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RESEARCH ARTICLES

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Expression profiles suggest a role for Pax7 in the establishment of tectal polarity and map refinement

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Abstract The role for *Pax7* in establishing tectal polarity and map refinement was authenticated by gene expression studies in vivo and in vitro. Throughout development (stages E2–E12 were examined) a rostral^{low}-caudal^{high} and dorsal^{high}-ventral^{low} *Pax7* expression gradient was detected immunohistochemically in the chick optic tectum, indicating a role for *Pax7* in establishing tectal polarity. Chick retino-recipient tectal cells positive for *Pax7* also co-expressed *ephrin-A2*, a molecule involved in the establishment and refinement of the retinotopic map. In vitro, *PAX7* up-regulated *ephrin-A2* when transfected into undifferentiated P19 cells; cells became negative for both Pax7 and ephrin-A2 protein following treatment with anti-sense oligonucleotides. These results suggest that in addition to being involved in the early establishment of tectal polarity, *Pax7* plays a later role in retino-tectal map formation and refinement.

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Introduction

The optic tectum, or its mammalian homologue the superior colliculus, differentiates from the alar plate of the mesencephalon and receives retinal fibres in a precise retinotopic manner. Several transcription factors including Otx2, En and Pax2/5 as well as fibroblast growth factor 8 (Fgf-8) secreted from the isthmus, the organising centre at the midbrain-hindbrain boundary, function in a positive feedback loop to initiate regionalisation of the optic tectum and determine its polarity (Bally-Cuif et al. 1995; Crossley et al. 1996; Song et al. 1996; Araki and Nakamura 1999; Shamim et al. 1999). Several recent experiments indicate that the transcription factor, Pax7, may be included in the set of factors that function to define the tectal region.

Evidence for the role of *Pax7* in central nervous system (CNS) patterning and tectal regionalisation originated from expression studies that show early, restricted *Pax7* expression in the neural tube, where it is thought to pattern the dorso-ventral axis of the developing nervous system (Jostes et al. 1990; Stoykova and Gruss 1994; Tanabe and Jessell 1996; Kawakami et al. 1997). Furthermore, during early brain development and regionalisation *Pax7* expression is confined to the dorsal mesencephalon and the tectum anlagen (Jostes et al. 1990; Stoykova and Gruss 1994; Kawakami et al. 1997). The key role of *Pax7* in tectal differentiation was demonstrated by misexpression studies which show that *Pax7* induces formation of an ectopic tectum in the diencephalon (Matsunaga et al. 2001). Moreover, ectopic tectum formation induced by isthmus transplantation results in up-regulation of *Pax7* together with *En*, *Pax2/5* and *Fgf8* in the ectopic tectum (Nomura et al. 1998; Araki and Nakamura 1999). Taken together, these experiments suggest a role for *Pax7* in tectal differentiation and the establishment of its polarity.

In later stages of tectal development, when the characteristic laminae differentiate, *Pax7* expressing cells con-

centrate in the retino-recipient layer, the stratum griseum superficiale (*sgfs*) (Stoykova and Gruss 1994; Kawakami et al. 1997). Furthermore, when *Pax7* was misexpressed in the diencephalon, the resultant ectopic tectal swelling exhibited a well defined laminar structure that was innervated by retinal fibres (Matsunaga et al. 2001). The transition from region- to cell-specific expression led Kawakami et al. (1997) to suggest two different roles for *Pax7*, namely early regionalisation of the tectum followed by later differentiation of cells of the *sgfs* layer.

Cells within the *sgfs* layer also express *ephrin-A2*, a molecule implicated in establishing tectal topography (Cheng et al. 1995) and thus retino-tectal map formation. Complementary retinal and tectal gradients of Eph receptors and their ephrin ligands are considered to define topography; nasal^{low}-temporal^{high} retinal gradients of EphAs (Eph-A3 and/or Eph-A5) interact with a rostral^{low}-caudal^{high} gradient of tectal ephrin-A2 and a caudal gradient of ephrin-A5 (Drescher et al. 1995; Tessier-Lavigne and Goodman 1996; Hornberger et al. 1999). In addition, the dorsal^{high}-ventral^{low} tectal gradient of ephrin-A2 (Cheng et al. 1995; Marin et al. 2001) may act in concert with graded EphB/ephrin-Bs to define the dorso-ventral axis (Braisted et al. 1997; Mann et al. 2002). The similar restricted expression patterns of *Pax7* and *ephrin-A2* within the *sgfs* layer of the developing visual system (Kawakami et al. 1997; Marin et al. 2001) suggest that *Pax7* is involved in retino-tectal map formation (Ziman et al. 2001a).

Here, to test the possibility that *Pax7* is involved in establishing tectal polarity we investigated the chick tectal expression profile of *Pax7* from embryonic day (E) 2 to 12. Using immunohistochemistry, we show that *Pax7* exhibits a spatial and temporal graded expression profile at E2 similarly to that observed for *En*, which is considered to be the earliest known marker of tectal polarity (Logan et al. 1996). Furthermore *Pax7* expression continues to be expressed in a rostral^{low}-caudal^{high} and ventral^{low}-

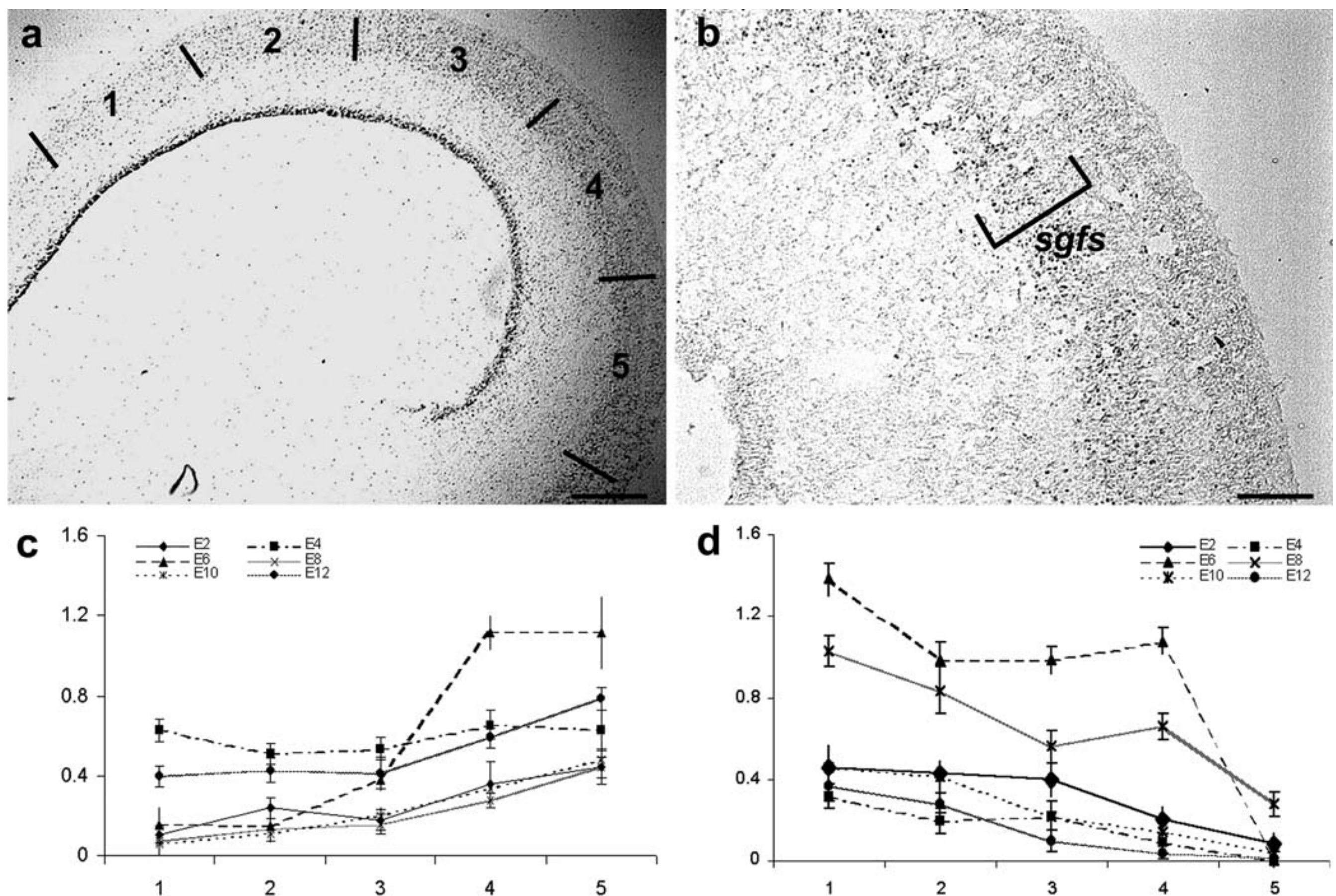

Fig. 1a–d Pax7 immunoreactivity in developing chick tecta. **a** A representative section showing graded Pax7 immunoreactivity across the rosto-caudal axis of an E8 sagittally sectioned chick tectum. *Scale bar* 250 μm. **b** A representative section showing Pax7 immunoreactivity in an E12 coronally sectioned chick tectum, with *Pax7* expression being concentrated in the *sgfs* layers. *Scale bar* 100 μm. **c** A rostral^{low}-caudal^{high} Pax7 gradient in the *sgfs* layer of the tectum. The tectum was sectioned into five areas along the rostro-caudal axis (1 being the most rostral and 5 the most caudal) and the average intensity of the Pax7 immunohistochemical reaction in each section was quantified and graphed in Excel. The rostral^{low}-caudal^{high} gradient was apparent from E2 onwards and was significant (p<0.05) at each stage except at E4 and E8 (p>0.05). **d** A dorsal^{high}-ventral^{low} Pax7 gradient in the tectum. The tectum was sectioned into five areas along the dorso-ventral axis (1 being the most dorsal and 5 the most ventral) and the average intensity of the Pax7 immunohistochemical reaction in each section was quantified and graphed in Excel. There was a significant dorsal^{high}-ventral^{low} gradient at each stage from E4–E12 (p<0.05 at each stage) except at E2 (p>0.05) when only a single layer of cells is present in the tectum

dorsal^{high} trend throughout development. Moreover, we suggest that *Pax7* plays a role in retino-tectal map formation and refinement as it regulates *ephrin-A2* expression in a cell autonomous manner; in vivo during retinotopic map formation and refinement, *Pax7* and *ephrin-A2* are co-expressed in cells of the *sgfs* layer and in vitro *PAX7* transfection up-regulates *ephrin-A2*.

Some of these results have been presented in abstract form (Thomas et al. 2001).

Methods

Preparation and immunohistochemical analysis of chick embryos

For the in vivo studies, chick eggs (Leghorn) were incubated at 37–38°C. Staged embryos (Hamburger and Hamilton 1951) were terminally anaesthetized by hypothermia at E2, 4, 6, 8, 10 and 12. Brains were removed and cryosectioned at 20 μm in the sagittal or coronal plane to reveal the rostro-caudal or dorso-ventral tectal axis (n=5 animals per stage). Material was processed with antibodies directed against Pax7 (1/10; mouse monoclonal; DSHB). Antibody binding was detected using a biotin-streptavidin-peroxidase system (Dako) and metal-enhanced diamino-benzidine (DAB, Pierce). Controls without primary antibody were negative.

Experimental procedures followed guidelines of the National Health and Medical Research Council of Australia and were approved by the Animal Ethics Committee, University of Western Australia.

Quantification of *Pax7* expression

Intensity of Pax7 immunoreactivity was quantified using a Leucia image analysis system. The tectum was divided into five equal sections along the rostro-caudal (1 being the most rostral and 5 the most caudal) (Fig. 1a) or dorso-ventral (1 being the most dorsal and 5 the most ventral) axis and five separate intensity measurements for each section were taken and averaged to give the average intensity reading per section. Once tectal laminae could be observed, intensity of Pax7 immunoreactivity was measured in the dorsal layers where Pax7 expression was concentrated (Fig. 1b). Levels were normalised against adjacent negative ventral staining regions. For each axis, three to five sections from each animal were analysed and standard errors calculated. At each developmental stage, expression of *Pax7* along the rostro-caudal and dorso-ventral axes were analysed by performing a one-way ANOVA (SPSS).

Immunofluorescence

For the co-expression study we processed sections as before and analysed both *Pax7* and *ephrin-A2* (1/50; rabbit polyclonal; Zymed) expression, detecting the signal by immunofluorescence using anti-mouse IgG-FITC (Oxford Biotechnology) and biotinylated anti-rabbit IgG followed by streptavidin-TRITC (Serotec). Tissue was examined on a fluorescence microscope (Leitz Diaplan with a Nikon DXM 1200 digital camera). Control experiments lacking primary antibodies showed no immunofluorescence.

In vitro transfection and antisense studies

For in vitro experiments, mouse P19 cells were chosen as a standard model to study gene expression associated with neuronal differentiation (Ziman et al. 2001b; Wei et al. 2002). Cells were plated at a density of 1×10^5cells/ml and incubated in DMEM containing 10% fetal calf serum and 2 mM L-glutamine, at 37°C in a 5% CO_2 atmosphere. Full-length *PAX7b* cDNA (Vorobyov et al. 1997; Ziman et al. 1997; Ziman et al. 2001b), isolated from human skeletal muscle, was subcloned into the HA-tagged *pHM6* vector (Roche), transfected into P19 cells using lipofectamine-2000 (Gibco) and resultant stable clones selected using G418 and re-plated (Ziman et al. 2001b). After 48 h, cells were fixed with 4% paraformaldehyde for immunohistochemistry or harvested for RNA isolation. As controls, we examined untransfected P19 cells, cells transfected with vector alone or cells transfected with a full-length *Pax6* cDNA transcript (Ziman et al. 2003). *PAX7*-transfected cells were also treated with fluorescein-tagged antisense or sense oligonucleotide for 24 h. The antisense and sense oligonucleotides (Geneworks) were directed to the mRNA translational start site of *PAX7* (Bernasconi et al. 1996). The sequences were:

Antisense: 5'-AGGGCCGCCATTCTTGC-3'

Sense: 5'-GCAAGAATGGCGGCCCT-3'.

It is possible that transfection with exogenous human *PAX7* also up-regulates endogenous mouse *Pax7* in P19 cells; therefore, mouse *Pax7* nomenclature will be used except when referring specifically to the human *PAX7* transcript.

In vitro immunohistochemistry

Immunoreactivity for Pax7, the neuronal markers neurofilament-H (NovaCastra), β-tubulin (Promega) and neural cell adhesion molecule (NCAM, DSHB) or ephrin-A2 were assessed separately on three or more clones. Antibody dilutions were: Pax7 1:10; ephrin-A2 1:50; neurofilament-H 1:200; β-tubulin 1:200; and NCAM 1:20. Immunoreactivity was detected using a biotin-streptavadin-peroxidase system and visualised with DAB. Controls containing no primary antibody were negative. Extent of neural differentiation as assessed by neurofilament expression was quantified and expressed as a percentage by counting the number of neurofilament immunopositive cells in a random sample of cells (n=250), repeated in three clones. Intensity of ephrin-A2 immunoreactivity was quantified using an Optimus image analysis system. Random cell sampling (n=45) from three distinct areas on each slide was repeated in three clones. Analysis was carried out using Student's t-tests with a 95% confidence interval.

RT-PCR

For RT-PCR analysis of gene expression in transfected cells, RNA was isolated from cells, lysed in 2 ml of RNAzol (Life Technologies) and included in RT-PCR reactions (Titan One-Step RT-PCR, Roche) that contained primers for *Pax7* (E1 and E4), *ephrin-A2* (F1 and F2; Cheng and Flanagan 1994) or *neurofilament-M* (NFF and NFR; Levy et al. 1987). The *Pax7* primers were complementary to human and mouse *Pax7* sequences (Vorobyov et al. 1997; Seale et al. 2000). Thermal cycler conditions were as previously described (Ziman et al. 2001b). Primer sequences were:

E1: 5'-TACCAGGAGACCGGGTCCATC-3';

E4: 5'-TCCGAACTTGATTCTGAGC-3'.

F1: 5'-AGGTTTCAGGTGAGCGCTGTG-3';

F2: 5'-CATCTTCACCAGTAACAGCTC-3'.

NFF: 5'-CAGCAGTTGGAAAATGAACTTC-3'; and

NFR: 5'-CTTCTCGACCTTGATTTCCTCCTTGACAGC-3'.

All experiments were repeated on three or more clones. Results are described qualitatively rather than analysed quantitatively since mRNAs were either present or absent.

Results

To further investigate the expression patterns of *Pax7* in the developing tectum we immunostained sagittal and coronal tectal sections from E2, 4, 6, 8, 10 and 12 chicks for *Pax7* and quantified the level of *Pax7* expression along the rostro-caudal axis and the dorso-ventral axis (in the superficial dorsal cell layers). We chose to examine embryos from E2, as the entire tectum differentiates from the mesencephalon region becoming clearly distinguishable from the remainder of the mesencephalic vesicle by E4. Our final time point was E12 by which time cell proliferation has effectively ceased (Cowan et al. 1968).

Pax7 expression during tectal development

Whilst at E2 the tectum is not distinguishable from the mesencephalic vesicle, *Pax7* expression occurs in a very distinctive pattern. In the rostro-caudal axis *Pax7* expression is expressed in a rostral^{low}-caudal^{high} gradient (F=5.982, p=0.038, Fig. 1c). Even though the tectum is not clearly distinguishable from the mesencephalon, *Pax7* expression occurred only in the dorsal regions of the mesencephalon, being completely absent in the ventral regions (Fig. 1d).

In E4 chicks the intensity of tectal *Pax7* expression increases compared to that observed in E2 chicks; however, its expression profile along the rostro-caudal axis is no longer graded, but instead is linear (Fig. 1c). Along the dorso-ventral axis, *Pax7* expression is present as a gradient (F=6.662, p=0.023, Fig. 1d) with *Pax7* expression occurring in the dorsal tectal regions only.

When chicks reach E6, there is a marked increase in tectal *Pax7* expression levels, with the caudal levels increasing dramatically such that a significant rostro-caudal gradient is established (F=13.840, p=0.007, Fig. 1c). Likewise along the dorso-ventral axis, *Pax7* expression increases dramatically in the dorsal regions and continues to decline in the more ventral regions, resulting in a significant dorsal^{high}-ventral^{low} gradient (F=50.716, p=0.001, Fig. 1d).

The caudal expression levels of *Pax7* are not as marked in E8 tecta; however, although not statistically significant, the trend of the *Pax7* expression profile is nevertheless towards a rostral^{low}-caudal^{high} gradient (Fig. 1c). Along the dorso-ventral axis a significant dorsal^{high}-ventral^{low} gradient is maintained (F=6.137, p=0.024, Fig. 1d).

In E10 tecta, there is little change in the *Pax7* expression levels compared to those observed in E8 tecta; however, the rostral^{low}-caudal^{high} gradient is re-established (F=10.551, p<0.001, Fig. 1c). Along the dorso-ventral axis dorsal *Pax7* expression levels decrease yet maintain a dorsal^{high}-ventral^{low} gradient (F=11.296, p=0.008, Fig. 1d).

The rostro-caudal expression levels are slightly increased in tecta of E12 chicks with the *Pax7* profile maintaining a rostral^{low}-caudal^{high} gradient (F=27.459, p<0.001, Fig. 1c). Along the dorso-ventral axis *Pax7* expression levels decrease but a strong dorsal^{high}-ventral^{low} gradient is still apparent (F=17.076, p=0.010, Fig. 1d).

In summary, at all stages of development, *Pax7* expression follows a rostral^{low}-caudal^{high} (Fig. 1c) and a dorsal^{high}-ventral^{low} (Fig. 1d) gradient although this profile does not always reach statistical significance.

Specificity of the Pax7 antibody has previously been determined by Western blot analysis (Kawakami et al. 1997; Ziman et al. 2001b).

Co-expression of *Pax7* and *ephrin-A2* in *sgfs* tectal neurons

The continual restriction of *Pax7* from region specific to a subgroup of postmitotic cells concentrated in the *sgfs* layers has led to the proposal that during development *Pax7* has a biphasic role, firstly in the tectal regionalisation and later in the specification of cells (Stoykova and Gruss 1994; Kawakami et al. 1997). To further investigate the role of Pax7 in the developing tectum, specifically in the formation of retino-tectal maps and map refinement, we examined whether *Pax7* expressing cells also expressed *ephrin-A2*, the ligand known to be involved in guiding retinal axons to their correct tectal locations (Cheng et al. 1995; Drescher et al. 1995; Marin et al. 2001). Immunofluorescence shows *Pax7* (Fig. 2a) and *ephrin-A2* (Fig. 2b) expression concentrated in cells of the

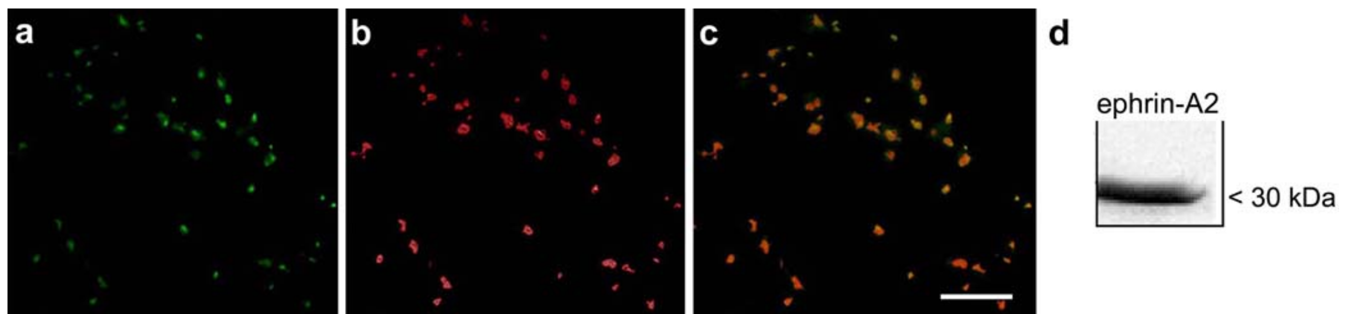

Fig. 2a–d Co-expression of *Pax7* and *ephrin-A2* in tectal *sfgs* cells. Chick tectum (E12) with **a** nuclear *Pax7* expression and **b** cell surface *ephrin-A2* expression in cells of the *sgfs*. Images are overlaid to show *Pax7* (*green*) and *ephrin-A2* (*red*) expression co-localised **(c)**. *Scale bar* 40 μm **(a–c)**

sgfs layers. Moreover, all *Pax7* (green) expressing cells within the *sgfs* layer co-express *ephrin-A2* (red), suggesting that Pax7 regulates *ephrin-A2* expression in a cell autonomous manner (Fig. 2c). Control experiments without primary antibodies show no fluorescence (results not shown, no signal). Antibody specificity for ephrinA-2 is confirmed by Western blot; a single band is obtained for aliquots of mouse brain (Fig. 2d).

Neural differentiation regulated by *Pax7* in vitro

Previous research demonstrated that when undifferentiated P19 embryonal carcinoma cells were transfected with *PAX7b*, the cells differentiated along a neurogenic lineage (Ziman et al. 2001b). Using this in vitro model the extent of *Pax7* and *ephrin-A2* co-expression was further investigated. Initially we confirmed the ability of *PAX7* to initiate neural cell differentiation. There is a dramatic change in cell morphology of transfected cells; cells no longer grow as aggregates but form a monolayer, become elongated and develop neurites (Fig. 3a) (Ziman et al. 2001b). Cells transfected with vector alone (Fig. 3b) or untreated cells (Fig. 3c) continue to grow as aggregates. Nuclear expression of *Pax7* is observed in transfected cells (Fig. 3d) but not in the control cells, those transfected with vector alone (Fig. 3e) or in untreated P19 cells (Fig. 3f).

Neural differentiation was assessed by expression of the neural markers neurofilament-H, β-tubulin and NCAM. *PAX7*-transfected cells become immuno-positive for all three neural markers both in their cell bodies and neurites (Fig. 4a–c). Cells transfected with vector alone (Fig. 4d–f) or untreated cells (Fig. 4g–i) show no expression of any of the three neural markers. The ability of *Pax7* to direct neural differentiation was further assessed by quantifying the number of *PAX7* stably transfected cells that underwent neural differentiation. The entire population of *PAX7*-transfected cells become immuno-positive for neurofilament-H (Table 1) indicating that all had differentiated into neurons. The result contrasts with differentiation of P19 cells into neurons, glia and fibroblast-like cells when induced to differentiate along a neurogenic lineage by retinoic acid treatment (Jones-Villeneuve et al. 1982; McBurney et al. 1988; Chen et al. 1999). We conclude that *PAX7* transfection directs and maintains P19 cells in a neurogenic lineage. These results were confirmed in more than three separate experiments.

Up-regulation of *ephrin-A2* in *PAX7* transfected cells

Having observed co-localization of *Pax7* and *ephrin-A2* expression in vivo we further investigated the ability of Pax7 to regulate *ephrin-A2* expression in the in vitro model system. To do so we assessed the expression of *ephrin-A2* in the *PAX7* transfected neurogenically differentiated P19 cells. The entire population of *PAX7*-transfected cells exhibit intense *ephrinA-2* up-regulation on the surface membranes of the cell body as well as on the neurites (Fig. 5a). Quantification confirmed a significant

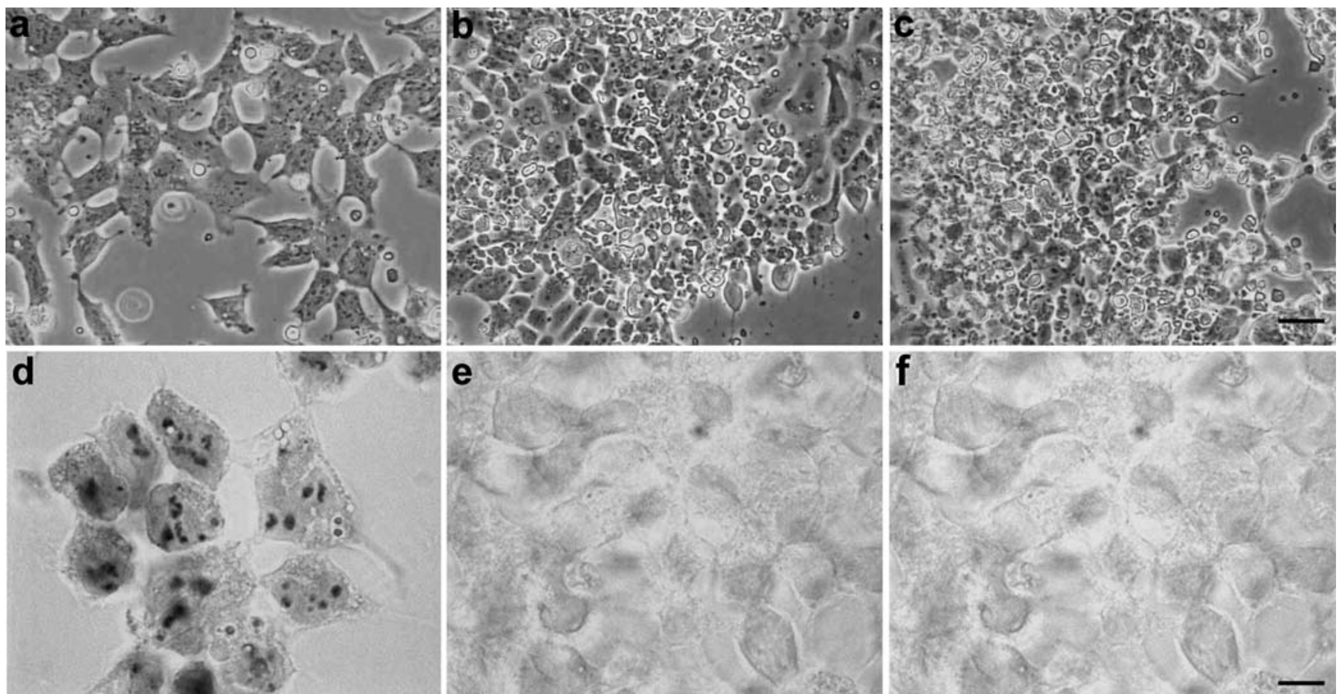

Fig. 3a–f Cell morphology and confirmation of *Pax7* expression in transfected cells. Phase contrast microscopy of cells transfected with *PAX7-pHM6* **(a)**, with vector alone **(b)** or untreated **(c)**. *PAX7* transfected cells form monolayers and differentiate along a neurogenic lineage as evident by the presence of neurite outgrowth. P19 cells transfected with vector alone or untreated continue to grow as aggregates. *Scale bar* 100 μm **(a–c)**. Pax7 immunoreactivity in the nucleus of transfected cells **(d)** but absent from those treated with vector alone **(e)** or from untreated cells **(f)**. *Scale bar* 20 μm **(d–f)**

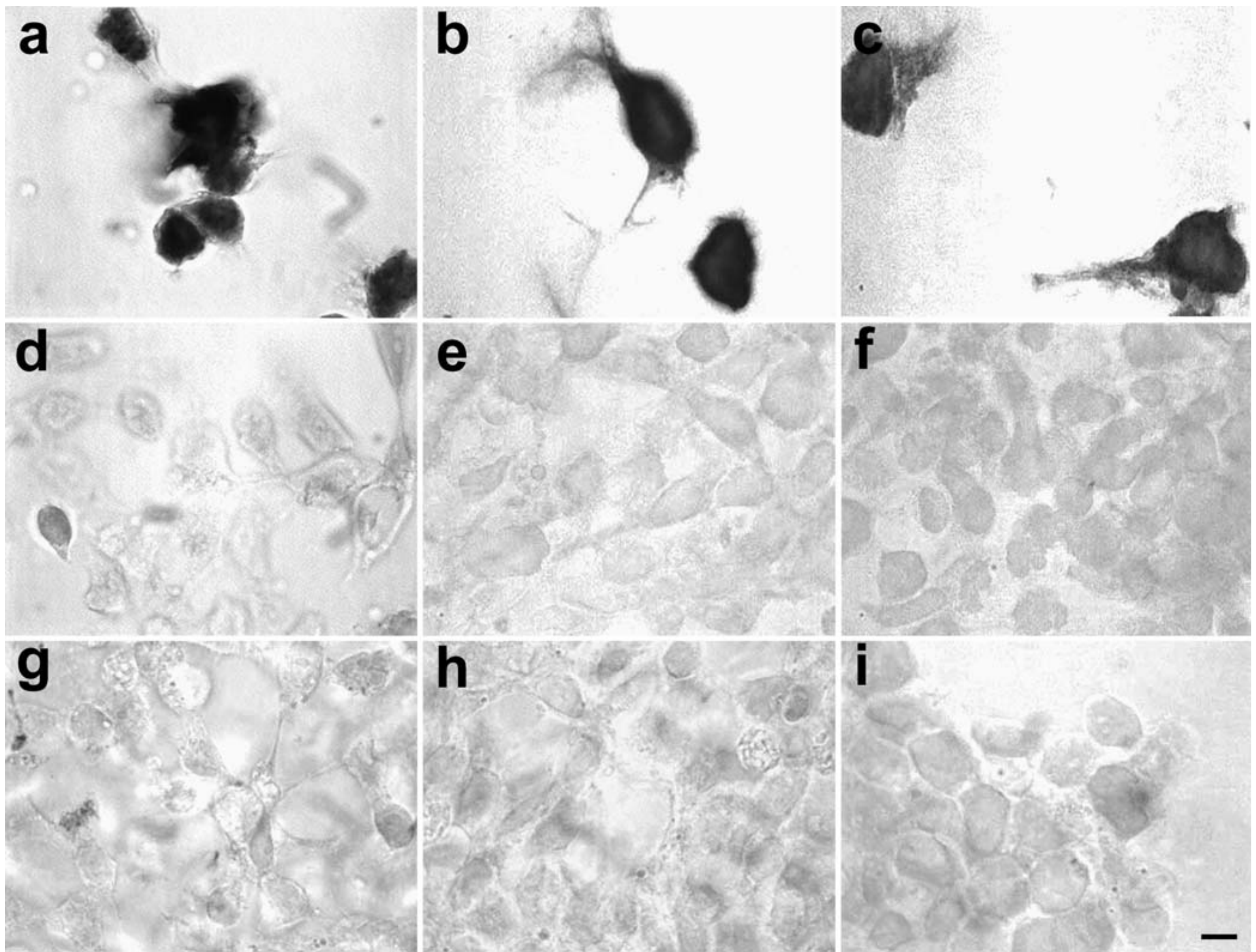

Fig. 4a–i Neural differentiation of *PAX7* transfected cells. Neurogenic differentiation demonstrated by neurofilament **(a)**, β-tubulin **(b)** and NCAM **(c)** immunoreactivity of the somal cytoskeleton in *PAX7* transfected but not in vector transfected **(d–f)** or untransfected cells **(g–i)**. This confirms that *PAX7* transfection induces the undifferentiated P19 cells to differentiate along a neurogenic lineage. *Scale bar* 20 μm **(a–i)**

increase in *ephrin-A2* expression in *PAX7* transfected cells (1.92±0.8; $p<0.0001$, Fig. 5g) as compared to cells transfected with vector alone (0.22±0.87, Fig. 5b, g). As before, results were confirmed in more than three separate clones.

Antisense studies

Antisense oligonucleotides were used to inhibit Pax7 protein expression in transfected cells. Cells treated with antisense oligonucleotides exhibit reduced *ephrin-A2* (Fig. 5c) and *Pax7* (Fig. 5d) expression whereas those treated with a control sense-oligonucleotide remain positive for ephrinA-2 (Fig. 5e) and Pax7 (Fig. 5f). Quantification of immunoreactivity indicates a significant decrease in *ephrinA-2* expression in antisense-treated cells when compared to untreated transfected ones ($p<0.001$, Fig. 5g) or those treated with sense oligonucleotides ($p<0.001$, Fig. 5g), indicating a link between *Pax7* and *ephrinA-2* expression. Experiments were repeated on three separate clones.

Table 1 Proportion of *PAX7* transfected cells differentiated along a neurogenic lineage. By counting the number of neurofilament-H immuno-positive cells present in stably transfected cell cultures, the percentage of cells that differentiated along a neurogenic lineage was calculated

Cell type	Total number of cells counted	% of neurofilament positive cells
P19 cells transfected with *PAX7b*	250	100%

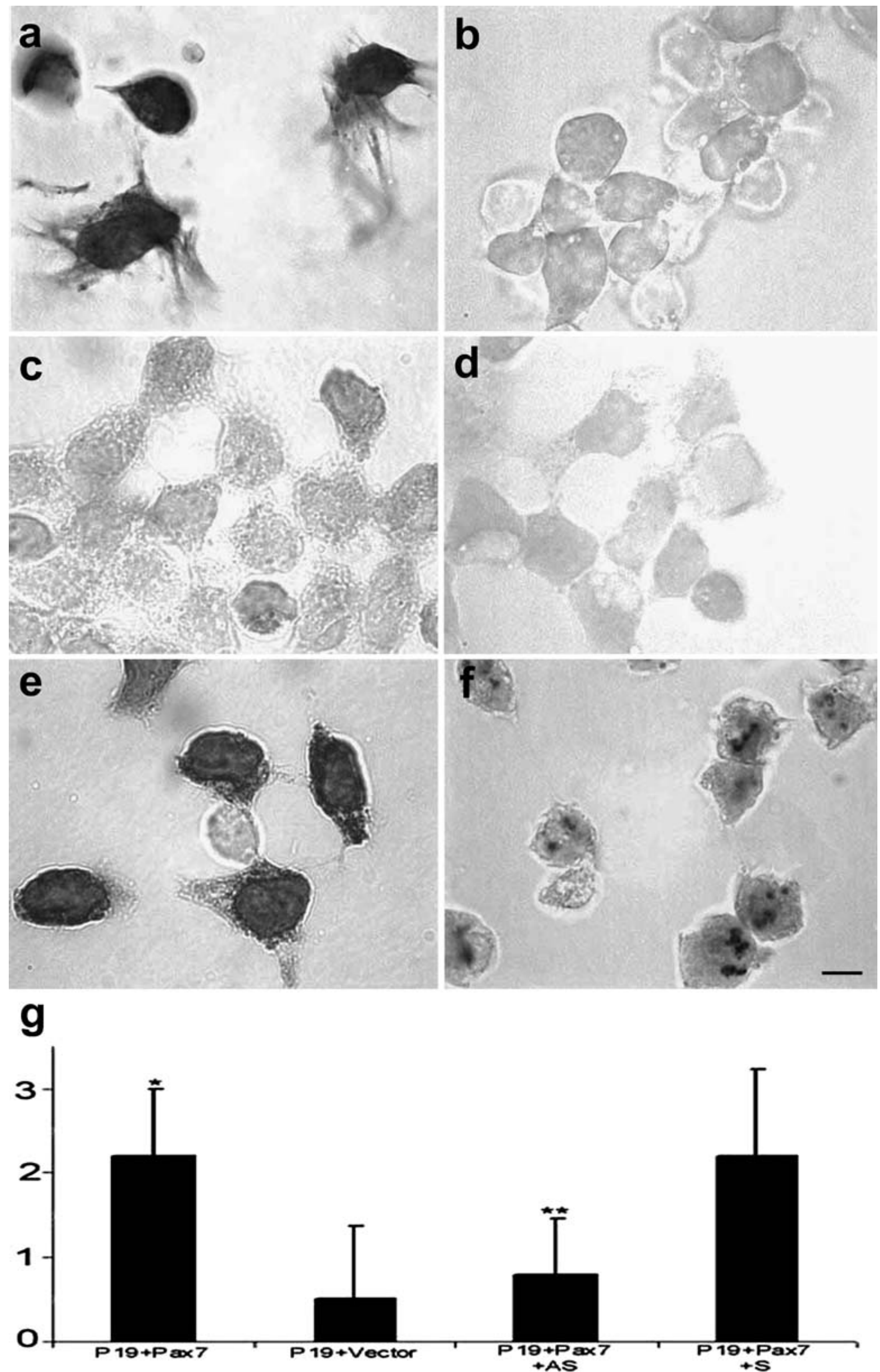

Fig. 5a–g *Ephrin-A2* expression in vitro. Ephrin-A2 immunoreactivity in cells transfected with *PAX7* **(a)** but not in those transfected with vector alone **(b)**. Loss of protein levels of ephrin-A2 **(c)** and Pax7 **(d)** in *PAX7* transfected cells treated with a *Pax7* antisense oligonucleotide. Ephrin-A2 **(e)** and Pax7 **(f)** protein levels remained unchanged in *PAX7* transfected cells treated with a sense oligonucleotide. Histogram showing intensity of ephrin-A2 immunoreactivity in cells transfected with *PAX7* or with vector alone, and after oligonucelotide treatment **(g)**. Results are an average of measurements from three separate clones. *Error bars* represent standard error. *Significant difference between *PAX7* and vector transfected groups (p<0.001); **significant difference between antisense treated and sense treated or non-treated *PAX7* transfected groups (p<0.001). *Scale bar* 20 μm **(a–f)**

Neural differentiation and up-regulation of *ephrin-A2*

In order to confirm that *ephrin-A2* up-regulation is a separate event from entry into the neurogenic lineage we assessed *ephrin-A2* expression in P19 cells transfected with *Pax6*. It has previously been demonstrated that P19 cells transfected with *Pax6* enter the neurogenic lineage (Ziman at el. 2003). RT-PCR using primers specific for *ephrin-A2* and *neurofilament-M* demonstrated that in P19 cells induced to differentiate along a neurogenic lineage by transfection with *Pax6* (Fig. 6b, lane 2) no *ephrin-A2* expression is observed (Fig. 6c, lane 3). Cells transfected

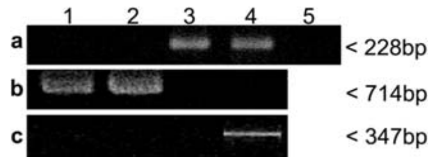

Fig. 6a–c RT-PCR for *Pax7*, *neurofilament-M* and *ephrin-A2* in the in vitro P19 cell model. **a** RT-PCR demonstrating *Pax7* expression (expected 228 bp product size) in transfected cells (two separate clones, *lanes 3, 4*); results were negative for untreated (*lane 1*) or vector transfected cells (*lane 2*), or those transfected with *Pax6* (*lane 5*). **b** RT-PCR demonstrating *neurofilament-M* expression (expected size 714 bp) in *PAX7* and *Pax6* transfected cells (*lanes 1, 2,* respectively) but not vector transfected or untreated cells (*lanes 3, 4,* respectively). **c** RT-PCR demonstrating *ephrin-A2* expression (expected size 347 bp) in cells transfected with *PAX7* (*lane 4*) but not with *Pax6* (*lane 3*) or with vector alone (*lane 2*) or in untreated cells (*lane 1*)

with vector alone do not enter the neurogenic lineage (Fig. 6b, lane 3) nor do they express *ephrin-A2* (Fig. 6c, lane 2). Therefore we conclude that the up-regulation of *ephrin-A2* is an event separate from neurogenesis.

RT-PCR results

Confirmation of immunohistochemical results was obtained by RT-PCR using *Pax7, neurofilament-M* or *ephrin-A2* gene specific primers, which give rise to products of the expected sizes: *Pax7*, 228 bp; *neurofilament-M*, 714 bp; *ephrin-A2*, 347 bp. *Pax7* expression is observed in transfected cells (Fig. 6a, lanes 3, 4, two separate clones) but not in untreated cells (Fig. 6a, lane 1), vector transfected cells (Fig. 6a, lane 2), or those transfected with *Pax6* (Fig. 6a, lane 5). *Neurofilament-M* expression occurs in *PAX7* and *Pax6* transfected cells (Fig. 6b, lanes 1, 2, respectively) but not in vector transfected or untreated cells (Fig. 6b, lanes 3, 4, respectively). Expression of e*phrin-A2* as detected by RT-PCR is apparent in cells transfected with *PAX7* (Fig. 6c, lane 4) but not in cells transfected with *Pax6* (Fig. 6c, lane 3) or with vector alone (Fig. 6c, lane 2) nor in untreated cells (Fig. 6c, lane 1).

Discussion

Pax7 expression profiles during tectal development

A large body of experimental work links *Pax7* expression with tectal regionalisation and differentiation. The changing expression patterns of *Pax7* from region-specific to cell-specific suggests that during tectal development, *Pax7* has a biphasic role; early on in the differentiation and polarisation of the tectum and at later stages in the specification of specific subsets of cells involved in retino-tectal map formation and refinement. The results presented here are the first to quantify *Pax7* expression throughout tectal development. In the rostro-caudal axis we observed a weak rostro-caudal gradient at E2, uniform expression at E4, the establishment of a steep rostro-caudal gradient at E6, a continued rostro-caudal trend for *Pax7* expression at E8, and well established gradients at stages E10 and E12. At all stages examined *Pax7* was expressed in a diminishing dorso-ventral gradient.

The rostro-caudal graded *Pax7* expression at E2 occurs concurrently with the establishment of rostro-caudal graded expression of *En*, the earliest known marker for tectal polarity (Logan et al. 1996). The importance of graded expression of transcription factors at E2 and the establishment of tectal polarity has previously been demonstrated. When the mesencephalic vesicle is reversed on the anteroposterior axis at E2, the En gradient re-adjusts to its original polarity and both the graded cytoarchitecture and pattern of retino-tectal projections developed normally (Martinez and Alvarado-Mallart 1990; Ichijo et al. 1990; Matsuno et al. 1991; Nakamura et al. 1994). However, when the mesencephalic vesicle is returned to its original orientation at E3, the En gradient fails to adjust and subsequently the cytoarchitecture and retino-tectal projections are inverted (Matsuno et al. 1991; Nakamura et al. 1994). The clear *Pax7* gradient observed here at E2 is in keeping with a possible role for *Pax7*, in conjunction with other genes, in the initial establishment of tectal polarity.

We also report a significant Pax7 gradient at E6. The elevated Pax7 levels observed at this stage may be related to an increase in cell proliferation that occurs at E6 (Cowan et al. 1968). The overall trend in cell proliferation and tectal development occurs such that the rostral tectal regions are more advanced than the caudal ones. The chronological gradient of cell proliferation parallels the subsequent invasion of the tectum by retinal axons (DeLong and Coulombre 1965). Thus cell proliferation, tectal cytoarchitectural development and tectal polarity appear to be intrinsically related.

The biphasic role for Pax7 in specifying tectal cells involved in retino-tectal map making is further supported by the continual trend for *Pax7* expression to occur in a rostral^{low}-caudal^{high} gradient of *sgfs* cells in later stages of tectal development (E8–E12). At E8 a crude retino-tectal map is first formed and *Pax7* expression in the *sgfs* cell layer also occurs in a rostro-caudal trend. By stages E10 and E12 when retinal axons have spread throughout the tectum and map refinement is taking place (DeLong and Coulombre 1965), *Pax7* gradients are clearly established, suggestive that Pax7 is involved in retino-tectal map formation and refinement.

Establishment and implications of the *Pax7* rostro-caudal gradient

The quantification of a graded *Pax7* expression pattern (this paper), together with earlier *Pax7* expression studies (Stoykova and Gruss 1994; Kawakami et al. 1997; Nomura et al. 1998; Marin et al. 2001; Matsunaga et al. 2001) implicate *Pax7* in the establishment of tectal polarity. Further support for this concept comes from transplant experiments and ectopic expression studies

showing *Pax7* expression in the diencephalon induces an ectopic tectum with distinct laminae that receive retinal projections (Nomura et al. 1998; Araki and Nakamura 1999; Matsunaga et al. 2001).

We do not know the upstream controllers in the tectum of the observed rostro-caudal *Pax7* gradient. In general, secreted factors are considered to determine graded expression of region-specific genes (reviewed in Nakamura 2001). The genes that define rostro-caudal tectal boundaries may play a role in generating graded *Pax7* expression along this axis. These include: at the rostral boundary, *Pax6* and *Grg4*; caudally from the isthmus, *Wnt1*, *Fgf8*, *En* and *Pax2*; and at the mid-hindbrain boundary, *Otx2* and *Gbx2* (reviewed in Nakamura 2001).

The above studies suggest that *Pax7* operates in conjunction with other tectal specific genes to regulate rostro-caudal tectal polarity. Previous studies implicating *En* in tectal differentiation, tectal polarity and the regulation of the graded rostro-caudal expression of *ephrin-A2* (Friedman and O'Leary 1996; Logan et al. 1996) suggest a close functional relationship between *Pax7*, *En* and *ephrin-A2*. However, the relationships between *En* and *Pax7* and *En* and *ephrin-A2* are likely to be indirect, since *Pax7* and *ephrin-A2* are predominantly restricted to cells of the *sgfs* (Cheng et al. 1995; Kawakami et al. 1997; Marin et al. 2001), whereas *En* expression is concentrated in cells surrounding the isthmus (Araki and Nakamura 1999; Shamim et al. 1999; Nakamura 2001). Moreover, in experiments where the isthmus is ablated, a transient tectum forms that expresses *Pax7* but not *En* (Nomura et al. 1998). Indicative of a further level of complexity, it appears that *Pax7* may reinforce its own expression via a positive feedback loop that includes sequential up-regulation of *En* and *ephrin-A2* (Araki and Nakamura 1999; Matsunaga et al. 2001). Thus *Pax7*, *En* and *ephrin-A2* may be required for the establishment and maintenance of the tectum and its polarity across the rostro-caudal axis.

Establishment and implications of the *Pax7* dorso-ventral gradient

We report here that *Pax7* is continually expressed in a strong dorsal^{high}-ventral^{low} gradient. It is likely that this gradient of *Pax7* expression is established by the secreted morphogenic factors such as sonic hedgehog (Shh) and bone morphogenic protein-4 (BMP-4), molecules concerned with overall dorso-ventral brain patterning (Ericson et al. 1995; Zhang et al. 2000; Sasagawa et al. 2002). Specifically, it is thought that *Pax7* expression is repressed ventrally by the action of Shh (Watanabe and Nakamura 2000; Nakamura 2001) and activated dorsally by BMP-4; concentration dependent relationships between *Pax7*, Shh and BMP-4 have been established experimentally (Goulding et al. 1993; Lee and Jessell 1999; Timmer et al. 2002).

Dorsal-ventral gradients in the neural tube are known to be important in the specification of CNS neuronal subtypes (Ericson et al. 1996; Briscoe et al. 2000; Timmer et al. 2002). The graded restricted expression profile of *Pax7* within the superficial (dorsal) layers of the tectum suggests that *Pax7* is important for specification of tectal laminae and subsets of neurons in a manner similar to its role in neural tube patterning (Tanabe and Jessell 1996; Mansouri and Gruss 1998).

Pax7 regulates *ephrin-A2* and retino-tectal map formation and refinement

Our suggestion that Pax7 is involved in retino-tectal map formation follows from a study (Ziman et al. 2003) implicating Pax6 in determining graded retinal expression of the topography inducing molecule *Eph-B2* (Braisted et al. 1998; Mann et al. 2002). Graded *Pax6* expression is also implicated in establishing circuitry in the developing cortex (Stoykova and Gruss 1994; Bishop et al. 2000). Similarly, our results demonstrating the co-expression of *ephrin-A2* in all *Pax7* expressing cells within the *sgfs* laminae of an E12 tectum strongly support our suggestion that Pax7 up-regulates *ephrin-A2* in a cell autonomous manner. This assertion was supported in vitro, by transfecting *PAX7* into undifferentiated P19 cells; the transfected cells differentiated along a neurogenic lineage and expressed *ephrin-A2*. Furthermore, using a previously characterised cell line in which undifferentiated P19 cells were induced to differentiate along a neurogenic lineage by the transfection of *Pax6* (Ziman et al. 2003), we have demonstrated that in these cells *ephrin-A2* expression was not up-regulated. Thus we confirm that up-regulation of *ephrin-A2* in *PAX7* transfected cells was a direct result of *Pax7* expression rather than a result of neural differentiation per se. The ability of Pax7 to directly regulate *ephrin-A2* is indicated by our finding that a consensus Paired Domain binding sequence for Pax proteins (Czerny et al. 1993; Chalepakis and Gruss 1995; Holst et al. 1997; Meech et al. 1999) exists in the promoter region of *ephrin-A2* at position –217 (accession number CAAA01139072).

The rostro-caudal and dorso-ventral graded expression profile of *ephrin-A2*, apparent from E3 and more strongly from E4 onwards to E15, was clearly demonstrated by Cheng et al. (1995). The similarity in the spatial and chronological expression profiles of *Pax7*, either quantified (this paper) or revealed qualitatively (Jostes et al. 1990; Stoykova and Gruss 1994; Kawakami et al. 1997), and *ephrin-A2* (Cheng and Flanagan 1994; Cheng et al. 1995; Marin et al. 2001), further supports our suggestion that *Pax7* is involved in the establishment of retino-tectal maps and map refinement through the up-regulation of *ephrin-A2*. The proposal could be investigated further by comparing expression profiles of *Pax7* and *ephrin-A2* in ectopic tecta induced by *Pax7* mis-expression or in *Pax7* null mice.

In summary, we infer that Pax7 plays a role in the early stages of development in the establishment of tectal polarity and at later stages contributes to the regulation of *ephrin-A2*, implicating the transcription factor in retino-tectal map formation and refinement. Further studies are required to examine the precise roles of transcription

factors such as Pax6 and Pax7 in establishing specific patterns of neural wiring throughout the developing brain. The information is likely to be important in designing strategies, including stem cell therapies, aimed at repairing specific brain circuits.

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