Photoperiodic regulation of cellular retinoic acid-binding protein 1, GPR50 and nestin in tanycytes of the third ventricle ependymal layer of the Siberian hamster

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Abstract

Tanycytes in the ependymal layer of the third ventricle act both as a barrier and a communication gateway between the cerebrospinal fluid, brain and portal blood supply to the pituitary gland. However, the range, importance and mechanisms involved in the function of tanycytes remain to be explored. In this study, we have utilized a photoperiodic animal to examine the expression of three unrelated gene sequences in relation to photoperiod-induced changes in seasonal physiology and behaviour. We demonstrate that cellular retinoic acid-binding protein 1 (CRBP1), a retinoic acid transport protein, GPR50, an orphan G-protein-coupled receptor and nestin, an intermediate filament protein, are down-regulated in short-day photoperiods. The distribution of the three sequences is very similar, with expression located in cells with tanycyte morphology in the region of the ependymal layer where tanycytes are located. Furthermore, CRBP1 expression in the ependymal layer is shown to be independent of a circadian clock and altered testosterone levels associated with testicular regression in short photoperiod. Pinealectomy of Siberian hamsters demonstrates CRBP1 expression is likely to be dependent on melatonin output from the pineal gland. This provides evidence that tanycytes are seasonally responsive cells and are likely to be an important part of the mechanism to facilitate seasonal physiology and behaviour in the Siberian hamster.

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Introduction

The cells of the ependymal layer of the third ventricular wall are regarded as an interface between the cerebrospinal fluid (CSF), the neuropil and the portal capillaries serving the pituitary gland. The apparent simplicity of this layer hides a potential functional complexity with significance for neuroendocrine interactions (reviewed in Rodriguez *et al.* 2005). Cells of the ependymal layer are not homogeneous and can be classified into ependymocytes, cuboidal cells with microvilli extending into the third ventricle and tanycytes. The tanycytes are concentrated at the base of the third ventricle, extending approximately one-third dorsally along the wall of the ventricle. Within this distribution there is regionalization of subtypes, with α tanycytes being regarded as the interface between the CSF and the neuropil, and β tanycytes as the interface between the CSF and the portal system (reviewed in Rodriguez *et al.* 2005). Although a precise role for any group of tanycytes is unclear, there is some evidence of transport between the CSF and the neuropil or the portal system involving caveola and clathrin mediated endocytosis and transcytosis (Tuma & Hubbard 2003, Peruzzo *et al.* 2004, Rodriguez *et al.* 2005), the most striking example of which is the transportation within the ependymal layer of insulin-like growth factor (I) (IGF-I) (Garcia-Segura *et al.* 1991, Fernandez-Galaz *et al.* 1997). Tanycytes are also a component of the hypothalamus–pituitary–thyroid hormone axis, being the only source in the brain of type II deiodinase responsible for the conversion of inactive thyroxine (T4) to active tri iodothyronine (T3) (Diano *et al.* 1998).

Tanycytes of the ependymal layer are characterized by long basal process, terminating in structures termed end-feet that make contact with portal blood vessels and neurons (reviewed in Rodriguez *et al.* 2005). In the rat and the mouse, some of these cells may possess cilia which extend into the ventricular space, while other cells are non-ciliated and may possess microvilli (Devarajan *et al.* 2005, Xu *et al.* 2005). Although the functional significance of the tanycytes in the ependymal

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layer is not at all well understood, it has been proposed that they act as a key element in the bidirectional movement of bioactive molecules between the brain and the CSF (Bruni *et al.* 1972, Vigh & Vigh-Teichmann 1998). Furthermore, recent evidence from a study in the rat suggests that they may be an important source of neural stem cells which give rise to differentiated neuronal cells that migrate to appropriate locations in the hypothalamus (Xu *et al.* 2005).

The Siberian hamster undergoes major physiological and behavioural changes to enable survival in the harsh climate of winter. This includes a large reduction in adipose stores, cessation of reproductive activity and a change in pelage colour (Badura & Goldman 1992, Gorman & Zucker 1995). The striking reduction in immunoreactivity of the intermediate filament protein, vimentin, on transition from long to short photoperiod exposure, accompanied by morphological changes in tanycyte end-feet surrounding gonadotropin-releasing hormone (GnRH) axon terminals terminating on the portal capillary supply (Kameda *et al.* 2003), is indicative of a role for the ependymal layer in seasonal responsiveness.

It has also been reported that the expression of mRNA for type II deiodinase is photoperiodically regulated in the ependymal layer of the third ventricle of the Djungarian hamster (Watanabe *et al.* 2004). These data have led us to the hypothesis that the tanycytes of the third ventricular ependymal wall are important in governing seasonal neuroendocrine responses.

In the search for photoperiodically regulated genes, which might provide the molecular basis for the underlying neuronal control of seasonal physiology and behaviour of the Siberian hamster, we have identified a number of genes that are up-or down-regulated by photoperiod in the dorsal medial posterior arcuate nucleus (dmpARC) (Barrett *et al.* 2005, Ross *et al.* 2004, 2005). These include components of the retinoic acid signalling pathway, RXRγ, RAR, CRABPII and CRBPI (Ross *et al.* 2004, 2005). However, CRBPI was also noted to be highly expressed in the ependymal layer with a potential to be photoperiodically regulated (Ross *et al.* 2004). In a capacity as a retinol transport protein, photoperiodic regulation of CRBPI would implicate the ependymal layer as a seasonal regulatory barrier to retinoic acid-dependent functions with possible consequences wider than dmpARC functions. Therefore, it was important to determine *bona fide* photoperiodic regulation and provide a clearer indication of which cells in the ependymal layer express CRBPI.

GPR50 is an orphan G-protein-coupled receptor with approximately 45% homology with melatonin receptors, but does not bind melatonin (Reppert *et al.* 1996, Drew *et al.* 1998). This is despite the presence of a histidine residue in transmembrane domain 5 of the receptor that has been demonstrated to be a key amino acid for the binding of melatonin in the MT1 receptor (Conway *et al.* 1997). We and others have previously noted high-level expression of GPR50 in the ependymal layer of rodent species (Drew *et al.* 1998, Vassilatis *et al.* 2003). GPR50 could therefore have a strong potential to influence signalling pathways in ependymal cells. In the context of photoperiodic regulation of ependymal cell function, this receptor is a candidate as part of a mechanism of sensing components of metabolic status in the CSF. In the absence of a ligand or an antibody for GPR50 to demonstrate *in vivo* expression of a receptor protein and only a description of mRNA expression by *in situ* hybridization, we took advantage of a GPR50 null mouse in which GPR50 coding sequence has been interrupted by a β-galactosidase (lacZ) coding sequence. This transgenic mouse translates the interrupted GPR50 mRNA sequence to produce a GPR50-lacZ fusion protein, which can be detected by the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal staining) and thereby implicate the presence of GPR50 protein.

Recent findings of neuronal stem cells in the third ventricle ependymal layer (Xu *et al.* 2005) prompted an investigation of the possibility for a seasonal regulation of stem cell progenitor cells in the Siberian hamster third ventricle ependymal cell layer. Nestin is a common marker for neuronal progenitor cells and was used to assess this possibility.

Materials and Methods

Animals

Male Siberian hamsters (*Phodopus sungorus*) were obtained from a breeding stock held at the Rowett Research Institute. All research using animals was licensed under the Animals (Scientific Procedures) Act of 1986 and received ethical approval from the Rowett Research Institute ethical review committee.

Hamsters were individually housed at a constant temperature of 20 °C with access to food and water available *ad libitum*. Hamsters held in long-day (LD) photoperiods were exposed to a 16 h light:8 h darkness cycle. Hamsters in short-day (SD) photoperiods were exposed to an 8 h light:16 h darkness cycle.

In a simple LD–SD comparison, hamsters were held in their respective photoperiods for 14 weeks before being culled at 3 h after lights on. Pinealectomy or sham operations were performed on Siberian hamsters held in LD under a general anaesthetic (ketamine (0·4 mg/kg), xylamine (2 mg/kg, i.p.)) as described (Schuhler *et al.* 2002) and subsequently transferred to SD. The testosterone replacement experiment has been described elsewhere (Ross *et al.* 2004), but briefly, after 13 weeks in either LD or SD, interscapular silastic capsules containing either crystalline testosterone or cholesterol were implanted and left in place for 5 days before hamsters were called. Testosterone was assayed using the DSL-4000 ACTIVE testosterone RIA kit (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). The experiment using pinealectomized (PNX) hamsters or steroid replacement in hamsters has been described previously (Ross *et al.* 2004).

All animals were killed by cervical dislocation. Brains were rapidly removed and frozen on dry ice and stored at −80 °C until required. Brains were sectioned on a cryostat at 15–20 μm for *in situ* hybridization analysis by film autoradiography.

Gene-specific probes

The amplification and cloning of a fragment of mouse GPR50 (previously referred to as the melatonin-related receptor) between transmembrane domains 3 and 7 have been described previously (Drew *et al.* 2001). Similarly, the details of cellular retinoic acid-binding protein 1 (*CRBP1*) gene fragment have also been described (Ross *et al.* 2004). A fragment of the nestin gene was amplified from mouse brain cDNA with the following primers: forward primer 5′-GCGGGGCGGTGC-GTGACT; reverse primer 5′-AAGGGGGAAGAGAAG-GATGTTGG (nucleotides 653 through to 1175 based on mouse nestin gene sequence -Genbank sequence number BC060693. The fragment was amplified with Taq DNA polymerase (Promega) with cycling conditions of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s for 35 cycles. The resultant amplified DNA fragment was cloned into pGEM-Teasy (Promega). The sequence and orientation of the PCR fragment in the vector was verified using Beckman–Coulter sequence chemistry on the CEQ 8000 DNA sequencer.

Generation of GPR50-lacZ mice

GPR50 knockout mice were generated by Deltagen Inc. (San Carlos, CA, USA; www.deltagen.com) and provided to Andrew Loudor under agreement, from mice purchased by Astra Zeneca (Alderly Edge, Cheshire, UK). A fragment (from bases 45 to 411) within the coding sequence of the *GPR50* gene was targeted with a construct containing the *Escherichia coli lacZ* gene encoding β-galactosidase and the neomycin-resistance sequence (Fig. 1). Genomic DNA isolated from the embryonic stem cell (ES) lines was digested with the restriction enzymes determined to cut outside of the construct arms. The DNA was analyzed by Southern hybridization, probing with a radiolabelled DNA fragment that hybridizes outside of and adjacent to the construct arm. Following ES cell selection, recombinant animals were generated, and F1 animals crossed to generate knockout genotypes. Animals were bred against a C57/B6 background for six generations. Animals were housed under a 12 h light:12 h darkness cycle at 22 °C and food water available *ad libitum*. Animals were perfused and the brain was removed, fixed in 2% paraformaldehyde for 6 h at 4 °C and cryoprotected in 30% sucrose overnight at 4 °C.

Cryoprotected sections (cut at 15 μm) were incubated at 32 °C overnight in X-gal solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, Triton 10% and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside in 0·1 M PBS) and protected from light throughout.

In situ *hybridization*

Plasmids were linearized with an appropriate restriction enzyme. Antisense or sense transcripts were synthesized with SP6, T3 or T7, as appropriate. For radioactive *in situ* hybridization, RNA transcripts were made in the presence of ^{35}S UTP (Perkin-Elmer, Buckinghamshire, UK).

In situ hybridization was carried out as described previously (Morgan *et al.* 1996). In brief, frozen brain sections mounted on glass slides were fixed in 4% paraformaldehyde in 0·1 M PBS, acetylated in 0·25% acetic anhydride in 0·1 M triethanolamine (pH 8). Radioactive probes (approximately 10^6 c.p.m.) were applied to the slides in 70 μl hybridization buffer containing 0·3 M NaCl, 10 mM Tris–HCl (pH 8), 1 mM EDTA, 0·05% tRNA, 10 mM dithiothreitol, 0·02% Ficoll, 0·02% polyvinylpyrrolidone, 0·02% BSA and 10% dextran sulphate. Hybridization was carried out overnight at 55 °C. Post-hybridization, slides were washed in 4×SSC (1×SSC is 0·15 M NaCl, 15 mM sodium citrate), then treated with ribonuclease A (20 μg/μl) at 37 °C and finally washed in 0·5×SSC at 55 °C. Slides were dried and apposed to Biomax MR film for 5–7 days.

Image analysis

Slides containing all the brain sections for a complete experiment were apposed to a single sheet of autoradiographic

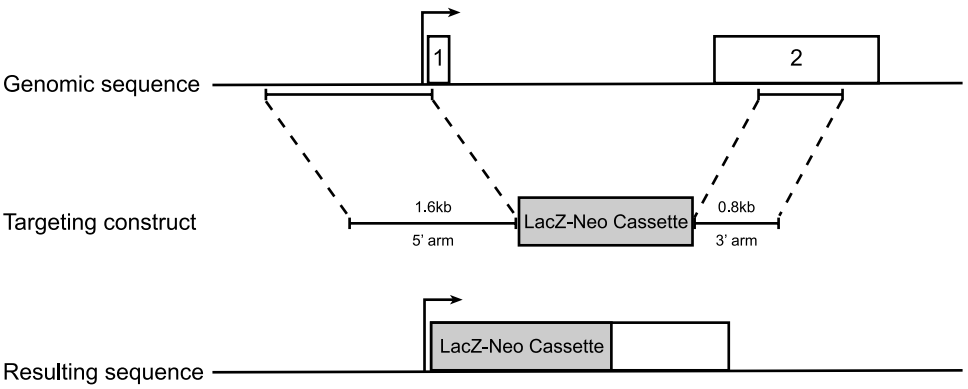

Figure 1 Targeted disruption of GPR50 by homologous recombination. The LacZ–Neo was inserted between bases 45 and 411 of the coding sequence covering the end of exon 1 and the beginning of exon 2. The arrow represents the transcription start site and the open boxes exon regions of GPR50 coding sequence.

film. Autoradiographic films were scanned at 600 dpi on a Umax scanner linked to a PC running Image-Pro PLUS version 4.1.0.0 analysis software (Media Cybernetics, Workingham, Berkshire, UK). For each probe, four sections spanning a selected region of the hypothalamus (approximately Bregma −2·54 to −1·82 mm) were chosen for image analysis. The substantial difference in gene expression levels in the two photoperiods did not allow the blinding of the image analysis operator to the treatment groups.

Integrated optical, density reading for each selected region was obtained by reference to a standard curve generated from the ^{14}C microscale. The integrated optical densities for each section of each animal were summated and an average (with S.E.M.) obtained for a specific treatment. The values of one treatment in an experiment were set to 100% expression value and other treatment values calculated accordingly.

Statistical analysis

The statistical tests applied in this study were *t*-tests, one-way or two-way ANOVA both with *post hoc* Tukey tests for multiple comparisons. Significance was taken as a $P<0·05$.

Results

Retinoic acid-binding protein CRBP1

In a series of experiments performed to examine the expression of components of the retinoic acid signalling pathway in relation to photoperiod in the dmpARC, we observed a difference for CRBP1 in the ependymal layer between hamsters held in LD and those held in SD for 14 weeks (Ross *et al.* 2004). Emulsion-coated slides reveal silver grains over the tanycytes and cells within the region of the median eminence (Fig. 2). Therefore, we performed a quantification of the *in situ* hybridization signal in the ependymal layer/median eminence in tissue sections of the experiments used for analysis of expression in the dmpARC (Ross *et al.* 2004). This analysis shows a >80% reduction in the expression of CRBP1 mRNA (LD 100±4·5% vs SD 16·1±1·8%, $P<0·001$, Fig. 3A), with most of the residual expression found in the median eminence. Expression of CRBP1 is also observed in the ependymal layer of the dorsal third ventricle (D3V) (Ross *et al.* 2004); however, expression is not influenced by photoperiod in these ependymal cells (100±7·2% vs SD 95±3·7% $P=0·46$, Fig. 3A). The expression of CRBP1 was quantified at 3-h intervals in the ependymal layer/median eminence of hamsters held in LD or SD for 14 weeks. As for expression in the dmpARC, there was a photoperiodic difference between LD and SD ($P<0·001$), no circadian regulation of CRBP1 in either LD or SD ($P=0·34$) and no interaction between photoperiod and time ($P=0·26$, Fig. 3B). Quantification of the expression of CRBP1 mRNA in the ependymal layer/median eminence in hamsters held in SD compared with hamsters that were PNX before exposure to SD, demonstrated a difference of approximately 80% (sham 23·2±6·2% vs PNX 100±7·7%, $P<0·001$), similar to that found in a LD–SD comparison (Fig. 3C).

Photoperiod-driven testosterone levels in the Siberian hamster can elicit significant changes in the regulation of follicle stimulating hormone (FSH), androgren receptors, steroid receptor co-activator-1 and neuropeptides (Simpson *et al.* 1982, Yellon 1994, Bittman *et al.* 1996, Tetel *et al.* 2004); therefore, we assessed whether reduced steroid background due to testicular regression in SD may have contributed to the SD-induced decline in CRBP1 expression by replacing testosterone in SD testes-regressed male hamsters.

Male hamsters were transferred to SD for 13 weeks, then implanted with silastic capsules releasing testosterone (T) or cholesterol (control) for 5 days. In the presence of the testosterone implant, testosterone levels were comparable with LD levels (plasma testosterone level 0·09±0·03 ng/ml control; 2·11±0·11 ng/ml T; 1·08±0·55 ng/ml LD animals; Ross *et al.* 2004). However, no effect of testosterone

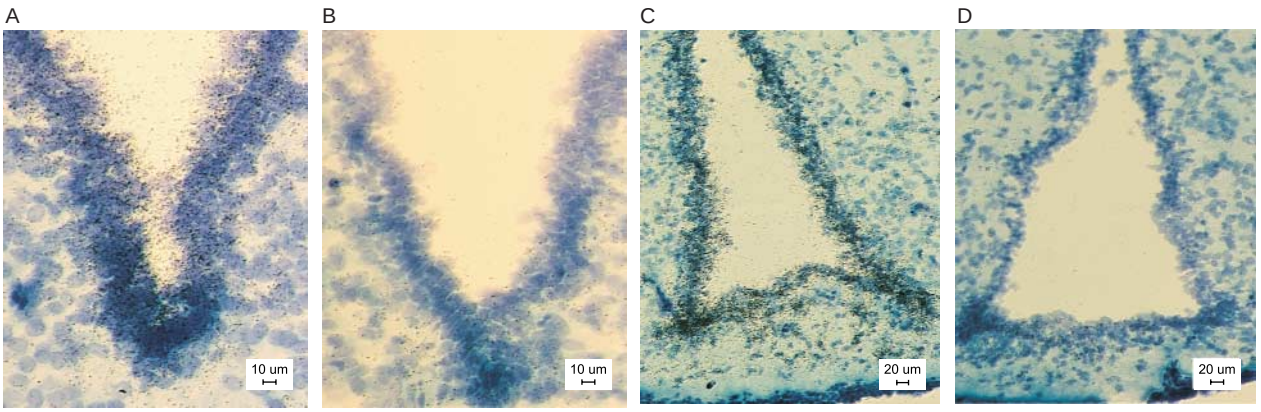

Figure 2 Emulsion-coated slides of *in situ* hybridized sections of Siberian hamster brain probed with a CRBP1 antisense riboprobe. (A) High power magnification of the caudal hypothalamic region, LD; (B) high power magnification of the caudal hypothalamic region, SD; (C) medial hypothalamic region, LD; (D) medial hypothalamic region, SD.

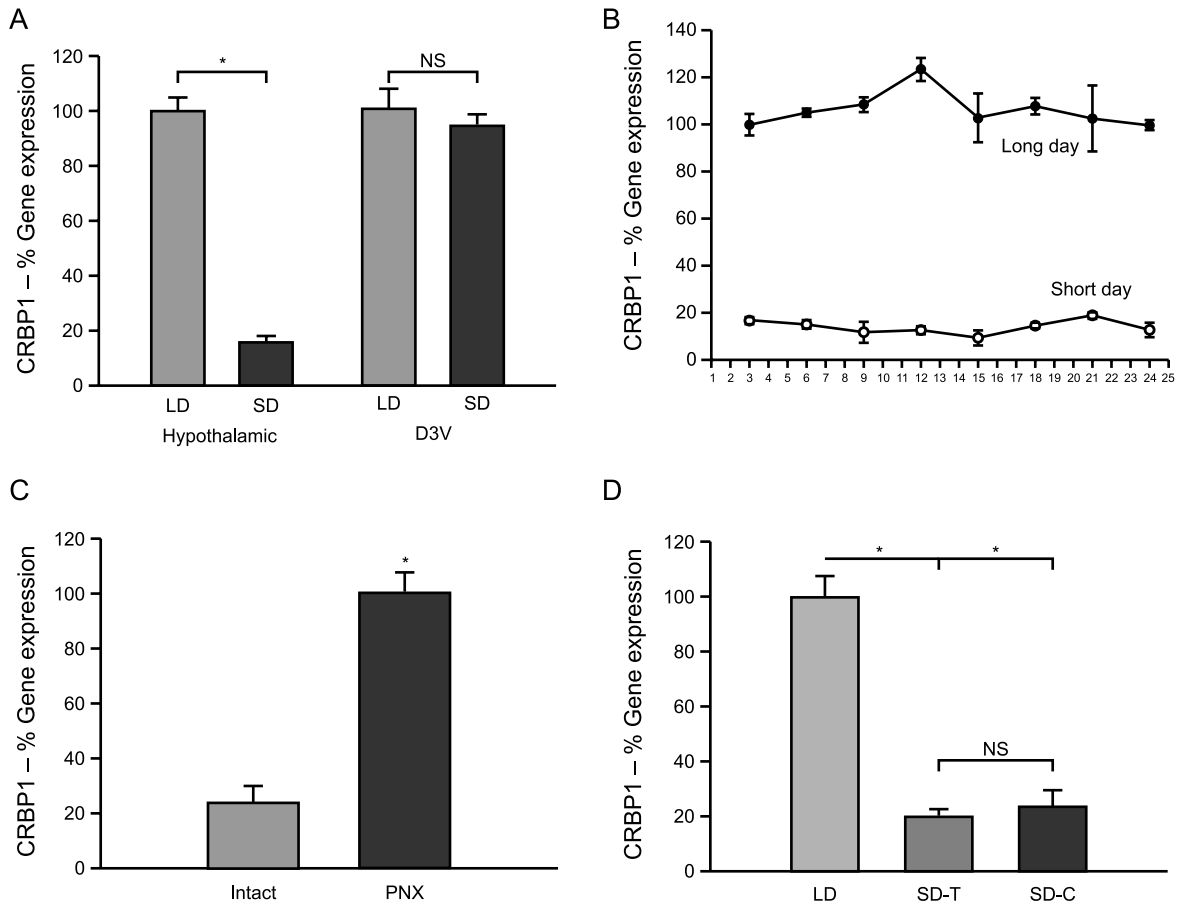

Figure 3 Image analysis of autoradiographs for CRBP1 expression in the ependymal layer. (A) Siberian hamsters were held in LD or SD for 14 weeks ($n=10$ per group); CRBP1 was quantified in the region of the ependymal layer of the third ventricle (hypothalamic) or the ependymal layer of the dorsal third ventricle (D3V). Statistical comparison was by means of a *t*-test. (B) Ependymal layer of Siberian hamsters in LD or SD for 14 weeks, culled every 3 h over a 24-h period ($n=4$ per time point, except SD 3 and 6 h time points when $n=3$). Statistical comparison was by means of a two-way ANOVA. (C) Ependymal layer of Siberian hamsters that were pinealectomized (PNX) or remained sham-operated (intact; $n=4$ per group). Statistical comparison was by means of a *t*-test. (D) Ependymal layer of Siberian hamsters held in LD or SD with testosterone (SD-T) or cholesterol (vehicle) releasing silastic capsules (SD-C; $n=8$ per group). Statistical comparison was by means of a one-way ANOVA. Means are shown as percent expression of either LD (A, B and D) or PNX animals (C) set at 100%±S.E.M. *$P<0{\cdot}001$; NS, not significant.

replacement (SD-T) was found over cholesterol-implant (SD-C) control animals (LD 100±8·1% vs SD-T 20·5±2·1% vs SD-C 24·2±5·9%, Fig. 3D).

Orphan receptor GPR50

In situ hybridization of brain sections of the Siberian hamster sectioned from the posterior hypothalamus (approximately Bregma −2·70 mm relative to mouse brain atlas, Franklin & Paxinos 1997) through to the medial preoptic nucleus (approximately Bregma −0·1 mm) revealed a restricted expression pattern of GPR50 in the ependymal layer. Expression of GPR50 was observed to extend from the earliest caudal hypothalamic sections through to the beginning of the retrochiasmatic nucleus (approximating to Bregma −0·94 mm) (Fig. 4A–C; retrochiasmatic area, data not shown). Beyond this region, no expression of GPR50 was observed in either LD or SD photoperiod. GPR50 expression was also noted in the cells of the median eminence (Fig. 4D and E).

GPR50 expression in the ependymal wall varied depending upon the location in the hypothalamus. mRNA for GPR50 was highly expressed in the entire lateral walls of the ventricle in caudal ARC sections of the hypothalamus (Figs 4C and 5C), but restricted to approximately the ventral third of the ependymal layer in rostral ARC sections.

Comparison of hamsters held in LD and SD reveal a marked decrease in the expression of GPR50 in SD (Fig. 4A and B). Dividing the ependymal layer into the medial (vertical ependymal wall of the third ventricle) and the ventral (floor of the third ventricle area), image analysis reveals the largest decline in expression in the medial portion (LD 100±3·0% vs

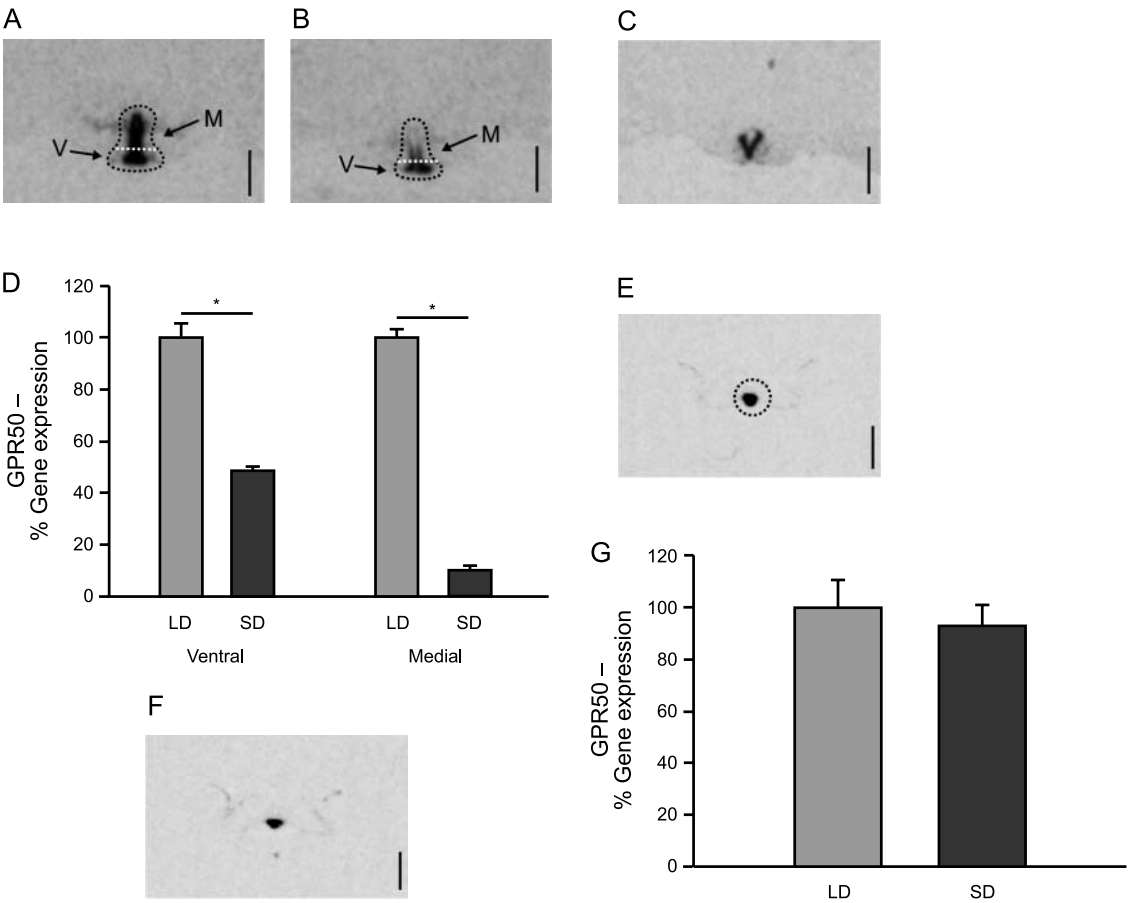

Figure 4 *In situ* hybridization of a riboprobe for GPR50 to sections of a hamster brain. (A) Medial hypothalamic region, LD; (B) medial hypothalamic region, SD; (C) caudal hypothalamic region, LD; (D) image analysis and quantification of the autoradiographic signal in the medial and ventral/median eminence region of the ependymal layer. The dashed black line highlights the area of quantification, with 'M' and 'V' (separated by white dashed line), indicating the medial and the ventral regions of the ependymal layer quantified by image analysis. Quantification was done on four sections spanning Bregma −2·54 to −1·82 mm that were consistently represented on all slides; (E) Rostral hypothalamic region showing subfornical organ, LD; (F) as (E) in SD; (G) expression in the subfornical organ quantified by image analysis highlighted by the dashed line in (E). Means are shown as percent expression of LD animals set at 100%±S.E.M. $n=6$ per group. Statistical comparison was by means of a *t*-test. *$P<0{\cdot}001$. Scale bar=1 mm.

SD 10·5±1·4%, $P<0{\cdot}001$), whereas the decline in the ventral portion (including the median eminence) was less (LD 100±5·5% vs 48·6±1·3%, $P<0{\cdot}001$, Fig. 4D).

In the anterior hypothalamic sections of the hypothalamus, GPR50 expression was observed in the subfornical organ. However, in contrast to the ependymal layer, no photoperiodic regulation of GPR50 was evident in this structure (LD 100±10·7% vs SD 92·8±8·1%, $P=0{\cdot}6$, Fig. 4E–G).

Emulsion-coated sections demonstrated the presence of silver grains over the cells constituting the ependymal layer (Fig. 5). Individually labelled cells in the median eminence were also observed and showed reduced expression in SD photoperiod (Fig. 5D and E). The emulsion-coated sections also reveal silver grains extending out into the ventricular space (Fig. 5D) and may suggest that mRNA for GPR50 is transported along cilia that may be found on tanycytes (Xu *et al.* 2005), before or during translation to protein. Morphologically, cells expressing GPR50 in the ventral third of the ependymal wall have an elongated appearance and can be distinguished from cells in the dorsal region of the ependymal wall where GPR50 is not expressed, which appear cuboidal, suggesting that these cells are of a different type (Fig. 6).

No verified antibody to GPR50 is available, therefore the presence of GPR50 protein in the ependymal layer of the third ventricle has been demonstrated with a transgenic knockout mouse for GPR50, where a part of the coding sequence of GPR50 has been replaced by a *lacZ* gene to generate a GPR50-lacZ fusion protein, allowing protein production to be visualized by X-gal staining. Figure 7 shows rostro-caudal brain sections of the transgenic mouse in the region of the third ventricle stained with X-gal. As can be observed, staining is obtained in cell

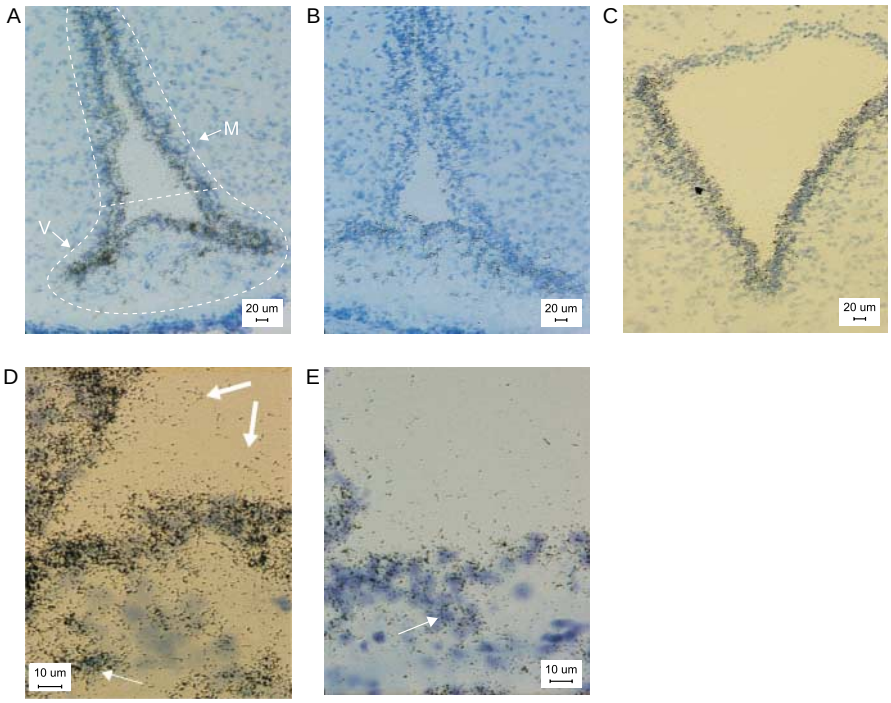

Figure 5 Emulsion-coated sections of an *in situ* hybridized ^{35}S-labelled antisense riboprobe for GPR50 to sections of hamster brain. (A) Medial hypothalamic region, LD. The dashed line highlights the approximate area quantified in Fig. 2 with 'M' and 'V' indicating the medial and the ventral areas quantified separately; (B) medial hypothalamic region, SD; a high density of silver grains is seen over cells of the ependymal layer in LD photoperiod animals whilst it is drastically reduced in SD photoperiod; (C) caudal hypothalamic region, LD; (D) higher magnification in the region of the floor of the third ventricle in LD; (E) higher magnification in the region of the floor of the third ventricle in SD. The expression in cells located in the region of median eminence is indicated by a thin arrow. The thick arrows indicate the silver grains observable in the third ventricular space.

bodies of the ependymal layer with a similar distribution to the mRNA found in the hamster.

Intermediate filament nestin

In situ hybridization with an antisense riboprobe for nestin mRNA reveals a similar distribution to GPR50 and CRBP1 in the ependymal layer. Nestin mRNA is present in the most caudal sections of the third ventricle (Fig. 8A) but is absent in sections beyond the retrochiasmatic nucleus (data not shown). However, in contrast to GPR50 and CRBP1, limited expression of nestin is observed in the region dorsal to the tanycyte layer (Fig. 8B and C). Analysis of mRNA expression in different photoperiods shows a large reduction in the signal for nestin mRNA hybridization in SD (LD 100±7·7% vs SD 20·4±6·1%, $P<0{\cdot}001$, Fig. 8B–D) in the tanycyte cell layer, with a residual expression in the upper region of the ependyma. Emulsion-coated slides reveal silver grains over cells in the ependymal layer and scattered cells in the neuropil of the arcuate nucleus. A limited number of cells in the median eminence are also coated with silver grains (Fig. 9).

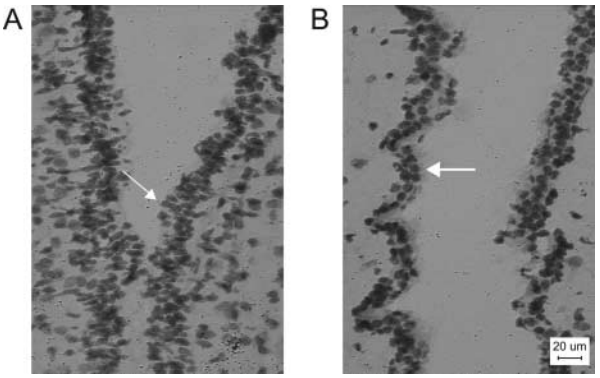

Figure 6 Toluidine blue stained hamster brain sections in the region of the ependymal layer of the third ventricle. (A) Ependymal cells in the region where GPR50 expression is observed. The cells are elongated in this region with a diffuse architecture to ependymal wall (thin arrow); (B) ependymal cells in the region where GPR50 is not expressed. The cells are cuboidal in shape with an architecture that is more defined (thick arrow).

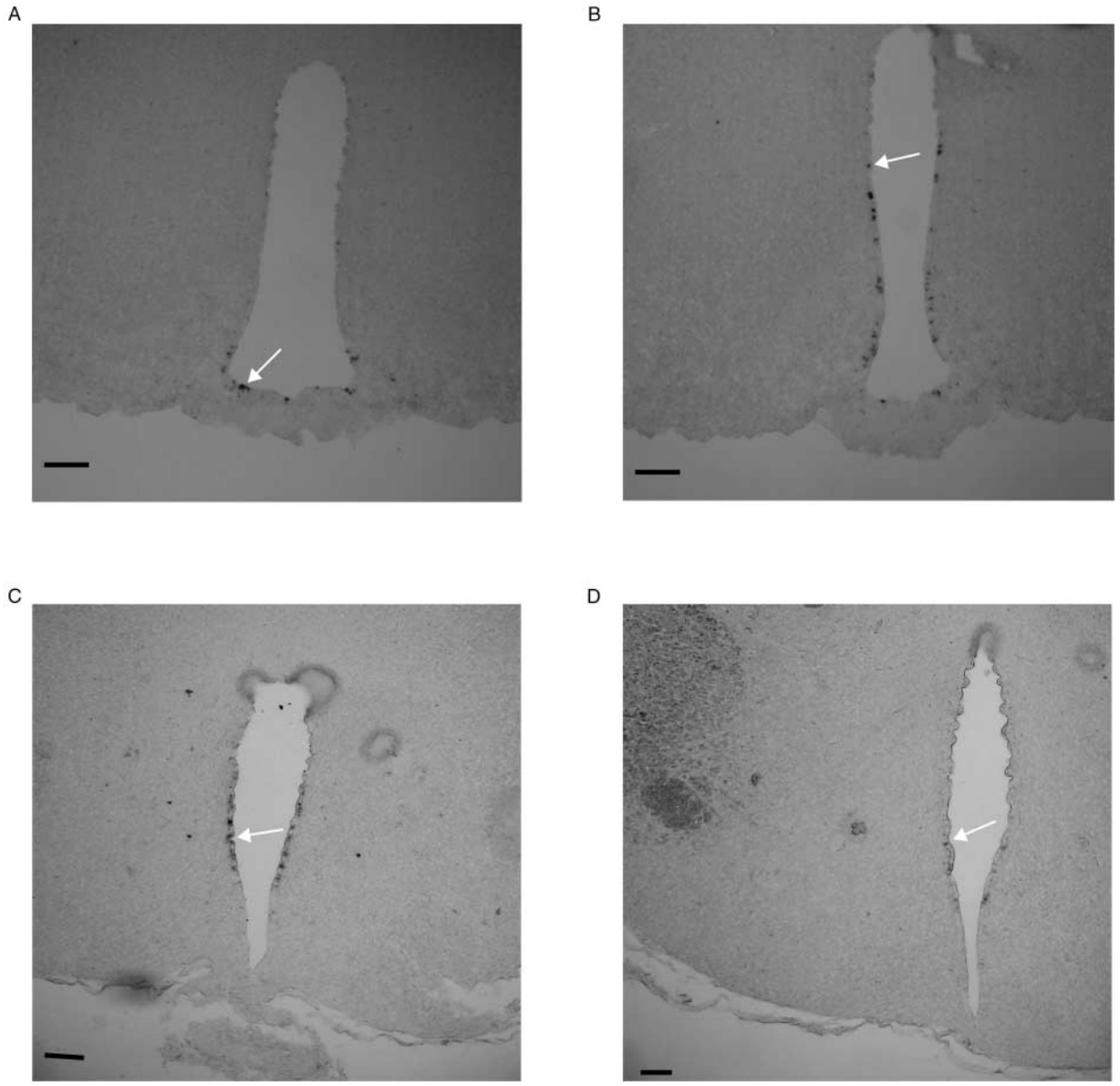

Figure 7 *GPR50* gene sequence was transgenically modified to express a *lacZ* gene and interrupt the expression of the GPR50 protein sequence. Subsequent staining with X-gal for LacZ protein expression reveals by proxy the location of GPR50 protein (example of labelling indicated by an arrow in each panel). As observed for in the *in situ* hybridization experiments, expression of GPR50 is observable in the ependymal layer of the third ventricle. The sections represent the rostro-caudal pattern of expression of GPR50 (A–D). Bar scale = 100 μM.

Discussion

In this study, we have investigated the photoperiodic regulation of three genes expressed in the ependymal layer of the third ventricle in the Siberian hamster, providing evidence that the ependymal layer is likely to be an important conduit to facilitate a seasonal photoperiod transition in behavioural and physiological states found in summer and winter photoperiods.

The three genes examined in this study encode diverse proteins, a retinoic acid transport protein, an orphan G-protein-coupled receptor and an intermediate filament protein and show a similar distribution in the ependymal layer. All three were found to be expressed along the length of the lateral walls in the most caudal sections of the third ventricle. However, the dorsal wall of the ventricle in these caudal sections shows only limited expression of CRBP1, GPR50 and nestin (Figs 2A, 5C and 9A). In more rostral

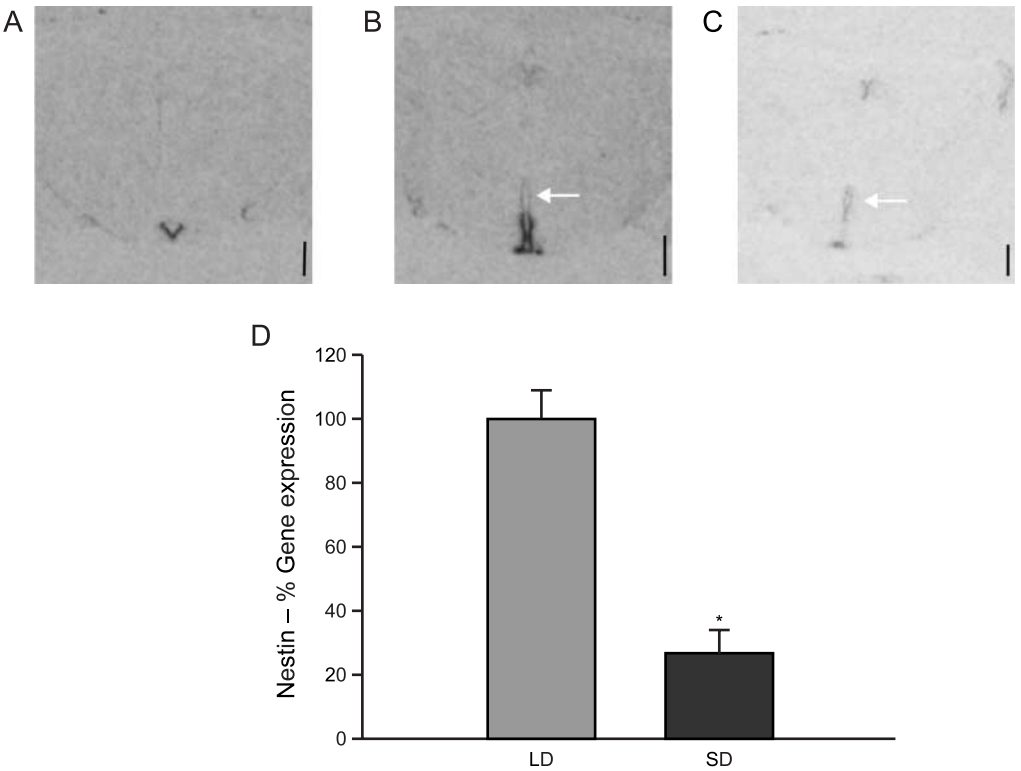

Figure 8 *In situ* hybridization of a riboprobe for nestin to sections of a hamster brain. (A) Caudal hypothalamic region, LD; (B) medial hypothalamic region, LD; (C) medial hypothalamic region SD; (D) image analysis and quantification of the ependymal layer autoradiographic signal in LD or SD photoperiods. Quantification was done on four sections spanning Bregma −2·54 to −1·82 mm that were consistently represented on all slides. Means are shown as percent expression of LD animals set at 100%±S.E.M. $n=6$ per group, statistical comparison was by means of a *t*-test, $^*P<0{\cdot}001$. Scale bar=1 mm. A faint signal for nestin gene expression can be observed in the region dorsal to the tanycyte cell layer but not in the region regulated by photoperiod (indicated by an arrow in (B) and (C)).

sections, both CRBPI (Fig. 2C) and GPR50 (Figs 4A and 5A) are found in the ventral region of the ventricular wall, the extent of which varies along the length of the hypothalamic region of the third ventricle. This is where studies in hamsters, rats and mice indicate that specialized ependymal cells called tanycytes are located (Kameda *et al.* 2003, Rodriguez *et al.* 2005). In the Siberian hamster these cells in the ventral third of the ependyma can be distinguished morphologically by toluidine blue staining (Fig. 6). However, nestin also appears to be weakly expressed in the dorsal aspect of the third ventricle (Fig. 8B and C). The cells in the upper two-thirds (ependymyocytes) with weak expression of nestin appear as a single layer of cells with a cuboidal morphology that contrasts with the cells on the ventral lateral walls which appear less organized and the layer more diffuse (Fig. 6).

Using the mouse brain atlas as a guide (Franklin & Paxinos 1997), the expression of these transcripts closely follows the anatomical distribution of hypothalamic arcuate nucleus and median eminence, implicating a close association of the cells expressing CRBP1, GPR50, and nestin with the surrounding neuronal structures and pituitary portal system.

CRBP1

In experiments designed to quantify CRBP1 expression in the region of the dmpARC (Ross *et al.* 2004), we noted that the expression of CRBP1 in the ependymal layer may be photoperiodically regulated. Quantification of CRBP1 in

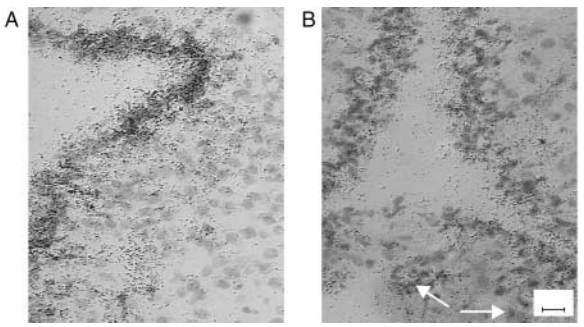

Figure 9 Emulsion-coated slides of *in situ* hybridized sections of hamster brain sections probed with a nestin riboprobe. (A) Caudal hypothalamic region, LD; (B) medial hypothalamic region, (LD). Arrows indicate cells in the median eminence labelled by a ^{35}S-labelled nestin antisense riboprobe. Scale bar =10 μM.

the ependymal layer of brain sections in these experiments (Ross *et al.* 2004), now clearly demonstrates a regulation of CRBP1 in the ependymal layer. Recent studies have identified an important role for retinoic acid in the central nervous system including identification of a number of retinoic acid-regulated genes important to neuronal function and synaptic transmission (reviewed by Lane & Bailey 2005), and regulation of GnRH transcription and peptide release (Meredith *et al.* 1998, Cho *et al.* 2001). Retinoic acid is also implicated in dopamingeric neuron activity through regulation of tyrosine hydroxylase and dopamine β-hydroxylase, with consequences on reward, emotion and locomotor activities (Lane & Bailey 2005). Some or all of these systems are potential targets for regulation in seasonal mammals.

The juxtaposition of the ependymal layer to the dmpARC and our earlier studies demonstrating expression of members of the retinoic acid signalling pathway in the dmpARC (Ross *et al.* 2004) suggest that ependymal CRBP1 may function as a transport protein facilitating delivery of retinoic acid to the dmpARC. This would be consistent with the concept of the ependymal layer acting as a gateway for bioactive molecules from the CSF or median eminence. Evidence includes tract-tracing studies linking tanycytes involved in T3 synthesis to arcuate nucleus neurons connected to thyrotrophin releasing hormone (TRH) synthesising cells of the paraventricular nucleus and the ability of tanycytes to transport dopamine, IGF-I and tracers such as wheat germ agglutinin (Scott *et al.* 1974, Fernandez-Galaz *et al.* 1997, Diano *et al.* 1998, Peruzzo *et al.* 2004). Therefore, the regulatory mechanism involving retinoic acid in the dmpARC may need to be reviewed in the light of the dramatic regulation of CRBP1 in the ependymal layer. These data suggest that a part of the mechanism involved in retinoic-dependent responses in the dmpARC could depend upon ependymal signalling.

GPR50

Analysis of the rostro-caudal expression of GPR50 within the ependymal layer by *in situ* hybridization revealed that GPR50 expression is limited to the ventricular wall caudal to the retrochiasmatic nucleus, extending to the most caudal sections cut at the posterior hypothalamic region. This restricted distribution suggests that the ligand for GPR50 could differentially affect tanycytes in a manner that is important for the cells adjacent to the ependyma in this region.

In SD, there was a substantial reduction of GPR50 mRNA expression indicating that this receptor is important for LD physiology of the cells of the ependymal layer. Densitometric analysis of the *in situ* hybridization signal in each photoperiod shows a smaller loss of GPR50 expression in the cells on the floor of the third ventricle and median eminence. Emulsion-coated slides indicate that the loss occurs in all cells that constitute the measured region and is not confined to the cells of the ependymal wall. The photoperiodic regulation of GPR50 is not a general phenomenon in these animals as the other major site of GPR50 expression, the subfornical organ, showed no change with photoperiod.

Using a transgenic knockout mouse for GPR50 designed to generate a GPR50-lacZ fusion protein in place of a functional GPR50 receptor, we were able to demonstrate by X-gal staining that the transcript for GPR50 is translated and therefore likely to yield a protein product *in vivo* (Fig. 7). Therefore, one can conclude that the ependymal cells of the third ventricle lining the extent of the arcuate nucleus are able to sense the ligand for GPR50, suggesting that the ligand for orphan GPR50 should be present in CSF of the third ventricle.

X-gal staining observed in the transgenic mouse (Fig. 7) demonstrates a similar distribution of GPR50 in the ependymal wall when compared with the hamster, albeit with a more sparse distribution than *in situ* hybridization data from hamster, mouse and rat (Drew *et al.* 2001, Vassilatis *et al.* 2003). This may be due to one of several reasons, including sensitivity of the staining, incorrect folding of the GPR50-lacZ fusion protein or post transcriptional regulation.

In the absence of a ligand, the function of GPR50 is uncertain. It has been reported that polymorphisms in GPR50 are associated with altered triglyceride and high density lipoprotein (HDL) cholesterol levels (Bhattacharyya *et al.* 2006), and a deletion mutation is associated with bipolar affective disorder and depression (Thomson *et al.* 2005). The former finding is interesting in the context of seasonal energy metabolism, but we cannot yet state the significance of SD down-regulation of this receptor.

It has recently been demonstrated that GPR50 can heterodimerize with melatonin MT1 and MT2 receptors, suppressing high-affinity binding to MT1 but not to the MT2 receptor (Levoye *et al.* 2006). However, melatonin receptors are not known to be expressed in ependymal cells (Schuster *et al.* 2000, Song & Bartness 2001) and therefore cannot function in the capacity of a regulator of melatonin receptor function in these cells.

Nestin

Nestin is an intermediate filament protein characteristic of dividing and migratory neuroepithelial cells during embryogenesis and is found in cells occupying subventricular zones (ependymal layer) in the adult brain (Wiese *et al.* 2004). Nestin-positive cells in the adult rat third ventricular ependymal layer have been demonstrated to differentiate to cells with neuronal phenotypes (orexin positive) and migrate to appropriate areas in the hypothalamus (Xu *et al.* 2005). These and other findings have led to the view that nestin expression is a putative marker for neuronal stem cells in the adult mammalian brain (Kronenberg *et al.* 2003, Wiese *et al.* 2004, Scheffler *et al.* 2005). However, it remains to be determined whether such regenerative processes play a role regulating the seasonal phenotype of the Siberian hamster.

A more likely role for the seasonal variation in nestin mRNA expression may be related to a function of the cells (tanycytes) in which it is expressed in LD. Intermediate

filament proteins are mobile and dynamically regulated proteins are important in cytoskeletal architecture (Helfand *et al.* 2004). Nestin is unable to form intermediate filament networks independently due to an N-terminal domain that is shorter than required to participate in filament formation (Herrmann & Aebi 2000). A study using glial fibrial acid protein and a vimentin double knockout mouse, and a study using chemical cross-linking to examine intermediate filament interactions, strongly suggest that intermediate filament proteins, vimentin and nestin co-polymerize both *in vitro* and *in vivo* (Eliasson *et al.* 1999, Steinert *et al.* 1999). Since vimentin is known to be strongly regulated by photoperiod in the Siberian hamster (Kameda *et al.* 2003), the SD down-regulation of vimentin and nestin intermediate filament proteins may be linked to the retraction of tanycyte end-feet associated with GnRH neurons and consequently to the SD-induced cessation of GnRH release (Kameda *et al.* 2003).

In summary, the cells in the ependymal layer of the third ventricle in the Siberian hamster demonstrate marked photoperiodic regulation of gene expression. Our data, together with evidence in the literature of photoperiodic regulation of type II deiodinase in the ependymal layer of Djungarian hamsters (Watanabe *et al.* 2004), type II and type III deiodinase regulation in the ependymal layer of quail brain (Yasuo *et al.* 2005) and an involvement of the choroid plexus in circannual control of a hibernation-specific protein complex in the brain of chipmunks (Kondo *et al.* 2006), would suggest that pathways regulated in the ependymal layer and the blood brain barrier could play a significant role in seasonal physiological and behavioural responses.

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