indicated (for the cortex P=0.0008, for the POa P=0.004). Example of a control (C) and a COUP-TFI overexpressing (D) brain slice after 60 hours of incubation. The vectors were focally injected into the MLGE region. Note that in the control situation (C), a few cells were found in the cortex (C'), while the majority remain compacted in the injection point. (D) After injection of high levels of COUP-TFI into the MLGE region, the GFP-positive cells are more dispersed (arrowheads) around the injection point, and a more pronounced contingent of cells migrates in the dorsal and ventral directions. MZ, marginal zone; Cx, cortex.
methods for details). In all the cases examined, the COUP-TFI-injected sides showed a statistically significant increase in the number of cells in the cortex, compared with the control side (Fig. 6B-D). Ventral migration was observed only in caudal sections in which the constructs were focally injected into the subventricular zone of the MLGE region (Fig. 6A). In controls, 24% and 17% of GFP-positive cells were scored on average in the cortex and in the POa, respectively (Fig. 6B), whereas the non-migrating cells were maintained in compact clusters next to the injection site. The injection of a CMV-COUP-TFI-IRES-GFP construct into the contralateral side of the same slices resulted in GFP-positive cells that were more dispersed in the mantle layer (arrowheads in Fig. 6D), and that were still in the process of migrating dorsally towards the marginal zone of the cortex and ventrally towards the POa (arrows in Fig. 6D). Moreover, a higher number of GFP-positive cells were scored in the cortex and in the POa (72% and 46%, respectively), compared with the control side. Thus, in the presence of higher levels of COUP-TFI, injected cells showed significantly increased spreading and migrating, compared with the normal situation (Fig. 6B), suggesting that COUP-TFI modulates cell migration in the basal telencephalon.

Ectopic expression of COUP-TFII in the ganglionic eminences induces a higher rate of migration into the intermediate zone of the cortex

Next, we tested whether COUP-TFII was also able to promote cell migration in the basal telencephalon. We generated an additional construct in which the COUP-TFII-IRES-GFP cassette was under the regulation of the CMV promoter. The control CMV-GFP- and CMV-COUP-TFII-IRES-GFP-expressing constructs were injected into the subventricular zone of the striatum on opposite sides of E13.5 brain slices (n=10) (Fig. 7A). The histogram in Fig. 7B shows that in the presence of ectopic expression of COUP-TFII, the average number of GFP-positive cells found in the cortex (60%, of a total number of 467 cells) was significantly higher than in the control situation (32%, of a total number of 875 cells). An example of an injection performed in the whole striatum is shown in Fig. 7C,D. As expected, a high number of GFP-positive cells migrated out of the injection point into the marginal and intermediate zones of the neocortex. After injection of the COUP-TFII-expressing construct, a statistically significant increase in the GFP-positive cells was found uniquely in the intermediate layer of the neocortex, and no GFP-positive cells were ever identified in the marginal zone (arrowheads and asterisk in Fig. 7D, respectively). However, and by contrast to previous experiments, no GFP-positive cells were ever located ventrally in the POa region, even if we focally injected the construct into the MLGE region (data not shown). Thus, ectopic expression of COUP-TFII in the basal telencephalon promotes migrating neurons to undertake a preferential route into the intermediate zone of the neocortex by avoiding the marginal zone, suggesting a cell-autonomous property of COUP-TFII in guiding cortical neurons into a specific layer of the cortex.

Discussion

Interneurons can migrate dorso-ventrally from the telencephalon to the diencephalon

Cell migration within the telencephalon can be subdivided
broadly into radial, and non-radial or tangential. While radial migration uses the radial glia as a scaffold upon which cortical neurons migrate to the developing cortical plate, GABAergic interneurons originate in the GEs and use tangential migration to migrate over long distances to reach their destinations in the cortex (Marin and Rubenstein, 2003; Rakic, 1990).

In this report, we have identified a novel migratory path, which to our knowledge has never been reported before. Using grafting experiments, DNA electroporation and tracer injections, we have shown that cells located in the interganglionic region, where the MGE and LGE fuse together, have the capability of migrating ventrally towards the pre-optic and hypothalamic regions of the diencephalon (Figs 3-5). As this path originates from the interganglionic region at specific caudal levels, it is very likely that it has been missed in other studies using similar approaches (Anderson et al., 2001; Jimenez et al., 2002; Lavdas et al., 1999; Wichterle et al., 2001). The novelty of this path is twofold: first, cells migrate tangentially from dorsal to ventral, along the subventricular zone of the MGE, i.e. in opposite directions with respect to the MGE-derived cortical interneurons; and second, these cells exit the basal telencephalon and migrate into the basal diencephalon. These cells are different from the gonadotropin-releasing hormone neurons, which originate in the olfactory placode and migrate along the vomeronasal nerve fibres until they reach the POa and hypothalamus (Wray et al., 1994; Yoshida et al., 1995). Migration of GABAergic neurons from the GEs to the dorsal thalamus has been observed in human brains; however, rodent embryos did not reveal a similar migratory pathway (Letinic and Rakic, 2001). With the help of retroviral cell tracing, Marin and collaborators (Marin et al., 2000) have shown that cells emanating from the MGE and the adjacent POa/anterior entopeduncular area express the transcription factor Nkx2.1 and migrate to the developing striatum, where they differentiate into local circuit neurons. It would be interesting to find out whether Nkx2.1 is also involved in the opposite migratory path described in this report.

Furthermore, we have shown that cells originating from the CGE migrate via different streams into the basal diencephalon, and in particular into the hypothalamus (Fig. 5B). As opposed to the MLGE transplant, in which the stream of ventrally migrating cells is quite compact, cells migrating out of the CGE take a medial and latero-ventral route. We do not know the fate of these ventrally migrating cells. In-vivo ultrasound-guided labelling or transplantation (Nery et al., 2002; Wichterle et al., 2001), in which total cortices are manipulated in toto and characterized at later stages, would help us to understand the final destinies of these migrating cells.

**Is the basal telencephalon only chemorepulsive?**

Transplantation experiments and co-culture assays have shown that the POa and hypothalamic regions prevent interneurons from migrating in a ventral direction by secreting repulsive molecules that are responsible for the dorsal migration of interneurons towards the cortex (Marin et al., 2003; Wichterle et al., 2003). However, the cues mediating this action have not been identified yet. Here, we show that cells originating from more dorsal and caudal regions are capable of migrating ventrally towards the POa/anterior hypothalamus. How do we reconcile these different results? First, the previous studies showed that the majority of interneurons that originated from the MGE avoided migrating in ventral directions. This is what is expected for a circumscribed graft in a section where the two GEs are well separated (Marin et al., 2003) (data not shown). However, the dorsal-to-ventral cell migration that we describe in this report originates from a restricted region, the interganglionic sulcus, at more caudal levels. It is plausible that in order to follow the migration that originates in the MGE and LGE, this region was not included in other studies. Therefore, we hypothesize that cells originating from different rostro-caudal levels can have different responses to the same environment by expressing position-dependent receptors. Accordingly, COUP-TFs are not expressed in ventrally migrating cells at more rostral levels, where the two GEs are well separated (data not shown). Thus, the POa and hypothalamus can have different roles, repulsion versus attraction, according to the type of cells they encounter.

**A novel role for COUP-TFs in neuronal migration in the developing forebrain**

COUP-TFs are orphan nuclear receptors of the steroid/thyroid superfamily shown to be involved in neurogenesis, axogenesis and neural differentiation (Park et al., 2003). In the cortex, COUP-TFI is mainly known for its role in regionalization and in guidance of thalamocortical projections (Zhou et al., 1999; Zhou et al., 2001), whereas COUP-TFII is a fundamental player in angiogenesis and heart development (Pereira et al., 1999). In this report, we have provided the first insights into the functional roles of COUP-TFI and COUP-TFII in neuronal migration in vivo. In a previous report, it was shown that COUP-TFI could regulate cell adhesion mechanisms required for the differentiation of embryonal carcinoma cells (Adam et al., 2000). Because of the substrates on which cells were plated, it was suggested that COUP-TFI could modify the synthesis of ECM molecules, making cells autonomous for migration and spreading. Our expression data in vivo and our functional data in organotypic cultures support these results in a context in which cell migration assumes a fundamental role in achieving neuronal diversity. By using these different approaches, we have shown that expression of COUP-TFs in highly motile neurons, such as GABAergic and reelin-positive cells (Fig. 2); we have then confirmed the co-localization of COUP-TFI in migrating neurons in a series of grafting experiments (Figs 3-5); and finally, we have demonstrated a role of COUP-TFs in modulating cell migration in a gain-of-function approach (Figs 6, 7). Our data also demonstrate that COUP-TFs can control directional migration. Indeed, cells expressing higher levels of COUP-TFs (and thus GFP) not only tend to spread and lose their aspect of aggregates, but also follow specific migratory pathways (dorsal and/or ventral) that are intrinsic to cells expressing COUP-TFs (Fig. 6, Fig. 7D).

Therefore, we hypothesize a dual role for COUP-TFs. First, they should regulate short-range cues, such as ECM and/or adhesive molecules, and in this way modulate the neighbouring environment. Changes in a permissive environment would allow random dispersion of migratory cells. Indeed, in the absence of COUP-TFI, GABAergic cells do migrate into the cortex, although in a less organized fashion than in the normal situation (M.S., unpublished). Therefore, we favour the hypothesis that COUP-TFI is involved in modulating cell migration, more than inducing cells to initiate migration.
Second, we propose that COUP-TFs control diffusible guidance molecules, and/or their receptors, involved in cell migration. Indeed, ectopic expression of COUP-TFI led to the correct directional migration of the targeted cells, i.e. dorsal into the cortex and ventral into the POa (Fig. 6D), suggesting that cells over- or mis-expressing COUP-TFI were able to respond to COUP-TFI-dependent guidance cues. More striking is the behaviour of MGE-migrating cells after mis-expression of COUP-TFI. In this case, neurons that were committed to migrate into the marginal and intermediate layers of the neocortex (see control in Fig. 7C) migrated at higher rates solely into the intermediate layer, avoiding specifically the marginal layer (Fig. 7D), suggesting that COUP-TFII regulates guidance cues required for migration into the intermediate zone of the neocortex. This behaviour is in accordance with the COUP-TFII expression pattern and the grafting experiments described in this study. Indeed, COUP-TFII-positive cells in the marginal zone are Cajal-Retzius cells, as seen by the co-expression with reelin, and it is now well established that Cajal-Retzius cells originate exclusively from the pallium (Hevner et al., 2003; Meyer et al., 1999; Takiguchi-Hayashi et al., 2004).

Conclusions

In summary, our data show a novel role for the COUP-TF nuclear receptors in tangential migration in the developing forebrain. COUP-TFI has previously been reported to be required in axon guidance in the telencephalon and romboencephalon (Qiu et al., 1997; Zhou et al., 1999). Here, we show that COUP-TFs are involved in modulating the rates and directions of tangentially migrating cells from the basal telencephalon to the cortex, and from the basal telencephalon to the basal diencephalon. Interestingly, the cells follow a migratory path that is dependent on the expression of COUP-TFI and COUP-TFII, suggesting an intrinsic role for COUP-TFs in guiding migrating cells towards their target region. Finally, we speculate that COUP-TFs are regulators of a series of factors, such as short-range, non-diffusible contact guidance cues and long-range, diffusible guidance cues.

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References


