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Chapter 2

Per-like immunoreactive neurons within the brain of *Xenopus* innervate both the retina and pineal organ

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Summary

Antibodies directed against *Drosophila per*-protein (peptide-S) were used in immunoblot and immunocytochemical studies to look for possible *per* homologues in the central nervous system of *Xenopus laevis*. Immunoblots of eye and brain were made at two opposite times of day (3 h before lights on and 1 h before lights off). In brain extracts antibodies bound several proteins (40–50 kD; 180 kD; 114 kD). In eye extracts a doublet of 21 kD was identified. None of these immunoreactive bands showed a consistent day:night difference. In immunocytochemical studies, neurons were labelled within the telencephalic septum, hypothalamus and thalamic-subhabenular areas of the central nervous system. Immunoreactive fibres enter the pineal gland and terminate close to the photoreceptor cells. Fibres were also identified within the optic nerve, running through the inner plexiform layer and terminating in the inner nuclear layer of the retina. While the functional relationship of vertebrate *per*-like antigens to *Drosophila per*-protein remains unclear, our results show that anti-*per* antibodies recognize cells and fibres associated with brain areas which receive inputs from the retina and pineal, and brain areas that project to retinal and pineal neurons.

Introduction

In a classic study 100 years ago, Ramón y Cajal (1893) described fibres entering the frog retina (efferent or centrifugal fibres). However, the existence of retinopetal projections to the anurans eye remained controversial. More recent immunocytochemical studies, modern tract-tracing methods and physiological experiments have confirmed Cajal's original findings and demonstrated that these projections appear to be Famide and substance P immunoreactive (see Repérant *et al.*, 1989). Central projections to the amphibian pineal (pinealopetal or centrifugal fibres), have also been identified and

appears to be AChE-positive (Wake *et al.*; 1974, Korf 1976). In this study we describe centrifugal fibres to the retina and pineal of *Xenopus* using antibodies raised against *Drosophila per*-protein (peptide-S).

Polyclonal antibodies against a small domain of the *per* coding sequence (peptide-S) were used by Siwicki *et al.* (1988) to localize *per* protein expression in adult wild-type flies (*Drosophila melanogaster*). Several sites within the central nervous system (CNS) express *per*, including neurons in the lateral protocerebrum, the nuclei of photoreceptors, and neuroglia throughout the CNS. Significantly, levels of *per* protein and its mRNA have been shown to fluctuate in a circadian manner (Hardin *et al.*, 1990; Zerr *et al.*, 1990). These and other data (see Hall, 1990) support the view that *per* protein may play a key role in the organization of circadian rhythmicity in *Drosophila*. In the vertebrates, anti-*per* antibodies have labelled various cells within the CNS. In the rat several areas of the brain, including the suprachiasmatic nuclei (SCN), were immunolabelled (Siwicki *et al.*, 1992). In our unpublished results, we have used the same anti-*per* antibodies to examine immunostaining within the CNS of the hamster (same pattern as rat), the fish *Gambusia affinis*, the lizard *Anolis carolinensis*, and in this report we describe our immunocytochemical and Western blots results in the amphibian *Xenopus laevis*.

Materials and methods

Adults *Xenopus laevis* were obtained from suppliers, and entrained to a 12:12 L:D cycle (light on at 05:00, off at 17:00) for a period of 7–10 days prior to use in experiments. *Xenopus* were provided with a small amount of liver. Two different batches of affinity-purified antibodies were used in this study: 6-89 was used in immunocytochemical experiments, while S80-R was used in Western blots. They were purified as previously described (Siwicki *et al.*, 1988) from antisera obtained at two different times from the same rabbit, which had been immunized and boosted regularly with a 14-amino acid peptide (peptide-S) from the sequence of the *Drosophila per* protein.

Immunocytochemistry

Xenopus were anaesthetized with ether and transcardially perfused with heparin saline and then with Bouin's fixative. Animals were killed between 30 and 120 min after lights-off. Brains and eyes were dissected and post-fixed for 12 h in the same fixative and then transferred to 0.1 mol/l phosphate buffered saline (PBS) at pH 7.4 overnight. Tissue was dehydrated through graded alcohols, embedded in paraffin and serial 10 μm sections cut. Antibody 6–89 was used at a dilutions between 1/20 and 1/100, for 72 h at 4 °C. Localizations of the antibody:antigen complex was performed using avidin-biotin-peroxidase reagents from Vectastain Elite kit (Vector Labs). Sections were incubated for 10 min in 0.025 per cent diaminobenzidine (DAB) containing 0.03 per cent (w/v) peroxide, washed in distilled water and finally dehydrated and mounted in Permount (Fisher Scientific). Two types of immunocytochemical controls were performed. Tissue sections were processed for immunocytochemistry but replacing the primary antiserum with normal non-immune rabbit serum. Sections were also incubated with 'pre-absorbed' primary antibody. To pre-absorb the antibody, 0.5 mg of peptide-S was added to 1 ml of diluted antibody (1:50) and incubated at 4 °C overnight before adding to tissues sections.

Immunoblots

Xenopus were anaesthetized with ether, decapitated and the brains rapidly removed, wrapped in aluminum foil and frozen in liquid nitrogen, and stored at –70 °C until preparation for Western blotting. Tissue was collected at 02:15 (n = 3) and 16:30 (n = 3). Tissue samples were homogenized in ice-cold Laemmli sample buffer, fractionated on either 7.5 per cent or 10 per cent SDS polyacrylamide gels, and electroblotted onto nitrocellulose. Blots were incubated with affinity-purified anti-*per* antibody (S80-R), followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Vector Labs), as previously described (Siwicki *et al.*, 1992).

Results and discussion

In *Xenopus* brain and retina, a consistent pattern of immunostaining was produced in all animals examined. This immunoreaction appeared specific as preincubation of antibody with peptide-S (antigen), and substitution of the primary antibody with normal rabbit serum, abolished all immunolabelling. Strongly immunoreactive bipolar and multipolar perikarya (25 μm × 10 μm) were identified in the telencephalic septum, hypothalamus and in the thalamic-subhabenular areas. Those cells within the ventral telencephalic septum and medial septal nucleus were scattered along the nervus terminalis. In the hypothalamus, neurons were dispersed around the preoptic recess, outside the periventricular preoptic nucleus, within the suprachiasmatic area (Fig. 1) and in the postchias-

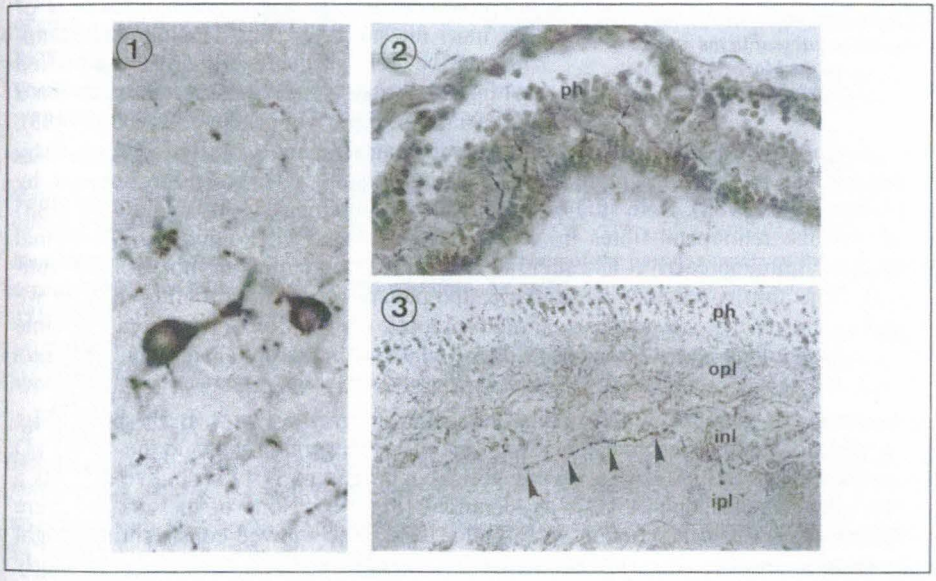

Fig. 1. Per-immunoreactive neurons in the suprachiasmatic area of the Xenopus hypothalamus. × 800. Fig. 2. Transversal section of Xenopus pineal; arrows indicate per-like fibres ending close to the pineal photoreceptor cells (ph). × 345. Fig. 3. Xenopus retina, arrowheads indicate a Per-immunoreactive fibre crossing the inner plexiform layer (ipl) ending in the amacrine layer of the inner nuclear layer (inl). Opl, outer plexiform layer; Ph, photoreceptors. × 400.

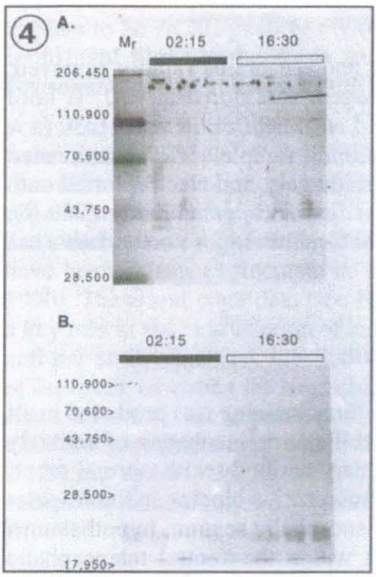

Fig. 4. Immunoblots of proteins extracted from Xenopus brains (A) and eyes (B). Molecular weight standards are included in A(M_r); their positions are marked on the left of B. (The horizontal tear in the upper right corner of the blot in A, does not represent antigenic bands.)

matic commisures. These brain areas are known to receive major inputs from the retina (Riss *et al.*, 1963; Toth *et al.*, 1980) and seem to be a recipient area for pineal projections (Foster & Roberts, 1982; Kemali & De Santis, 1983). A few cells (three to five) were identified adjacent to the habenula. Immunoreactive fibres were observed throughout the major areas of the brain except the cerebellum. Fibres from the subhabenular areas entered the pineal and terminated close to the photoreceptor cells (Fig. 2), while other fibres were traced passing through the pineal connecting each side of the brain. The distribution of immunopositive subhabenular neurons is like that described by Wake *et al.* (1974) for the AChE-positive neurons with pinealopetal axons in *Rana ridibunda* and *Rana esculenta*, and in *Ambystoma tigrinum* described by Korf (1976).

A small number of immunoreactive fibres were identified within the optic nerve which entered the retina and ran through the inner plexiform layer, terminating at the innermost sub-layer of the inner nuclear layer (Fig. 3). Note that immunoreactive cell bodies were never identified within the retina, a structure known to contain a circadian oscillator (Besharse & Iuvone, 1983). The pattern of retinopetal projections of anuran amphibians was first described by Ramón y Cajal in 1893, and more recently, using immunocytochemical techniques, by Uchiyama *et al.* (1988). Both studies are in general agreement. Uchiyama *et al.* (1988) report that the retinopetal fibres in *Xenopus* were either FMRF-amide or *N*-terminal substance P immunoreactive, and seem to originate in the lamina terminalis. This retinopetal projection is remarkably similar to the pattern of *per*-like immunostaining we see in *Xenopus*. We observed the same septo-preoptic distribution of perikarya, the same cellular morphology and a similar distribution of fibres in the optic nerve and retina.

Immunoblots of *Xenopus* eye and brain extracts are shown in Fig. 4. In the brain (Fig. 4a), a series of immunoreactive bands were identified: several proteins in the 40–50 kD range, another of 180 kD, and a weakly antigenic protein of 114 kD. In eye extracts a labelled doublet of about 21 kD was identified (Fig. 4b). None of these bands were identified when anti-*per* antibody was omitted, and none showed consistent day:night differences in intensity. By way of comparison, rat brain extracts showed two strongly antigenic proteins of 111 kD and 160 kD (Siwicki *et al.*, 1992). The brain of the golden hamster was essentially identical to the rat, while the brain of the lizard (*Anolis carolinensis*) contained two strongly immunoreactive proteins at 113 kD and 96 kD (unpublished: Garcia-Fernandez, Siwicki, Foster). The antigens in *Xenopus* brain extracts are distinctly different in size, but in the absence of any more specific information about

these antigenic proteins, it is not possible to know whether or how they might be related to the *Drosophila per* protein. The sequence of the cloned *Drosophila per* cDNA predicts a protein of 127 kD (Citri *et al.*, 1987). Thus, inferences about the functional relationship of these vertebrate *per*-like antigens must await molecular studies of vertebrate genes homologous to *per*. In *Drosophila, per* protein are expressed rhythmically (Zerr *et al.*, 1990), our immunoblot studies provided no evidence of daily changes in the intensity of any of the *Xenopus* antigens labelled by the antibodies.

While the homology/functional relationship (if any) of vertebrate *per*-like antigens to *Drosophila per* protein remains unclear, the immunocytochemical results in *Xenopus* suggest that the antibodies recognize cells and fibres associated with: (a) brain areas which receive inputs from the retina and pineal and (b) brain areas that project to and, probably with a feedback function, modify the activity of retinal and pineal neurons.

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