A potential role for the OTX2 homeoprotein in creating early ‘highways’ for axon extension in the rostral brain

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SUMMARY

Brain pattern formation starts with a subdivision of the neuroepithelium through site-specific expression of regulatory genes and, subsequently, the boundaries between presumptive neuromeres may provide a scaffold for early formation of axon tracts. In the mouse forebrain, the transcription factor OTX2 is strongly expressed at several such boundaries. Combining dye tracing and staining for OTX2 protein, we show that a number of early fibre tracts develop within stripes of OTX2 expression. To analyse a putative influence of OTX2 on the expression of molecules involved in neurite growth, we generated several clones of NIH3T3 cells stably expressing OTX2 protein at varying levels. As shown by immunoblotting, Otx2 transfection affects the expression of a variety of cell and substratum adhesion molecules, rendering the cells a favourable substratum in neurite outgrowth assays. Among the molecules upregulated with increasing levels of OTX2 are NCAM, tenascin-C and DSD-1-PG, which also in situ colocalize with zones of OTX2 expression at boundaries. These data suggest that Otx2 might be involved in defining local substrata for axon extension in the forebrain.

Key words: Otx2, Forebrain, Axon tract, Zona limitans intrathalamica, Mouse, Adhesion molecule

INTRODUCTION

During brain development, growing axons are guided by external cues, comprising both membrane- or extracellular matrix-bound molecules, and diffusible factors, which are matched by corresponding receptors on the axons themselves. In recent years, many of these cues have been discovered (for review see Tessier-Lavigne and Goodman, 1996), while we are only beginning to understand how their precise spatial and temporal expression pattern is controlled on the transcriptional level. There is evidence, for the most part still indirect, that the same sets of regulatory genes that are thought to confer positional and cell type identity on developing brain regions, might as well control axon pathfinding. It has often been noted that pioneer axons grow along boundaries between early subdivisions of the brain (e.g. rhombomeres, prosomeres), which coincide with boundaries for the expression of regulatory genes, and studies of zebrafish and mouse mutants have recently provided a direct link between homeobox and pax gene expression, and axonal elongation and pathfinding (Lumsden and Keynes, 1989; Krauss et al., 1991; Wilson et al., 1993; Boncinelli, 1994; Macdonald et al., 1994, 1997; Puelles, 1995; Shimamura et al., 1995; Torres et al., 1996; Mastick et al., 1997). Expression, or failure of expression, of these genes could influence many processes such as neuronal differentiation, expression of axonal receptors for guidance molecules, or of guidance molecules in neurons and early glial cells. Restricted expression of a variety of extracellular and membrane-associated adhesion molecules, with potential influences on axon growth, has been demonstrated at such borders (Figdor and Stern, 1993; Heyman et al., 1995; Matsunami and Takeichi, 1995; Redies and Takeichi, 1996). In several cases, the genetic control of regulatory genes on such molecules has been confirmed (Jones et al., 1993; Valarché et al., 1993; Holst et al., 1997).

In the developing rostral brain of vertebrates, genes of the Otx and Emx families participate in defining early subdivisions and regional identities (Simeone et al., 1992; Finkelstein and Boncinelli, 1994). Around midgestation in the mouse rostral brain, at a stage of early axon tract formation, analysis of the expression patterns of Otx1 and Otx2 shows them to be strongly expressed in several areas known to correspond to the path of some early axon populations, such as the boundary between dorsal thalamus (DT) and ventral thalamus (VT), the region of the posterior commissure, the optic nerve and the olfactory nerve (Simeone et al., 1993; Mallamaci et al., 1996). In Otx2 heterozygous mutants, several early developing axon pathways are disturbed (Matsuo et al., 1995). Otx genes are therefore interesting candidates for controlling the expression of putative axonal growth and guidance cues.

In this study, we have combined detailed dye tracing studies on some early mouse forebrain axon tracts with
immunohistological labelling for the presence of OTX2 protein. We describe several fibre systems that grow, both longitudinally and transversely, in areas where OTX2 is strongly expressed. In parallel, we investigated if expression of Otx2 in a given cell line that does not normally express Otx2, can influence the expression of molecules that participate in controlling axon growth and guidance. To this end, cell lines stably transfected with Otx2 under control of a strong promoter were generated. In contrast to the parental cells, these cell lines present a favourable substratum for neurite growth. Moreover, several adhesion molecules that modulate axon growth, are found to be up- or downregulated in correlation with the level of OTX2 expression.

MATERIALS AND METHODS

C57/BL6 mice were raised in a timed-pregnancy breeding colony. Embryonic age was determined by appearance of a vaginal plug (E0).

Immunohistochemistry and fibre tracing

Embryos were fixed overnight in 4% paraformaldehyde (Merck)/0.1 M phosphate buffer (PB). For immunohistochemistry on whole mounts, brains of embryos were bisected and permeabilised 10 minutes at −20°C in methanol. Non-specific reactions were blocked by preincubation in phosphate-buffered saline (PBS) containing 0.5% Triton X-100 (PBT) and 10% normal goat serum (NGS), before incubation with the primary antibody in PBT containing 10% NGS. After washing in PBTB (PBT, 2% BSA), embryos were incubated with the appropriate secondary antibody. HRP staining was revealed using the Vectastain ABC kit (Vector), alkaline phosphatase with the Vector Blue kit (Vector). For 473HD and KAF14(1) Triton X-100 was used only during the preincubation.

For immunohistochemistry on sections, fixed brains were equilibrated in 30% sucrose (Sigma) in PB. Cryostat sections of 10 μm thickness were blocked in PBS, 0.1% Triton X-100, 10% NGS, incubated with the primary antibodies appropriately diluted in the same solution. After washing, secondary antibodies were applied to the sections. HRP staining was revealed using the Vectastain ABC kit.

Fibre tracing was carried out using the fluorescent dye 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine (DiIC18(3); Molecular Probes), using capillaries (50-100 μm thickness) coated with Dil and letting the dye diffuse at room temperature (RT) for 3-10 days. For double labelling, specimen were preincubated on ice in 0.5 mg/ml DAB, Tris 0.1 M, pH 8.2, followed by photoconversion (Sandell and Masland, 1988). Brains were then rinsed overnight in Tris buffer and processed for immunohistochemistry as described above.

Generation of NIH3T3 cell lines stably expressing Otx2

The pSG-Otx2 construct was generated by cloning a 900 bp complete coding sequence of Otx2 (reconstructed from a partial cDNA and genomic sequences, kindly provided by A. Mallamaci) into the EcoRI site of the mammalian expression vector pSG-5 (Green et al., 1988). NIH3T3 fibroblasts were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum (FCS) (all from GIBCO) in 5% CO2 in air humidified atmosphere and transfected at 50% confluence by the calcium phosphate precipitation method (Sambrook et al., 1989) with 18 μg of pSG-Otx2 (or pSG-5 as negative control) and 2 μg of the G418 selection plasmid pRSV-Neo per 10 cm dish. After 56 hours, the transfected cells were diluted 1:20, selected for 20-30 days with G418 at 1 mg/ml (Life Sciences), and then several clonal cell lines were isolated. The pSG-5-containing (mock-transfected) cells were selected only as bulk population. Transfected cells were routinely maintained in DMEM supplemented with 10% FCS at 37°C and 5% CO2.

RNase protection

For RNase protection analysis, an antisense strand transcription reaction with SP6 polymerase (Riboprobe Kit, Promega Biotec) was carried out in the presence of [32P]UTP (Amersham) in order to obtain a probe protecting 260 bp of the third exon of the mouse Otx2 gene. RNase protection experiments were performed using standard conditions (Arcioni et al., 1992).

Membrane preparation

Cell membranes were prepared as described (von Boxberg et al., 1993). The concentration of each membrane suspension was adjusted such that an aliquot, after diluting 1:15 in 2% SDS, would yield an optical density of 0.15 at 220 nm (Hitachi spectrophotometer). Membranes were then vacuum-sucked onto a 1 cm2 (uncoated) Nucleopore polycarbonate filter support, to yield a homogeneous carpet. For some experiments, membranes from different clones of cells were applied as alternate stripes onto filters, generally following the procedures described by Walter et al. (1987). For these experiments, the filters were preincubated with laminin (20 μg/ml).

Cell culture

Retinas from E14.5 mouse embryos were dissected in F12:DMEM medium (GIBCO). For single-cell cultures, they were washed in 1 mM EDTA in PBS (pH 7.2), then incubated in trypsin (0.25%, GIBCO) in PBS for 7 minutes at 37°C, and finally in PBS with 50 μg/ml DNAse I (Sigma) and 20% FCS. They were mechanically dissociated, washed in serum-free medium and plated onto monolayers of transfected cells at a concentration of 2x103 cells per chamber of an eight-chamber slide (Lab-Tek, Nunc). For retinal explant cultures, small pieces of retina were obtained using a micropipette (100 μm diameter), which were then deposited on the membrane carpets placed in 4-well plates (Nunc).

All cultures were incubated for 48 hours in F12:DMEM medium supplemented with transferrin 100 μg/ml, putrescin 100 μM, progesteron 20 nM, selenium dioxide 30 nM, insulin 25 μg/ml, 0.01% BSA (all from Sigma), penicillin (40 U/ml), and streptomycin (40 μg/ml) (GIBCO). Cultures were fixed with 4% paraformaldehyde/0.33 M sucrose in 0.1 M PB, washed in PBS, blocked for 1 hour at RT in PBS containing 10% NGS and 0.1% Triton X-100, and stained with an antibody directed against neuron-specific class III β-tubulin (TuJ1; 1:1000) using indirect immunofluorescence. For those experiments in which fibres were grown on striped membrane carpets, cultures were stained with the vital dye, Calcein AM (C1430, Molecular Probes) (6 μM, 30 minutes) added directly to the culture medium prior to fixation.

Electrophoresis, immunoblotting and immunocytochemistry on Otx2-transfected cells

Samples of membrane preparations from pSG-5 and Otx2-transfected cells, as well as from a suitable control tissue were solubilized in 9 M urea/5% mercaptoethanol or boiled in SDS-buffer without mercaptoethanol (for the Thy-1 immunoblot), diluted 1:1 in SDS-sample buffer, equilibrated to yield equal protein concentrations and subjected to SDS-electrophoresis followed by western blotting. Blots were saturated with 5% non-fat dried milk in PBS (1 hour) and incubated overnight in the various primary antibodies, followed by 2 hours incubation in alkaline phosphatase-labelled secondary antibodies (Jackson; 1:3000) and subsequent development in BCIP/NBT (Roth). Gold total protein staining was done with the AuroDye forte kit from Amersham.

In order to determine the relative level of expression of OTX2 in the different cell lines, cells were solubilized in 9 M urea/5% mercaptoethanol. Western blots of samples from three different
cultures for each clone were stained with anti-OTX2 antiserum (Mallamaci et al., 1996; 1:1500), followed by peroxidase-coupled secondary antibody (Jackson; 1:1000) and development in the ECL-system (Fierce). Signals on the X-ray films were quantified densitometrically (absorption per area covering the two OTX2 bands minus background of the film) using a BIOCOM system.

The primary antibodies used for immunoblotting include a rabbit polyclonal antiserum against OTX2 (used at 1:1500); a rabbit polyclonal antiserum against fibronectin (1:3000) (Sigma); a rabbit polyclonal antiserum against laminin (1:2000) (Sigma); RC-2, a mouse mAb against carbohydrate epitopes on radial glia (1:20); a rat mAb against mouse Thy-1.2 (1:20); a mouse mAb against the HNK1 epitope (1:100); a rabbit polyclonal antiserum against NCAM (1:1000); KAF14(1), a rabbit polyclonal antiserum against tenascin-C (1:1500); 473 HD, a rat mAb against DSD-1-PG (1:100); MRCD5, a rat mAb against R-cadherin (1:200) and MNCD2, a rat mAb against N-cadherin (1:500); a mouse mAb against thrombospondin-1 (1:250).

The following antibodies gave negative results on Western blots, except for the brain-tissue-positive controls: anti-PSA, anti-F3, anti-L1, anti-CD44 and anti-FORSE-1.

Data analysis
For single-cell cultures on monolayers, the length of the longest neurite, from the centre of each neuron to the growth cone, was determined, counting only neurites longer than 30 μm that emerged from an isolated neuron and did not make contact with other neurites or cells. For each type of monolayer, around 500 neurites were measured using an image analysis system (Optimas, Bioscan). The percentage of neurons with a neurite longer than a given length ‘x’ was plotted versus neurite length in CA-Cricket graph (Computer Associates). For retinal explant cultures on membrane carpets, the number of axons or axon fascicles, intersecting two circles drawn at a distance of 50 μm and 100 μm from the edge of each explant, was counted, and the explant diameter was determined in μm. Values for fibre outgrowth are given as ratio between the number of axon bundles and the explant diameter, multiplied by 100.

Statistical significance was determined by variance analysis (ANOVA) with a Fischer PLSD test using StatView II (Abacus Concepts).

RESULTS

OTX2 expression and formation of transverse and longitudinal tracts in the forebrain
Confirming previous descriptions (Simeone et al., 1993; Mallamaci et al., 1996), at embryonic day 10.5 (E10.5), OTX2-immunopositive cells are found throughout a large domain, including cortex, DT (prosomere p2), pretectal area (PT, p1) and mesencephalon (mes). As seen in whole-mount brains, we observed that the expression of OTX2 was specifically located, along the alar/basal plate boundary and caudally separated by a thin lighter zone, with one stripe continuing (anteriorly) underneath the VT, and the other (posteriorly) underneath the dorsal diencephalon and mesencephalon (Fig. 1A). As seen in frontal sections, the lighter stained zone at the ZLI corresponded to a fibre-rich area, located at the surface of the diencephalon, and surrounded by OTX2-expressing cells. Otherwise, the strong OTX2 expression at this and other boundaries corresponded to radial ‘streams’ of strongly stained cells extending from the ventricle up to the pial surface of the brain (Fig. 1C,D). As judged from the intensity of staining, it appeared that at the DT/VT border, these cells were more concentrated, and also expressed OTX2 at a higher level than within DT. In PT, the density of labelled cells was particularly high (Fig. 1C). At E12.5, OTX2 is also expressed by cells in the neuroretina, in the pigmented epithelium of the retina, including around the optic stalk, and more weakly within the optic stalk (Fig. 1B).

As determined from DiI-tracing experiments, at E11.5 and E12.5 several tracts develop within the forebrain. Axons from...
neurons labelled by small deposits of DiI in VT, near the DT/VT border, initially grow dorsoventrally along the ZLI (p2/p3 boundary) (Fig. 1E) and do not develop into DT. When arriving in a more ventral location, these fibres appear to defasciculate and form a plexus, and then grow longitudinally, in a posterior direction along the alar/basal boundary (Fig. 1F). Their growth cones progress within the fascicle of fibres in the centre of the ZLI, and some also wander around it but still maintaining their association with the OTX2-expressing cells (Fig. 1E). Retrograde labelling shows that most of these fibres originate from the dorsocaudal aspect of VT (Fig. 2B). Axons from more ventral aspects of VT grow (dorsoventrally) directly towards the alar/basal boundary, along which they then grow (Fig. 3, vt2 tract). Injections in the dorsocaudal aspect of DT label a large contingent of fibres that follow first the DT/PT (p2/p1) border and then the alar/basal boundary (Fig. 2A). Other pathways developed across prosomere boundaries. By E12.5, large numbers of neurons in DT, mostly from its rostral aspect, project directly across the DT/VT border and continue towards the internal capsule (Fig. 2C). We also identified a group of neurons just rostral to VT that project across the DT/VT border and over the dorsal half of DT (Fig. 3, ap tract). Fig. 3 shows a schematic representation of the location of these and some earlier-developing tracts (mesencephalic tract of the trigeminal nerve, tmesV; tract of the postoptic commissure, tpoc) (Easter et al., 1993).

**Characterisation of Otx2-transfected NIH3T3 cells**

We investigated whether transfection of Otx2 in NIH3T3 cells, which do not normally express OTX2, would affect their general neurite outgrowth-supporting properties. On Western blots of protein extracts from the Otx2-transfected cell clones the anti-OTX2 antibody reacted with two proteins bands at 36 and 39 kDa, corresponding to the bands identified using mouse embryonic superior colliculus as positive control (Fig. 4A,C). As expected, OTX2 staining was absent in mock-transfected cells. Among the different clones, OTX2 protein expression varied considerably while, for a given clone, the expression level was stable from one culture to another (n=3). The highest level of OTX2 protein was observed in the Otx2 cl.7 cells; the cl.10 presented a level of OTX2 approximately 2.5-fold, and cl.12 a level 7-fold, lower in comparison to cl.7. These data were also confirmed by RNAase protection on the Otx2-transfected cell clones, which exhibited basically the same differences in Otx2 mRNA expression among the three clones observed at the protein level (Fig. 4B). Immunofluorescence analysis of the Otx2-transfected cells showed their nuclei brightly stained by the OTX2 antiserum (Fig. 4C).

**Neurite outgrowth on Otx2-transfected versus mock-transfected cell lines in vitro**

To assess the neurite outgrowth-promoting properties of these cell lines, we tested the outgrowth of embryonic retinal neurites on the various Otx2-transfected cells, as well as on mock-transfected cells.

In a first series of experiments, we measured the length of neurites growing out from dissociated mouse retinal neurons, cultured on confluent monolayers of control (mock-transfected) and Otx2-transfected cells (clones 12, 10 and 7). Fig. 5A-C shows an example of process-bearing retinal ganglion cells on the respective monolayers. The morphology of the retinal cells is relatively simple, with most neurons extending a single major neurite. A comparison of the neurite length distributions on mock or Otx2-transfected cells is shown in Fig. 5D: neurite length was significantly increased on monolayers of Otx2-transfected cells, compared with growth on mock-transfected cells. Statistical analysis of the pooled data from four independent experiments yielded an average neurite length on pSG-5 (control) cells of 155±10 μm (mean ± s.e.m.), on Otx2 cl.12 cells of 233±8 μm (+50%; P=0.0001), on Otx2 cl.10 cells of 211±11 μm (+36%; P=0.0003), and 183±6 μm for Otx2 cl.7 (+18%; P=0.001). Interestingly, among the Otx2-transfected clones, neurite growth was best on those expressing a lesser amount of OTX2 protein. It should be mentioned that neurite growth on another cell line, stably transfected with a partial Otx2 cDNA (such that the protein contains the homeodomain, but lacks a large part of its C-terminal sequence) was not increased relative to the pSG-5 cells (data not shown).

In a second series of experiments, explants of retina were
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cultured on carpets of membrane preparations derived from these same cells. In this case, the difference in neurite growth on mock-transfected versus Otx2-transfected cells was even more pronounced: while, after 2 days in culture, retinal explants had extended only some sparse neurites on carpets of pSG-5 cell membranes, both Otx2 cl.12 and cl.7 membranes presented a better substratum for neurite growth (Fig. 6A-C). Moreover, corroborating the growth preferences observed on cell monolayers, growth on Otx2 cl.12 membranes again largely exceeded that on cl.7 membranes, in that neurites were longer and less fasciculated. Quantification of neurite growth was done by counting the number of axon bundles longer than 50 μm and longer than 100 μm, normalised for the diameter of each explant (Fig. 6D). Compared to axon growth on pSG-5 membrane carpets (n=50, five independent series of experiments), on Otx2 cl.12 carpets (n=68, five experiments) the number of axon bundles longer than 50 μm was increased by 500% (P=0.001), this increase being even more pronounced for fibres longer than 100 μm. As for the growth on Otx2 cl.7 membranes (n=50, five experiments), the difference to pSG-5 membranes was significant only for the number of axons longer than 100 μm (P<0.01). That fibres grew to longer lengths both on cells and on membranes from the OTX2-expressing cell lines than from control cells, but that among the various clones that we used, growth was better on those expressing a lesser amount of OTX2 protein was intriguing. Therefore, we performed another type of outgrowth assay in which membranes from Otx2 cl.12 and cl.7 cells were applied to filters as a carpet of alternating stripes (Walter et al., 1987). In these experiments, retinal axons grew on both cl.12 and cl.7 membranes, but showed a slight preference for growing in the stripes that contained cl.12 membranes (Fig. 6E).

OTX2 expression in NIH3T3 cells affects expression of cell surface proteins

The changes in substratum properties induced by Otx2 transfection of NIH3T3 cells led us to examine the expression of various cell adhesion and extracellular matrix molecules, using immunoblotting or immunohistological staining of the different clones. After culturing the Otx2-transfected cells for 3 days, cell membranes were prepared in the same way as for the neurite outgrowth assay, and then subjected to Western blot analysis. For those cases where cell surface protein expression was altered by Otx2-transfection, results are summarised in Fig. 7. Colloidal gold total protein staining (top left) is included to show that equal amounts of protein were loaded in each lane and that overall protein composition of membranes derived from the different clones does not vary to a large degree (but note some apparent differences especially in the high molecular weight range).

The expression pattern of several adhesion and extracellular matrix proteins was found to vary characteristic in correlation with the level of OTX2 protein expression. Among adhesion molecules of the Ig-superfamily, N-CAM and Thy-1 were found to be upregulated in correlation with the amount
of OTX2 expression in the transfected cell lines. This result is especially intriguing for NCAM, of which only the 140 kDa isoform is expressed by pSG-5 cells. While in clone 7 this isoform is highly upregulated, this clone moreover expressed the two other major isoforms (120 and 180 kDa). The level of OTX2 expression appeared also to influence calcium-dependent adhesion molecules: although present at low level in the transfected cells, R-cadherin was upregulated and N-cadherin was downregulated. Tenascin-C, DSD-1-PG and thrombospondin-1 were induced with increasing concentrations of OTX2. Fibronectin, a major component of the fibroblast cell surface, and to a lesser extent also laminin, were downregulated in Otx2-transfected cells with increasing amount of OTX2 expression.

The HNK1-antibody, reacting with carbohydrate epitopes on several cell adhesion molecules (Kruspe et al., 1984), and the RC-2 antibody, which marks carbohydrate epitopes on early radial glia (Misson et al., 1988), revealed more complex staining patterns: while being similar for pSG-5 and Otx2 cl.12 membranes, the pattern of cl.7 membranes differed considerably.

We investigated the in vivo expression patterns of several extracellular or membrane-bound growth/guidance molecules in the forebrain that were found to be upregulated in the cell lines. In chick, a strong expression of N-CAM at the ZLI has already been described (Figdor and Stern, 1993) and we found also that, in the mouse forebrain at E12.5, N-CAM-expressing cells are concentrated along the ZLI (and near the surface of DT; Fig. 8A). In addition, we found that tenascin-C was very strongly expressed at the ZLI and at the DT/PT border at the same age (Fig. 8B). DSD-1-PG was strongly expressed in two continuous stripes, bordering the ZLI and along the alar/basal boundary (Fig. 8D). This chondroitin sulfate proteoglycan is also present around the optic nerve (Fig. 8C).

DISCUSSION

Otx2 is part of a network of genes that are involved in specifying various regions of the rostral brain (Simeone et al., 1992; Millet et al., 1996; Acampora et al., 1997). A role of Otx genes in distinguishing adjacent mesencephalic and metencephalic regions has been recently proposed (Millet et al., 1996; Acampora et al., 1997). The ZLI, where Otx1 and Otx2 are strongly expressed, is suspected to have features of a local inducer and to have morphogenetic properties (Martinez et al., 1991; Marin and Puelles, 1994; Bally-Cuif and Wassef, 1995). Based on its pattern of expression, it has been suggested that Otx2 might be involved in regulating axonal growth in the murine forebrain (Simeone et al., 1993; Mallamaci et al., 1996). Results of knockout experiments in mice have not been informative on this aspect, since homozygous Otx2-null mutant mice lack neuroectoderm rostral to rhombmere 3 (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996), which reflects an earlier requirement of this gene in formation of head regions. In heterozygous mutant mice, it has, however, been found that some pathways are missing or abnormal (Matsuo et al., 1995).

In Drosophila, expression of the Otd gene (the homolog of the vertebrate Otx genes) in cells along commissures of the embryonic brain and ventral midline has been described, and defects in neuron survival and axonal pathways formation have been noted in mutants (Finkelstein et al., 1990; Klämbt et al., 1992; Wieschaus et al., 1992).

Several axon tracts develop within zones of strong OTX2 expression in the forebrain and midbrain

Confirming previous descriptions (Puelles et al., 1992; Easter et al., 1993; Figgod and Stern, 1993; Macdonald et al., 1994; Wolfer et al., 1994; Chédotal et al., 1995; Mastick and Easter, 1996), we found that around E12-E13 in mouse, while some pathways develop entirely within the neuroepithelium in the alar plate, far from borders of neuromeres, others develop along longitudinal or transverse borders (Fig. 3). In particular, the v1 axons, which form part of the tract of the zona limitans

Fig. 5. Neurite growth from retinal neurons cultured on Otx2-transfected cells. Morphology of neurons from E14 mouse retina cultured on monolayers of mock- (A), Otx2 cl.12- (B) and Otx2 cl.7- (C) transfected cell clones. Bar, 70 µm. (D) Distribution of retinal ganglion cell neurite length on monolayers of control- (black squares) and Otx2-expressing 3T3 cells of clone 12 (rhombs), clone 10 (circles) and clone 7 (triangles), plotted as percentage of neurons with longest neurite (y axis) longer than a given length (x axis).
intrathalamica, grow almost entirely among OTX2-expressing cells, along the ZLI, and more ventrally along the alar/basal border. The dt1a pathway, which includes axons that form the habenopeduncular tract (Puelles et al., 1992; Wolfer et al., 1994), grows first beside the PT, which at E12.5 strongly expresses OTX2 (and slightly later is delineated by a radial stream of OTX2-expressing cells), and then along the alar/basal border. On the contrary, the ap pathway, which probably includes axons from the perireticular nucleus (Mitrofanis and Baker, 1993), does not grow along any particular boundary region.

In addition to the vt1 and dt1 pathways (Fig. 3), it seems that the location of still other fibre tracts might be closely correlated to sites of strong OTX2 expression. Part of the trajectory of the tpoc might develop around E10.5 along the ventral and longitudinal stripe of strong OTX expression (ventral to DT), and tmesV appears to correspond, over its longitudinal path, to the longitudinal zone of strong OTX2 expression, underneath DT, PT and mesencephalon (Easter et al., 1993; and our unpublished findings). The posterior commissure also develops among or above cells that strongly express OTX2, such as is also the case for the anterior commissure (Simeone et al., 1993). When recapitulating the trajectories of these tracts (Fig. 3), they appear to delineate a system of local ‘highways’ for axon extension in the brain, which forms a relatively simple grid-like pattern. In several locations, other genes might play similar or complementary roles. Thus, the tpoc also follows, over part of its trajectory, a zone of strong Pax6 expression, as well as two bands of Dlx2 and Nkx2.2 expression (Mastick et al., 1997; Shimamura et al., 1995). In zebrafish, the nk2.2 expression pattern demarcates a narrow boundary zone where neurons that give rise to the tpoc and medial longitudinal fasciculus differentiate (Barth and Wilson, 1995).

Our double-staining experiments suggest that the growth cones of fibres around the ZLI and more ventrally progress in the immediate vicinity of cells in which OTX2 is strongly expressed. Areas of strong OTX2 expression thus appear to demarcate the trajectory of these fibres, within which some more fine-grained features should exist. The nature of cells expressing OTX2 at the ZLI and other regions in midgestation embryos is not well-defined. However, other studies have shown that OTX2 expression can occur, exclusively or conjointly, in proliferating neuroblasts, in young postmitotic neurons and in differentiating neurons, as well as in glial cells (Frantz et al., 1994; Mallamaci et al., 1996; Bovolenta et al., 1997). As assessed from their distributions, the OTX2-expressing cells at the ZLI might during later development populate restricted thalamic nuclei (see also Puelles et al., 1992). Finally, it should be stressed that a higher expression of OTX2 along the ZLI and alar/basal border is already visible in the neuroepithelium at E10.5, before some of the tracts that we describe are formed.

**Otx2-transfected cells present neurite outgrowth-supporting properties**

To investigate the potential role of OTX2 in defining local substrata for axon growth, we assessed whether and how Otx2 might regulate the expression of cell- and substratum adhesion molecules expressed by a given cell type, which would then be analysed both biochemically and by in vitro outgrowth assays. To this purpose, we permanently transfected the NIH3T3 cell line with a plasmid carrying the Otx2 cDNA. A similar approach has previously been undertaken to investigate the regulation of the β-amyloid gene by the Hox 3.1 gene (Violette et al., 1992). The fact that we obtained cell clones expressing varying levels of OTX2 protein enabled us to correlate potential changes in the expression of adhesion molecules with

![Fig. 6. Neurite growth of retinal explants cultured on membrane carpets prepared from Otx2-transfected cells. (A-C) Neurite growth from E14 mouse retinal explants, cultured on membrane carpets prepared from control (pSG-5)-transfected (A), and Otx2-transfected 3T3 cells expressing different levels of OTX2: Otx2 cl.12 (B) and Otx2 cl.7 (C). (D) Quantitative analysis. The graph shows the number of axon bundles extending on membranes derived from the different clones that exceed 50 or 100 μm in length, respectively, corrected for the explant diameter (see Methods). Error bars indicate mean values ± s.e.m. for five independent experiments. (E) Typical growth pattern of retinal fibers on a striped carpet of alternating membranes from Otx2 cl.12 and cl.7 cells. Bar, 100 μm.](image-url)
the level of OTX2 expression. A limitation of these experiments is that the genetic background of this cell line, which is of fibroblastic (mesenchymal) origin, probably does not allow expression of proteins that are normally foreign to these cells.

In both our assay systems, a strong change in the neurite outgrowth-supporting properties of the fibroblast cell line was induced by Otx2 transfection. First, the neurite outgrowth on monolayers of three Otx2-transfected clones exhibiting different levels of OTX2 protein, was better than on mock-transfected cells. Second, this difference in neurite growth, i.e. both length and density of neurites, was even more pronounced on Otx2-transfected versus control cell membranes. Although retina was used for these in vitro experiments, it should be stressed that we have obtained quite similar results using other neuronal populations, e.g. early embryonic (E12.5) thalamic neurons and postnatal (P5) cerebellar neurons (K. T. N.-B.-C. and P. G., unpublished data).

**Regulation of cell- and substratum-adhesion molecules in Otx2-transfected cells**

Using immunoblotting, we found that following Otx2 transfection of NIH3T3 cells, the expression levels of a number of known cell- and substratum-adhesion molecules were affected, including several molecules of the Ig-superfamily, the cadherin family and extracellular matrix molecules (for review see Hynes and Lander, 1992). These changes could only be detected for such proteins that were already, be it at low level, expressed by the cells of origin. However, we would like to point out that the presented list of molecules that are affected by Otx2 expression may not be exhaustive.

The upregulation of R-cadherin, and the downregulation of N-cadherin, is intriguing since cadherins, in specific combination, are important for specific tissue cohesion during development and may also govern the pathway choices of growing axons in the forebrain (for review, see Redies and Takeichi, 1996). Among the high molecular weight glycoproteins, both tenascin-C and thrombospondin-1, which can promote axonal growth, were found to be upregulated in Otx2-transfected cells. The upregulation of tenascin-C may seem contradictory to the recent finding of OTX2 repressing tenascin-C in transiently Otx2-transfected cells (Gherzi et al., 1997); this may be explained by a different action of Otx2 in stably versus transiently transfected cells (see e.g. Mettouchi et al., 1997). Finally, the upregulation of NCAM by cl.7, and expression of all three isoforms, seems also interesting, in view of its role in promoting axon growth and cell migration.

The observed changes in cell adhesion molecule expression in the transfected cells suggest that Otx2 could regulate their expression. Whether such changes are due to direct regulation by OTX2 of the transcription from the corresponding genes, and the relevance of these observations to in vivo functions of OTX2, remains to be studied. For NCAM, tenascin-C, as well as DSD-1-PG, which are colocalised with zones of strong expression, it should be stressed that we have obtained quite similar results using other neuronal populations, e.g. early embryonic (E12.5) thalamic neurons and postnatal (P5) cerebellar neurons (K. T. N.-B.-C. and P. G., unpublished data).
OTX2 expression in the embryonic forebrain, such a regulation is at least likely.

**Relevance to axonal growth**

The basic result of the neurite outgrowth assays on the transfected cells, is that all three Otx2-expressing clones were converted into a better substratum for neurite growth than untransfected cells. The immunoblot data show that fibronectin, a molecule that is expressed in high quantity by untransfected fibroblasts and which is a poor substratum for neurite growth than untransfected fibroblasts, is converted into a better substratum for neurite growth than untransfected fibroblasts. These immunoblot data are certainly upregulated. Although these immunoblot data are certainly upregulated. Such a fine-tuning of the expression of both growth-promoting, and growth-repulsive, molecules may well be fundamental in delimiting axonal pathways in vivo.

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