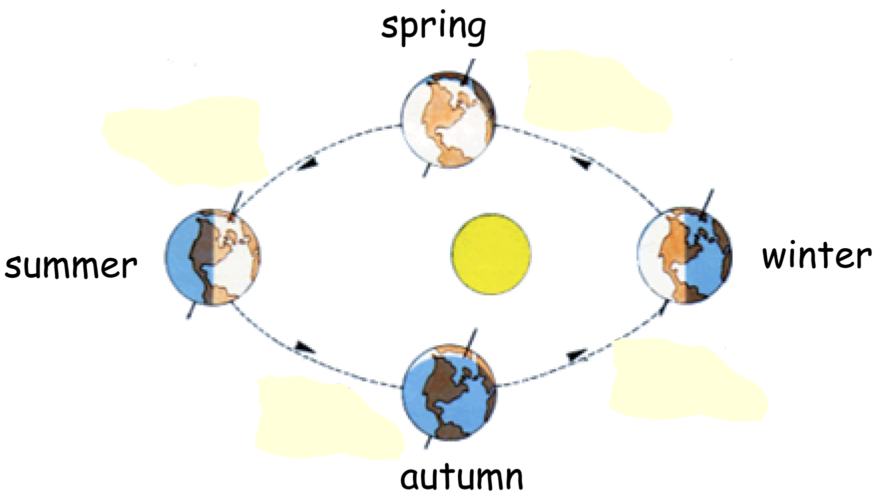

Life on earth is under the influence of basic environmental periodic changes such as day-night cycles or the fluctuation of seasons

Organisms have adapted to these rhythms in order to maximally benefit from the limited natural resources

The mechanism that keeps track of time, and therefore allows the organism to anticipate upcoming daily changes is termed

CIRCADIAN CLOCK

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Dip. Biologia

Circadian clocks are molecular time-keeping mechanisms that reside in a wide range of cell types in a variety of organisms

The key feature of a circadian clock is its ability to synchronize (***entrain***) to environmental time cues (so-called ***zeitgebers***; "time-givers") and to maintain rhythmic function when placed in constant conditions

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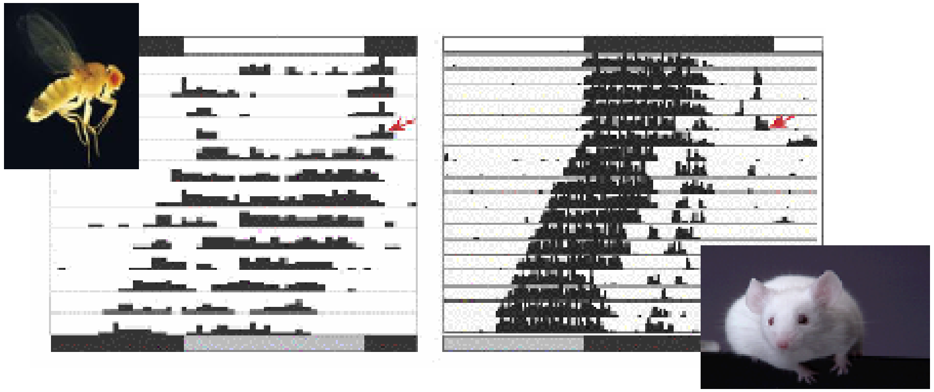
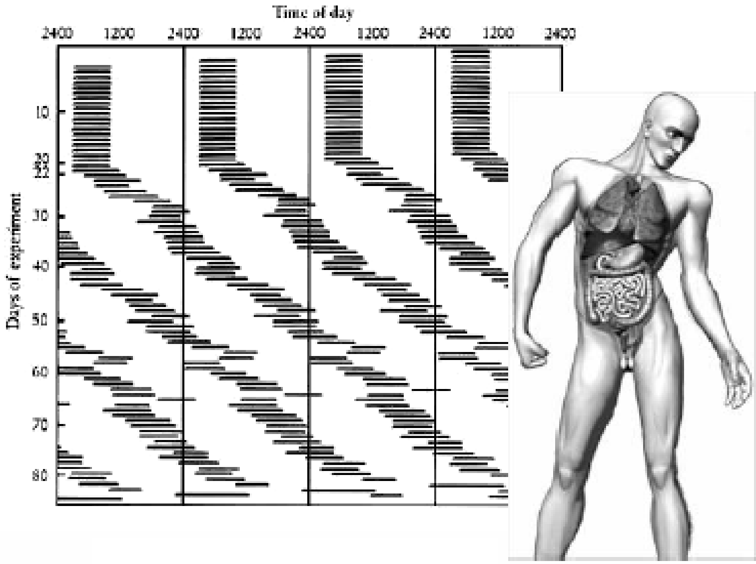
Endogenous clock systems
(circadian, circannual, ultradian)
are present in all organisms known.

They have evolved to measure time and to keep the organism in *entrainment* with the environment.

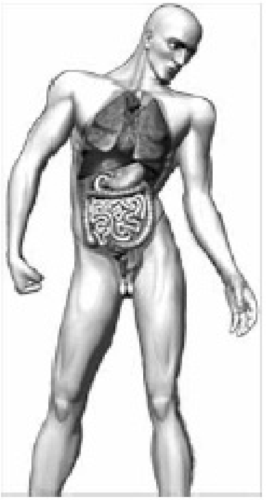
Light, feeding and temperature are the strongest *Zeitgebers* for circadian rhythms.

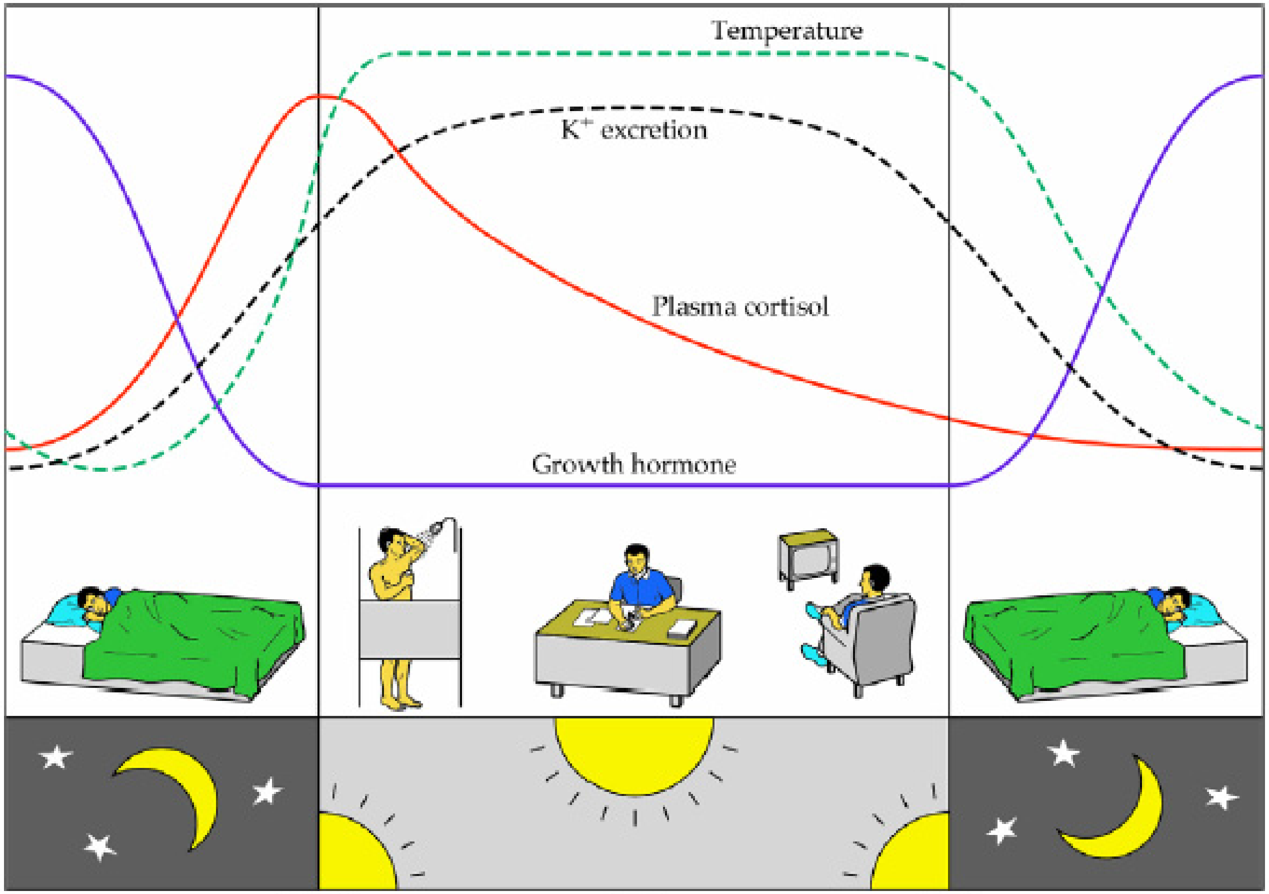
Biological rhythms are ubiquitous





Rhythms of body function in humans





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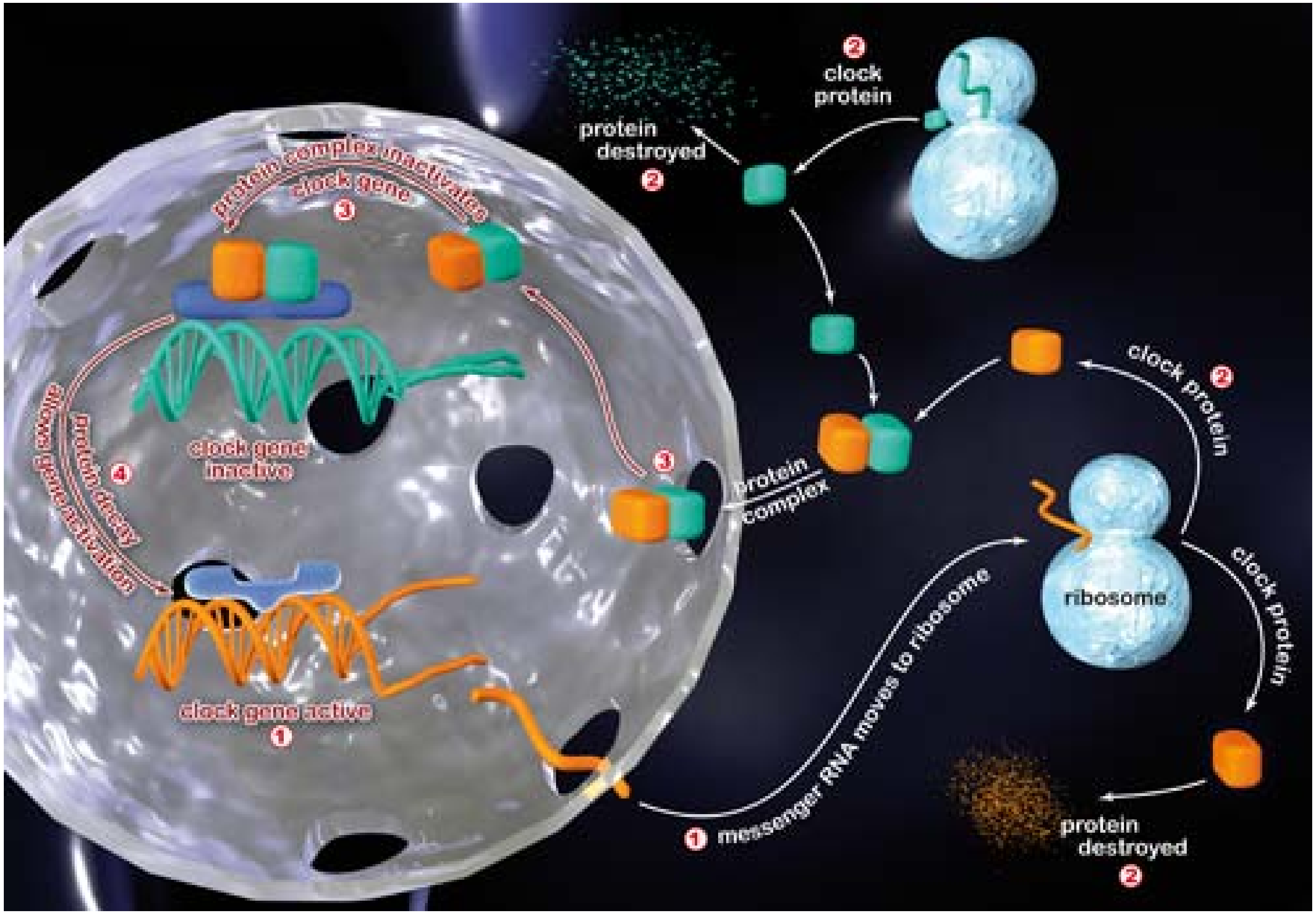
Circadian rhythms generation consists of interacting positive and negative transcriptional/translational feedback loops



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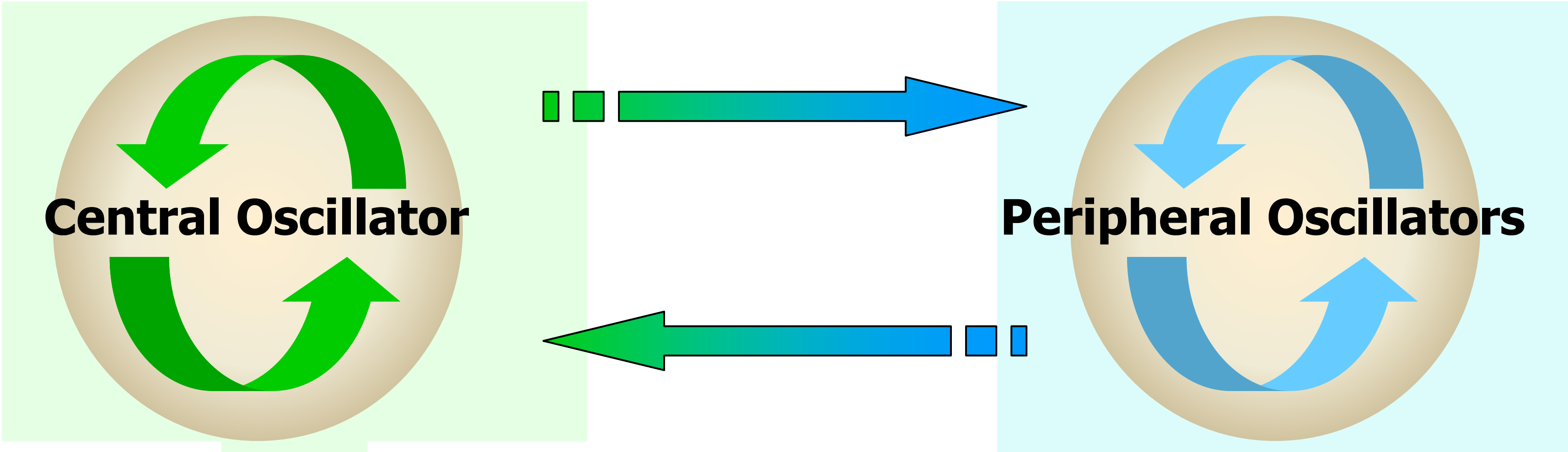
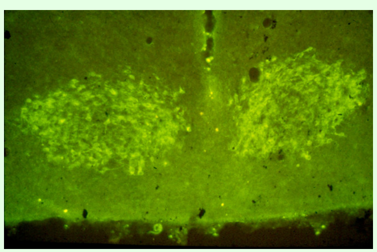
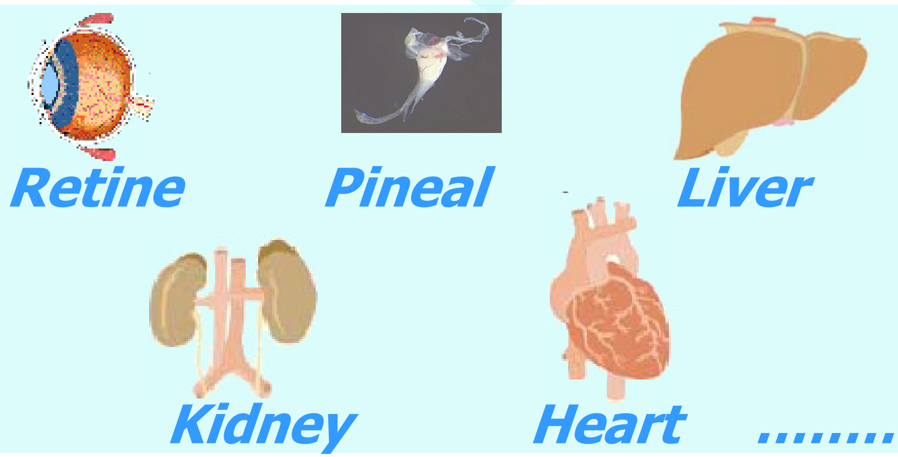
VERTEBRATE CIRCADIAN SYSTEM

MULTIOSCILLATORY SISTEM





Suprachiasmatic Nuclei of the Hypothalamus (SCN)



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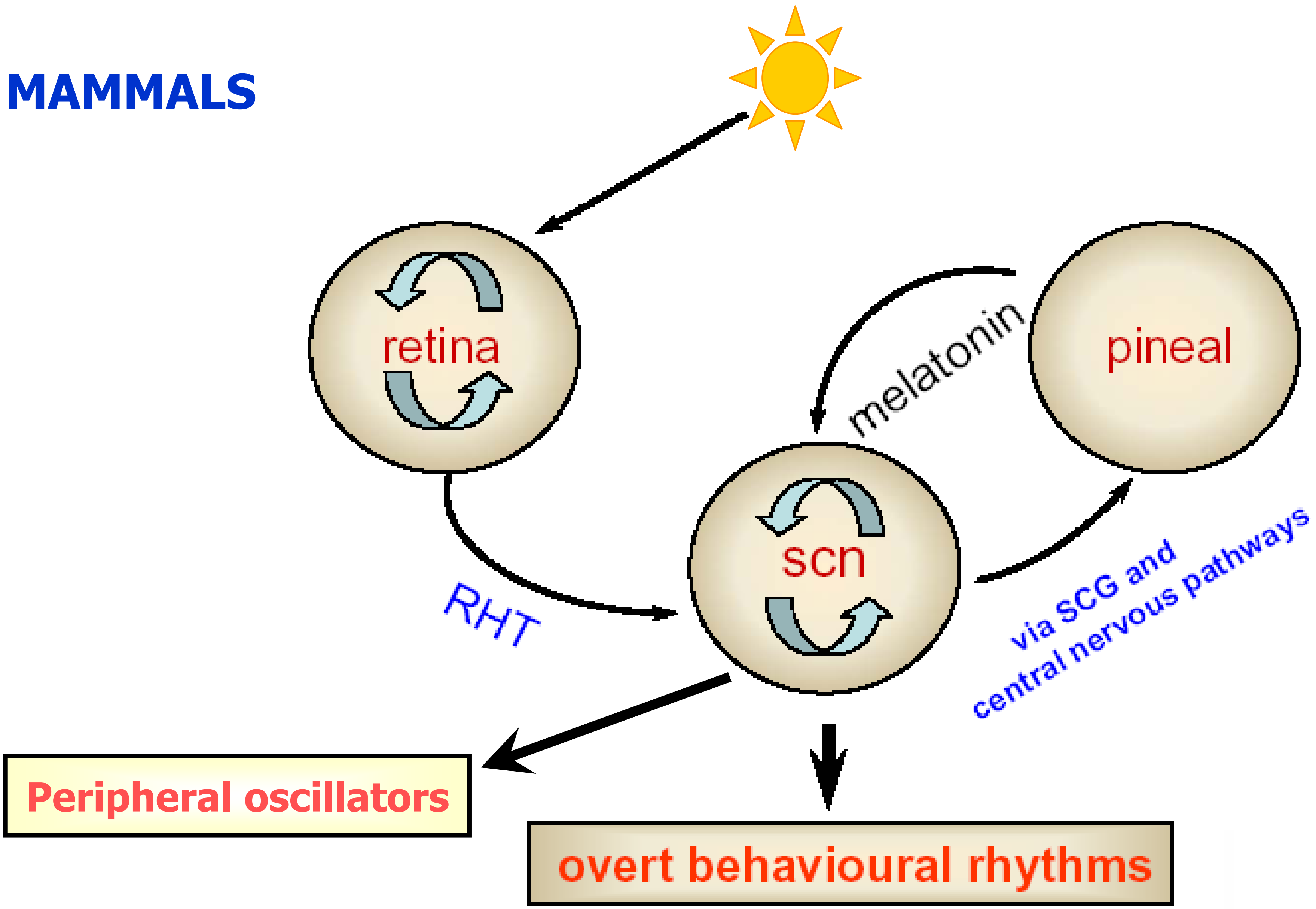
Key question:

How and where is the light signal , the most powerful zeitgeber, detected?

MAMMALS



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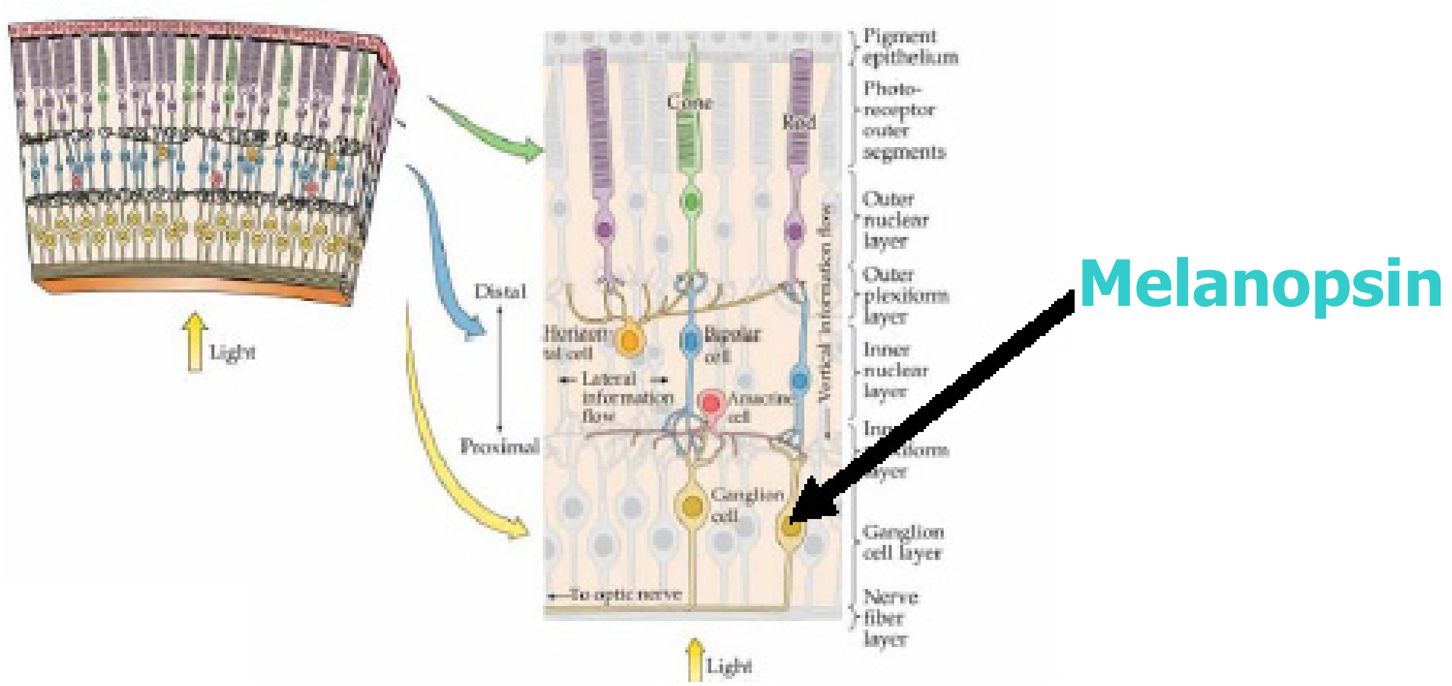
In mammals

Light input to the circadian system comes exclusively from the retina, mediated via a subset of ganglion cells that project to the suprachiasmatic nucleus.



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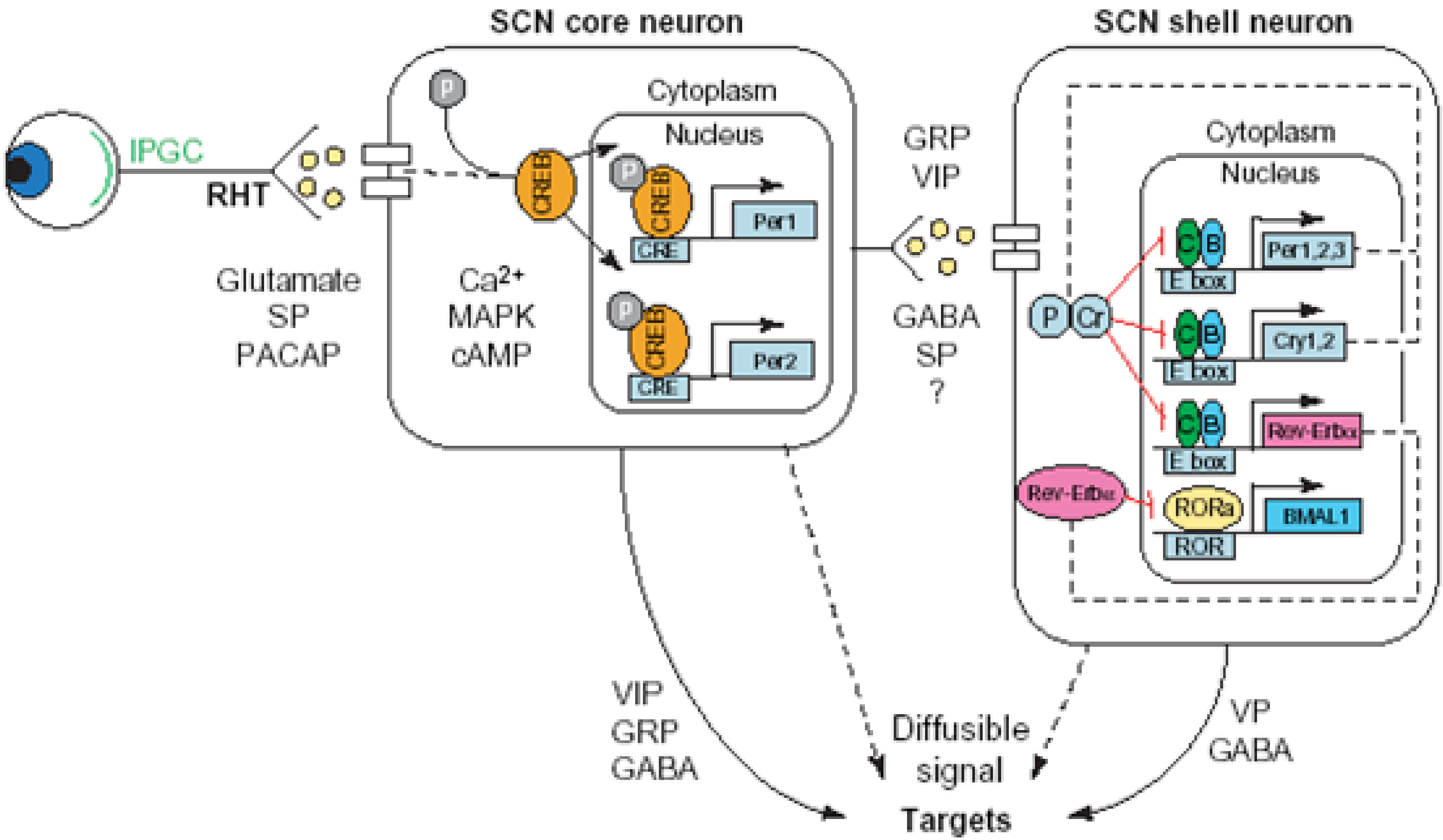

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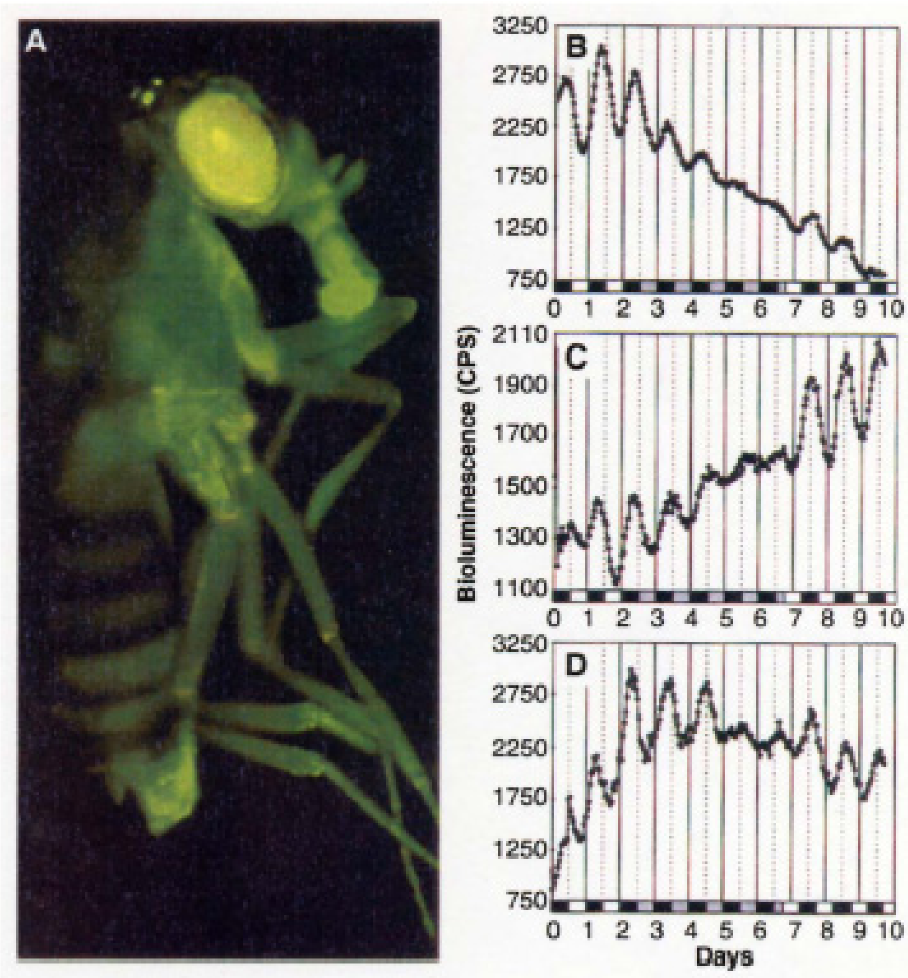
Light responsive circadian oscillators

Drosophila melanogaster



Head

Thorax

Abdomen

***Period* gene driven bioluminescence**

- present throughout the whole fly
- rhythms maintained in various body parts in culture

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Drosophila

Rhythmic clock gene expression can be found in any tissue

Rhythmic clock gene expression is maintained in culture

All cultured tissues are light responsive

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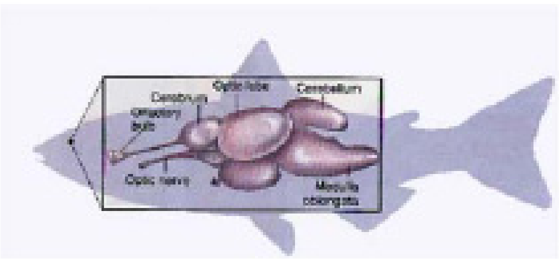
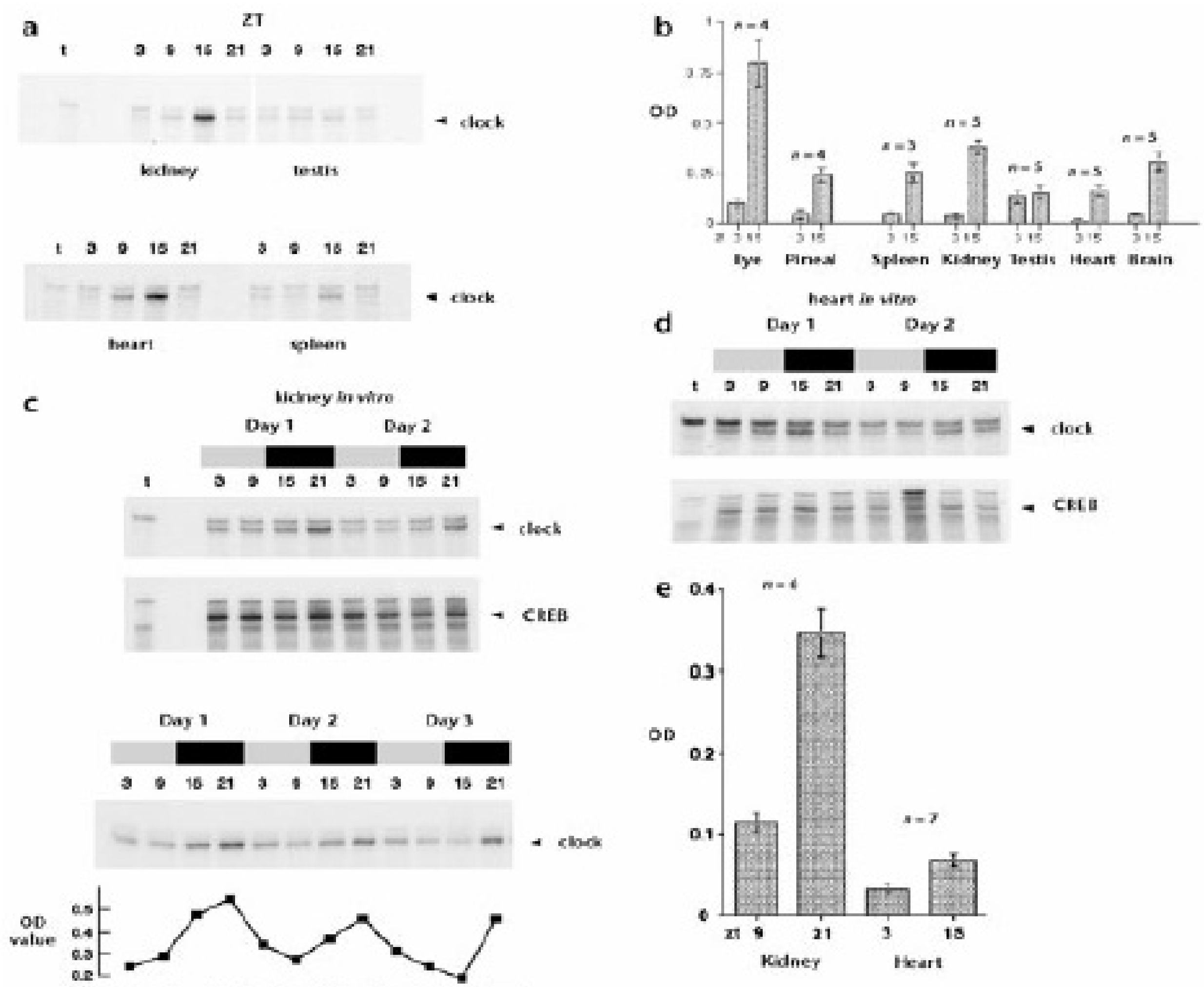
Light responsive circadian oscillators

ZEBRAFISH





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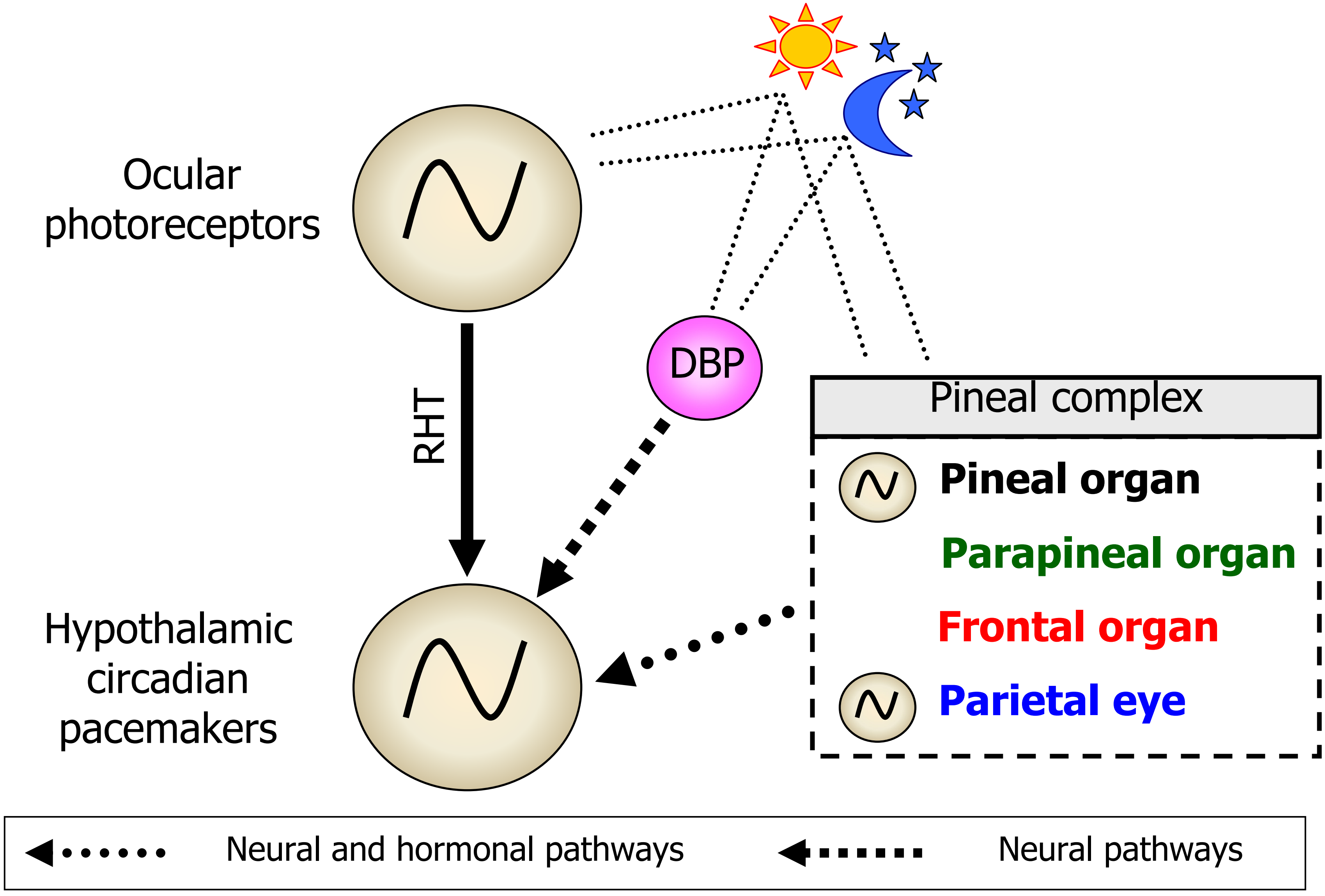
ZEBRAFISH

Rhythmic clock gene expression can be found in almost any tissue

Rhythmic clock gene expression is maintained in culture

All rhythmic tissues are directly light responsive

BRAIN CIRCADIAN PHOTORECEPTION



Bertolucci and Foà, 2004 CBI

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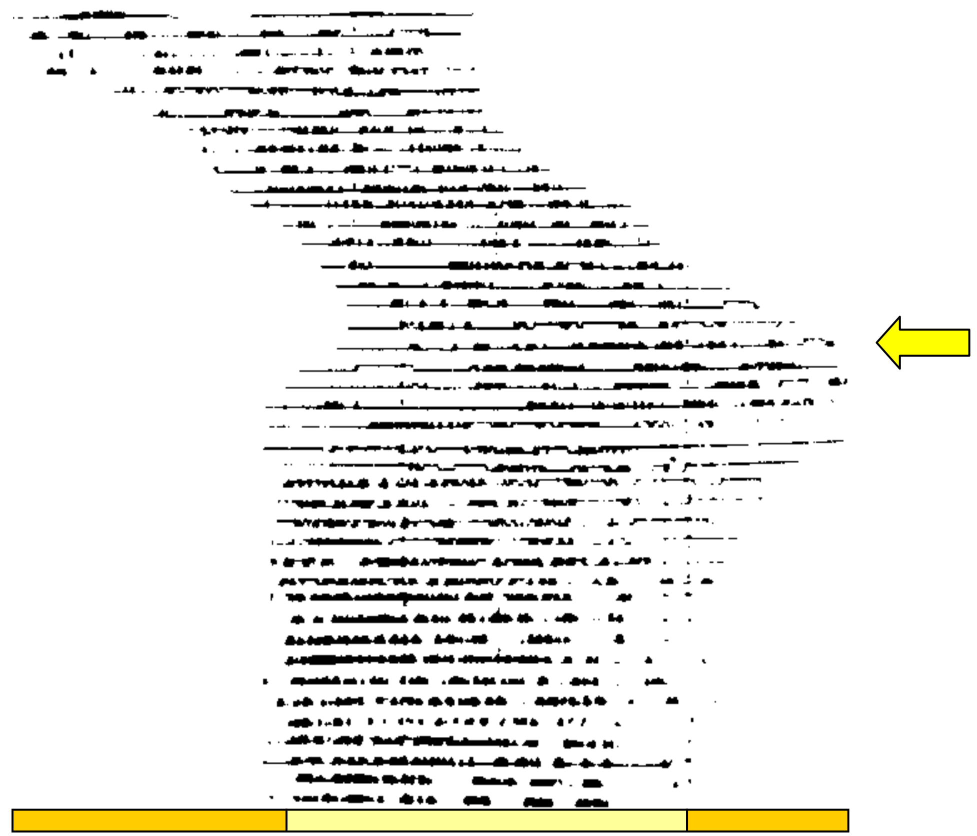
Podarcis sicula (Family Lacertidae)

- Phylogenetic position
- ectotherm hibernator
- interesting model for understanding circadian organization, its evolution, and its variability

Entrainment to LD cycles in reptiles

Podarcis sicula

PINX-RETX lizard entrained
to LD cycles



Foà *et al.*, 1993

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Opsins isolated and/or detected by in situ hybridization or immunocytochemistry with specific antiserum from extraocular photoreceptive structures

Melanopsin	Amphibians	*X. laevis*	Provencio et al., 1998
		D. rerio	Bellingham et al., 2002
	Teleosts	*G. morhua*	Drivenes et al., 2003
Pinopsin	Amphibians	*B. japonicus*	Yoshikawa et al., 1998
VA opsin (different isoforms)	Teleosts	*S. salar*	Philp et al., 2000
		D. rerio	Kojima et al., 2000
		C. carpio	Moutsaki et al., 2000
		P. altivelis	Minamoto and Shimizu, 2002
Rhodopsin	Birds	*C. livia*	Wada et al., 1998
	Teleosts	*P. altivelis*	Masuda et al., 2003
RH2 opsin	Reptiles	*P. sicula*	Pasqualetti et al., 2003
tmt-opsin	Teleosts	*D. rerio*	Moutsaki et al., 2003

Basal region of lateral ventricle

Anterior hypothalamus

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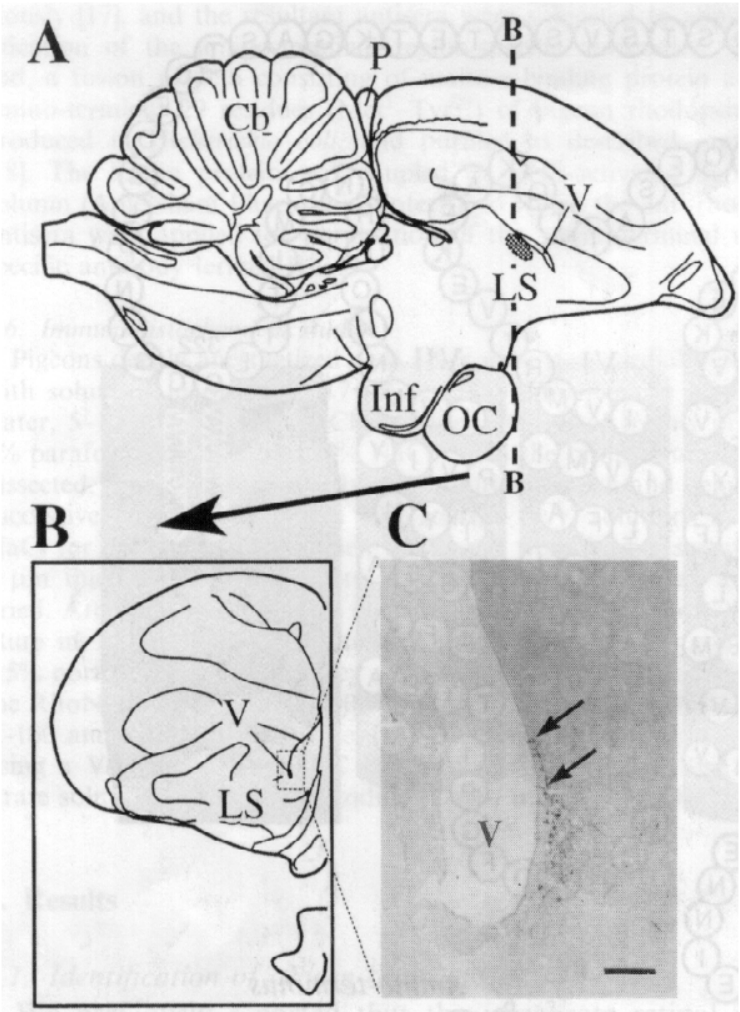
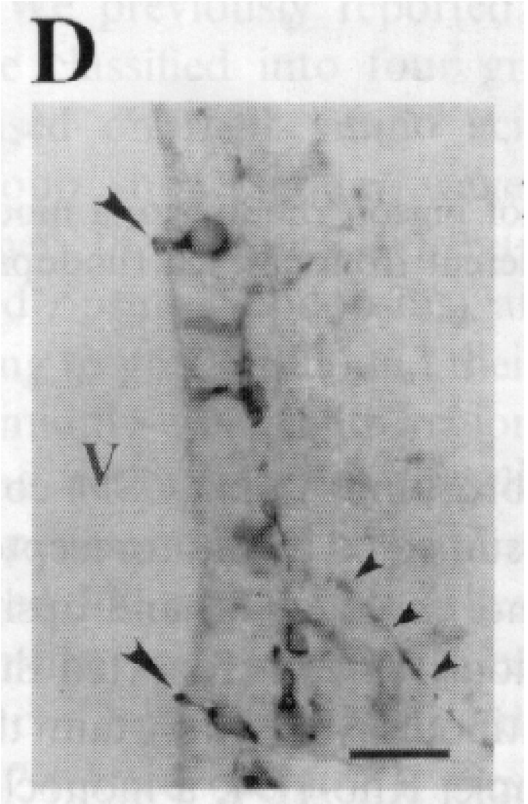
DEEP BRAIN PHOTORECEPTORS

Basal region of lateral ventricle in *Columba livia*



CSF-contacting neurons labelled with antiserum against rodopsin



V: telencephalic lateral ventricle

Wada et al., FEBS Letters, 1998

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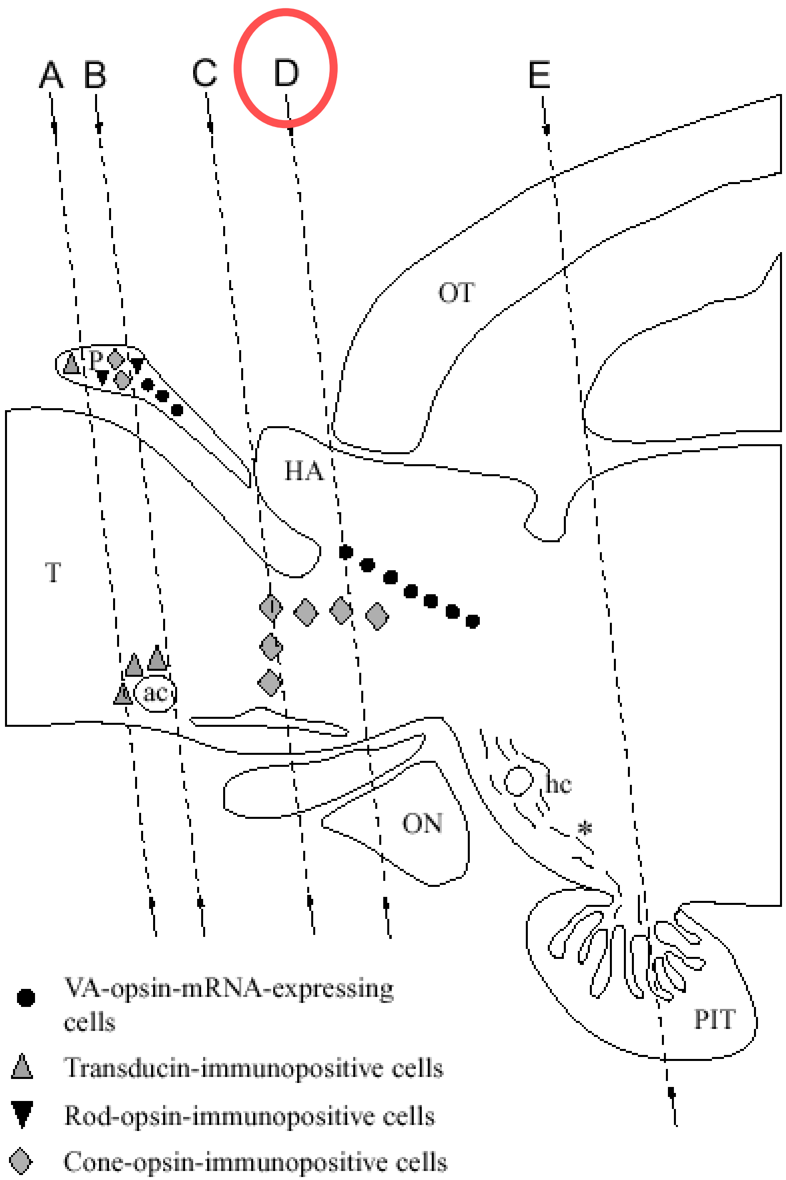
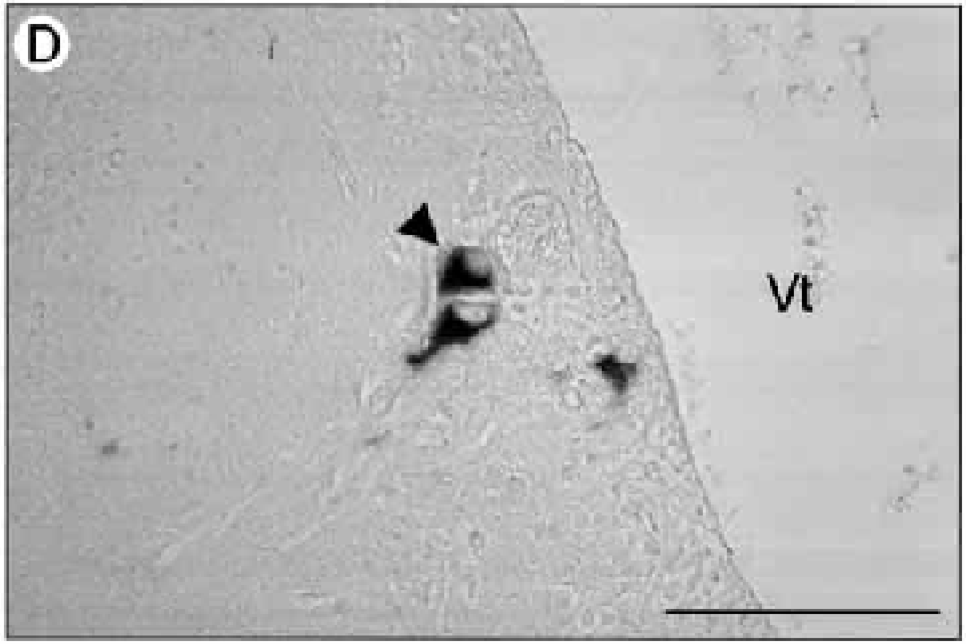
DEEP BRAIN PHOTORECEPTORS



Anterior hypothalamus of *Salmo salar*

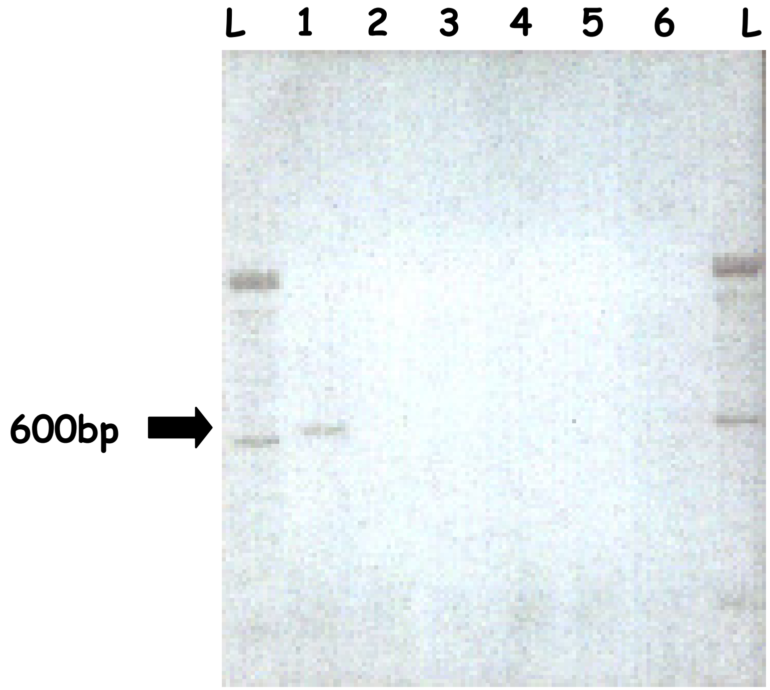
Neurosecretory cells labelled with antibody against cone-opsin



Vt: III ventricle

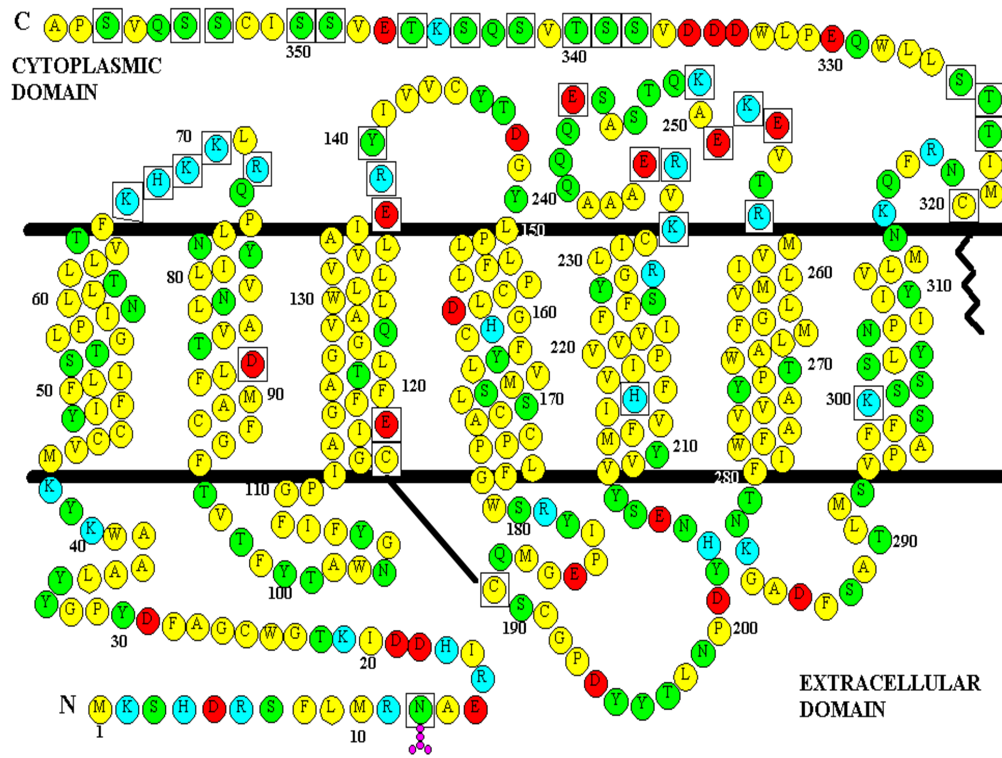
Philp et al., J. Exp. Biol., 2000

Identification of brain opsin in the lizard *Podarcis sicula*



L - ladder
1 - brain
2 - heart
3 - liver
4 - skin
5 - testicle
6 - negative control

RT-PCR in different tissues of lizards



AA Sequence of the brain opsin

Lys300 retinal
Glu138-Arg139-Tyr140 trasducin

Pasqualetti et al., 2003, *Eur, J, Neurosc.*

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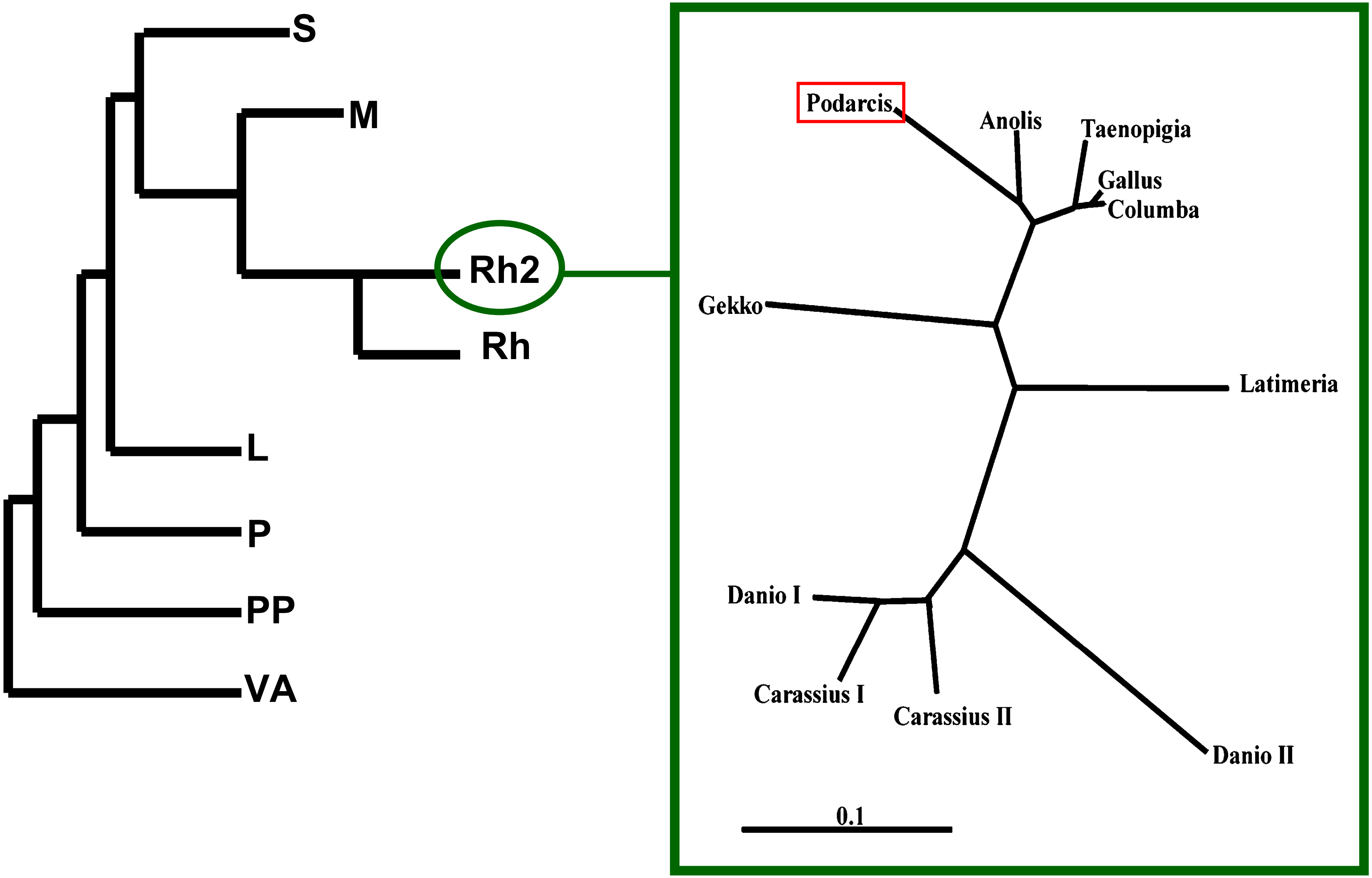
Rh2 Group
rhodopsin-like pigments (470-510 nm)



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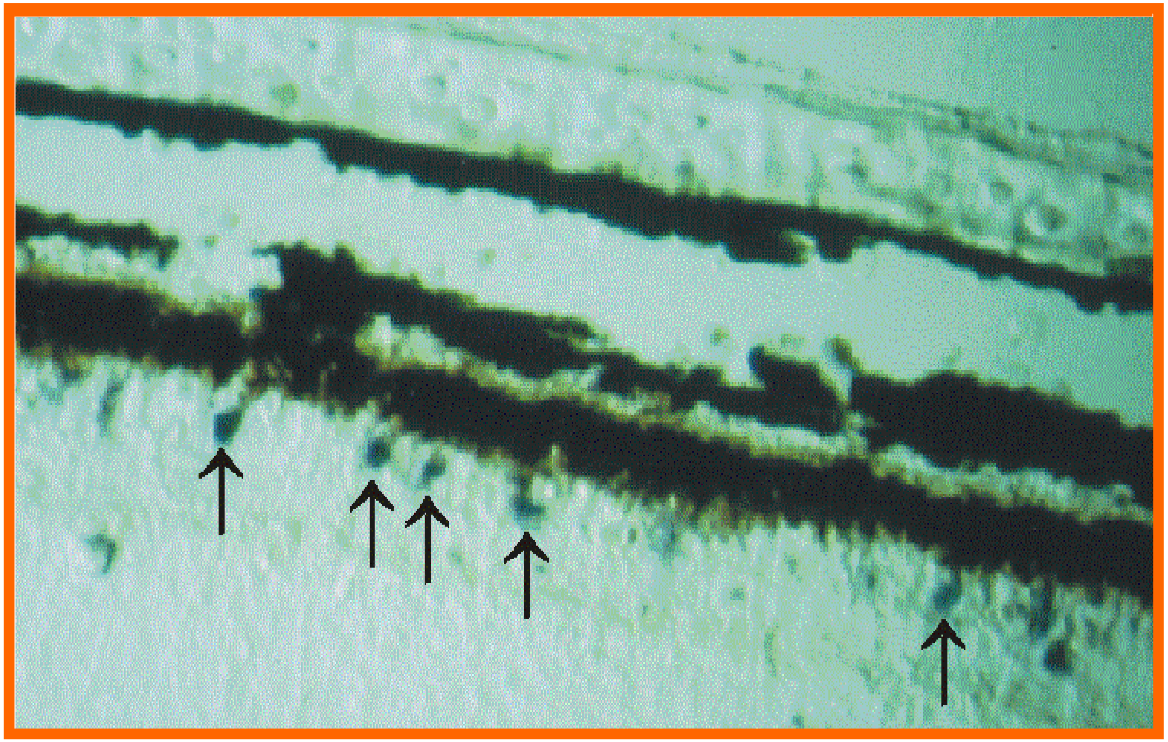
Podarcis sicula retinae
in situ Ibridization of RH2 opsin



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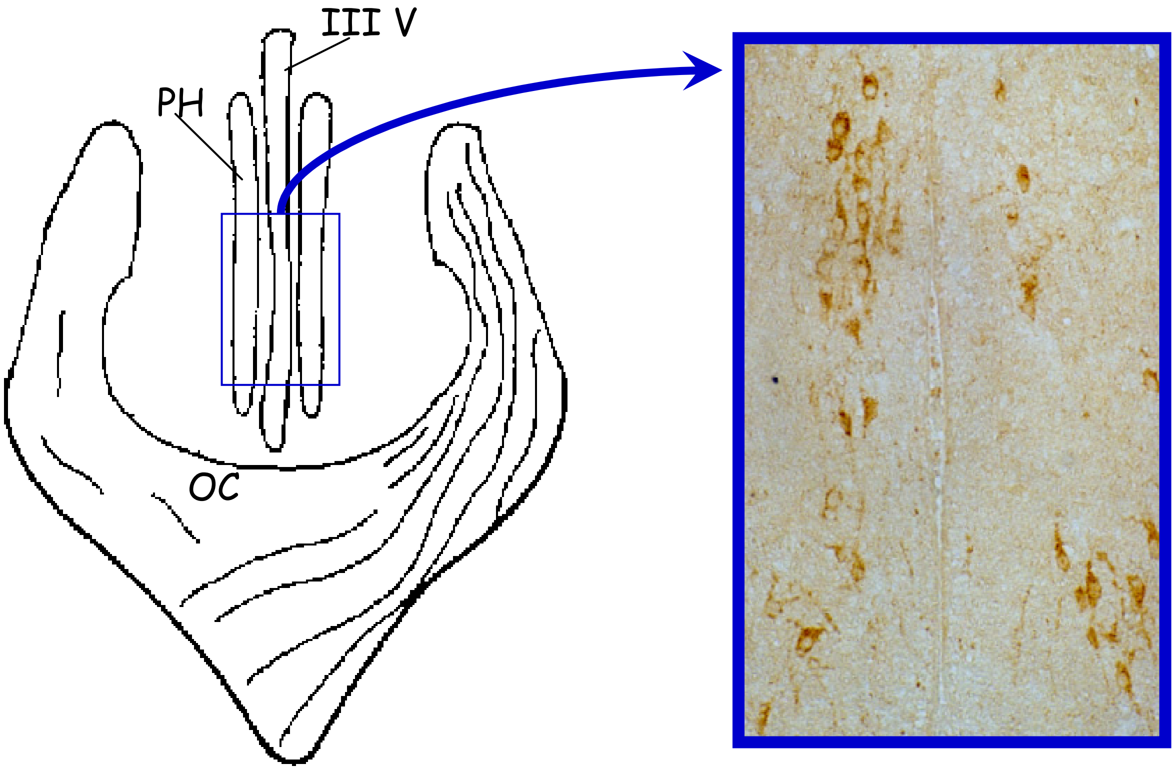
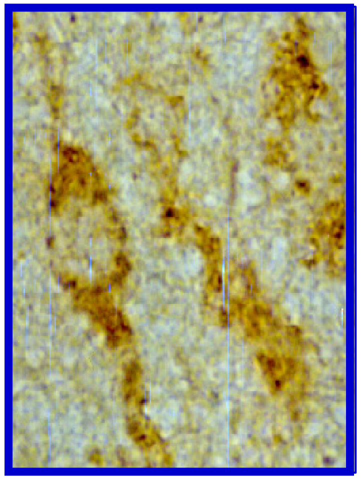
IMMUNOCYTOCHEMICAL ANALYSIS OF RH2 EXPRESSION IN THE BRAIN





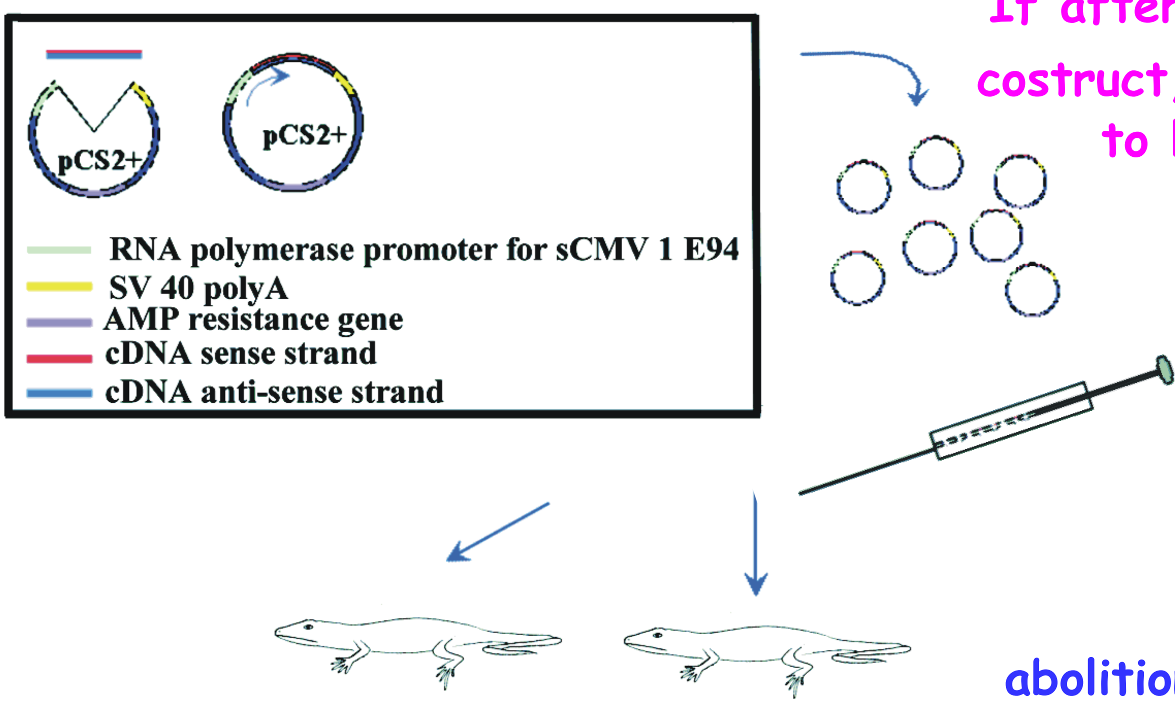
Anti-cone-opsin antiserum CERN 874 (specific for chicken Rh2 opsin) labelled neurons localized into the periventricular area (PH) of the hypothalamus, close to the border of the third cerebral ventricle

OC: optic chiasm

Pasqualetti et al., 2003, *Eur, J, Neurosc.*

ARE BRAIN CONE-OPSINS NECESSARY FOR PHOTIC ENTRAINMENT OF LOCOMOTOR RHYTHMS?

Post-transcriptional inactivation experiments by injecting an eukaryotic expression vector transcribing the antisense cone-opsin RH2 mRNA in the third cerebral ventricle of pinx–retx lizards previously entrained to an LD cycle



If after the injection with antisense costruct, CERN 874 completely failed to label cells within the PH

abolition of cone-opsins expression within the PH

Pasqualetti et al., 2003, *Eur, J, Neurosc.*

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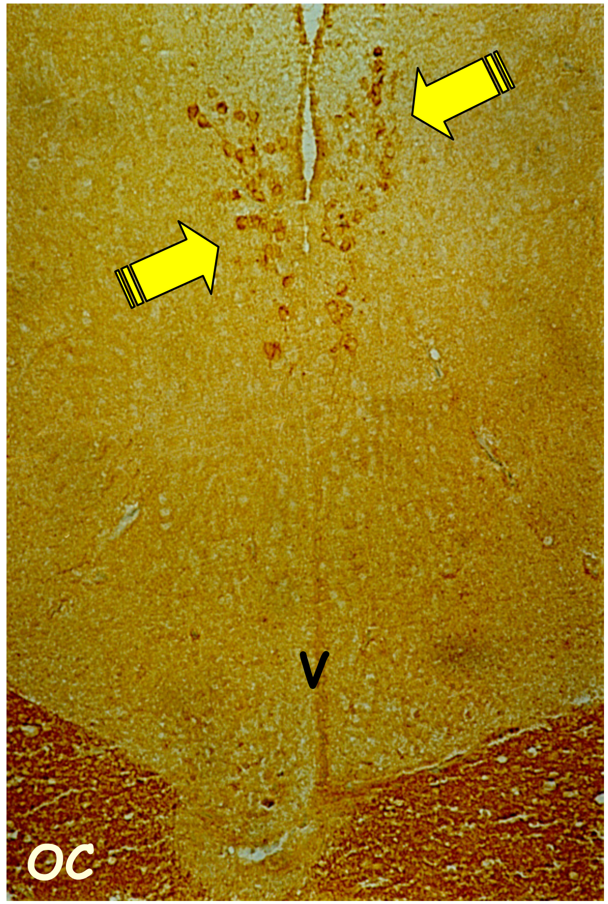
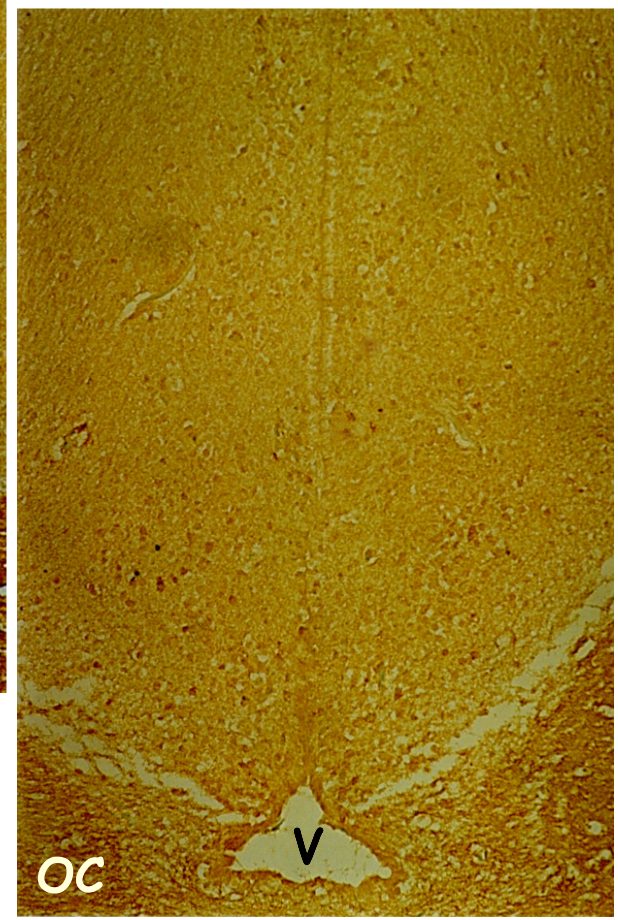
Histology

Sense



Antisense



OC: Optic chiasm

V: III ventricle

Pasqualetti et al., 2003, *Eur, J, Neurosc.*

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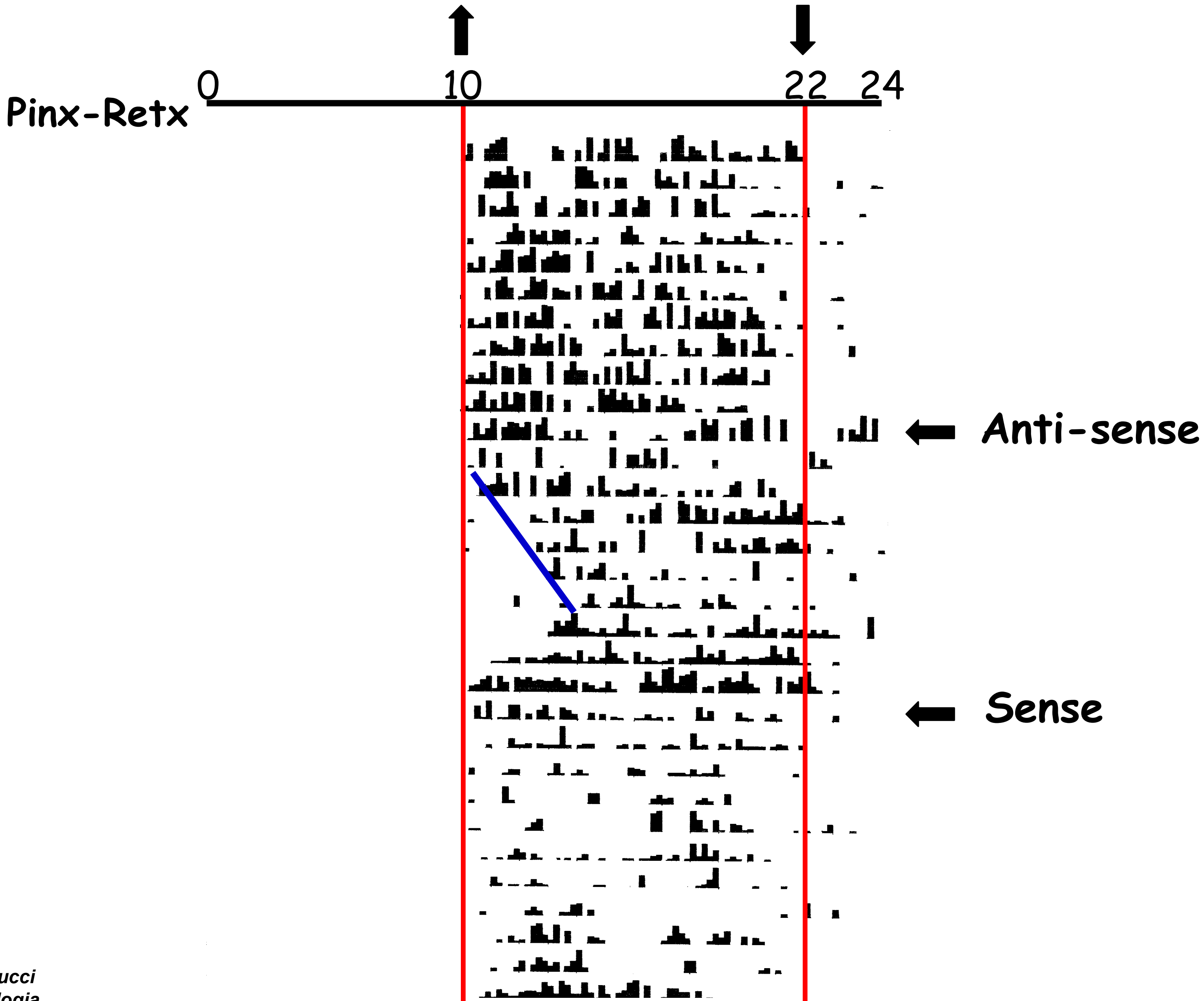
BEHAVIOURAL TESTS



Pasqualetti et al., 2003, *Eur, J, Neurosc.*

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BEHAVIOURAL TESTS

Pasqualetti et al., 2003, *Eur, J, Neurosc.*

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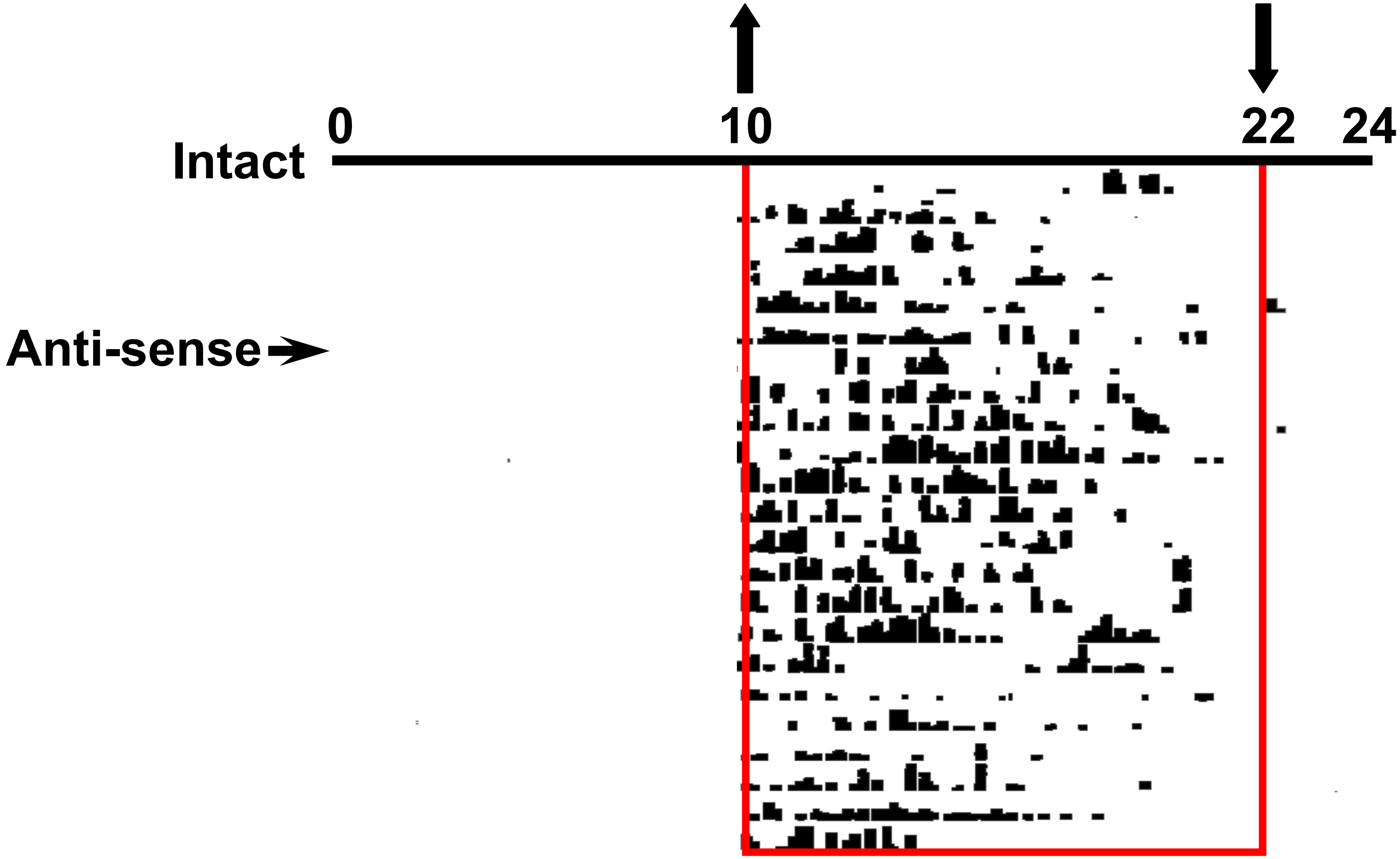
BEHAVIOURAL TESTS



Pasqualetti et al., 2003, *Eur, J, Neurosc.*

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Posttranscriptional inactivation experiments of endogenous brain cone-opsins mRNA demonstrate, for the first time in a vertebrate, that brain cone-opsins of lizards are part of a true circadian brain photoreceptor participating in photic entrainment of behavioral rhythms

Experiments don't allow us to exclude the possibility that an antisense construct inactivated cone-opsins different from Ps-RH2, since differences among sequences of cone-opsins are often limited to few amino acids. Furthermore, polyclonal antiserum CERN 874 could recognize other cone-opsins inactivated by antisense treatments

MELANOPSIN PINOPSIN RHODOPSIN … … … …

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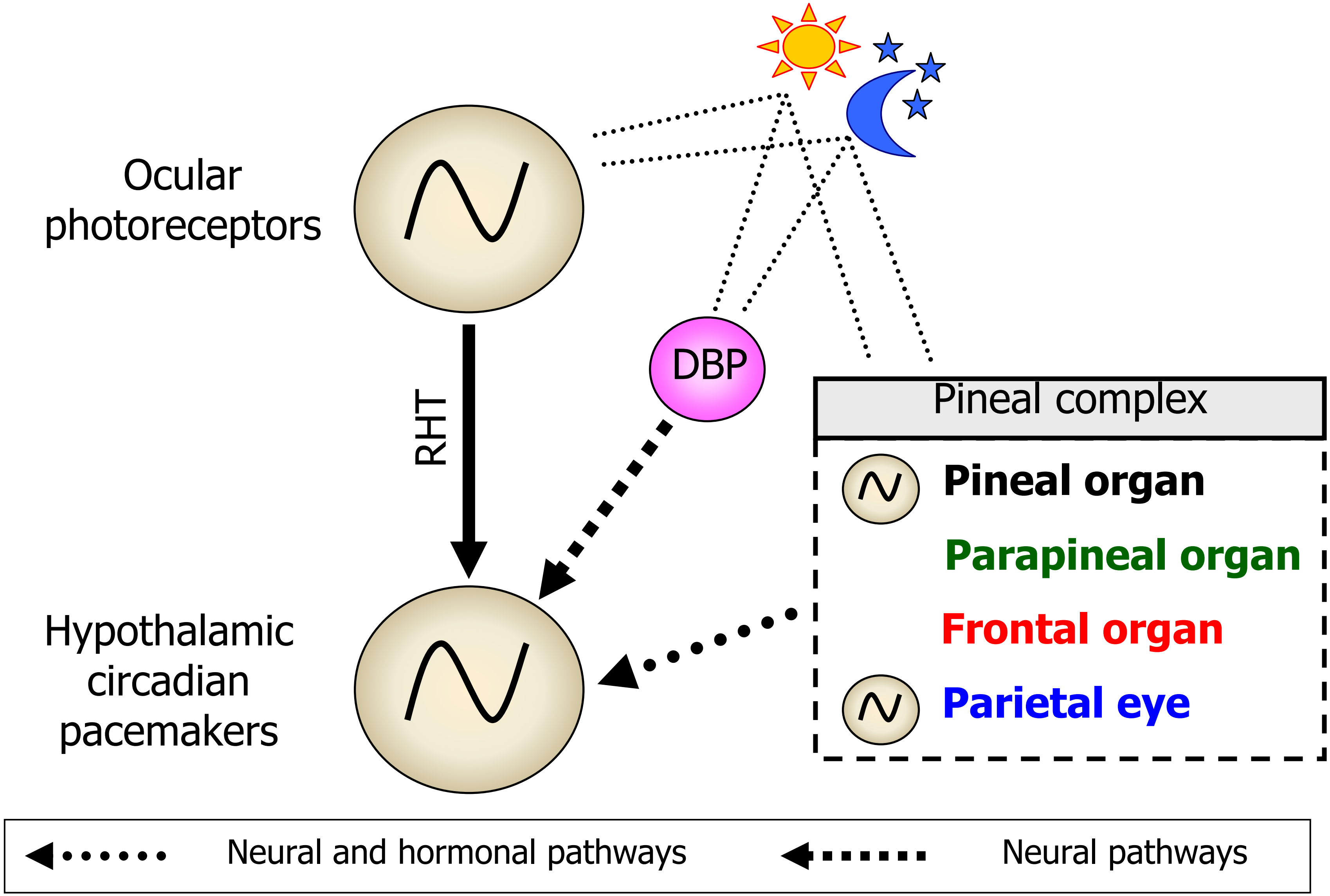
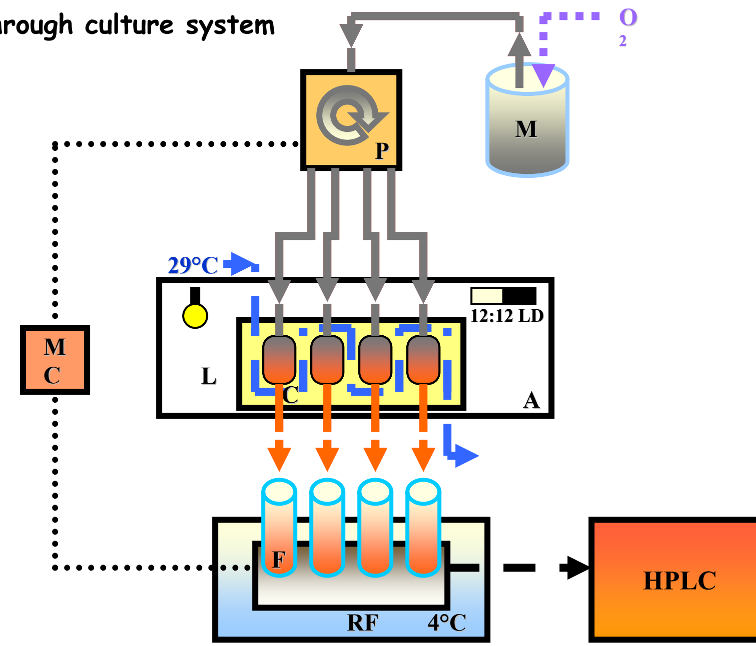
BRAIN CIRCADIAN PHOTORECEPTION

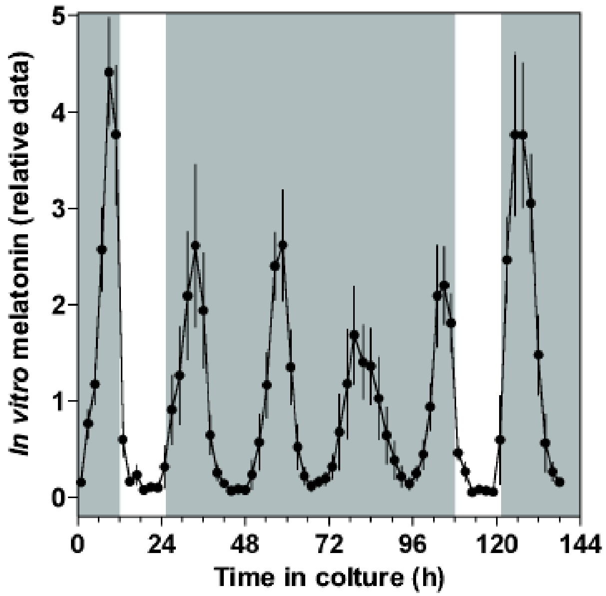


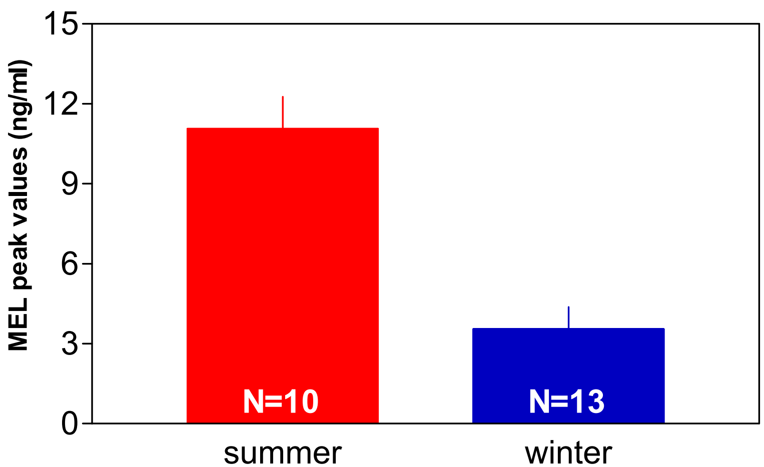
Bertolucci and Foà, 2004 CBI

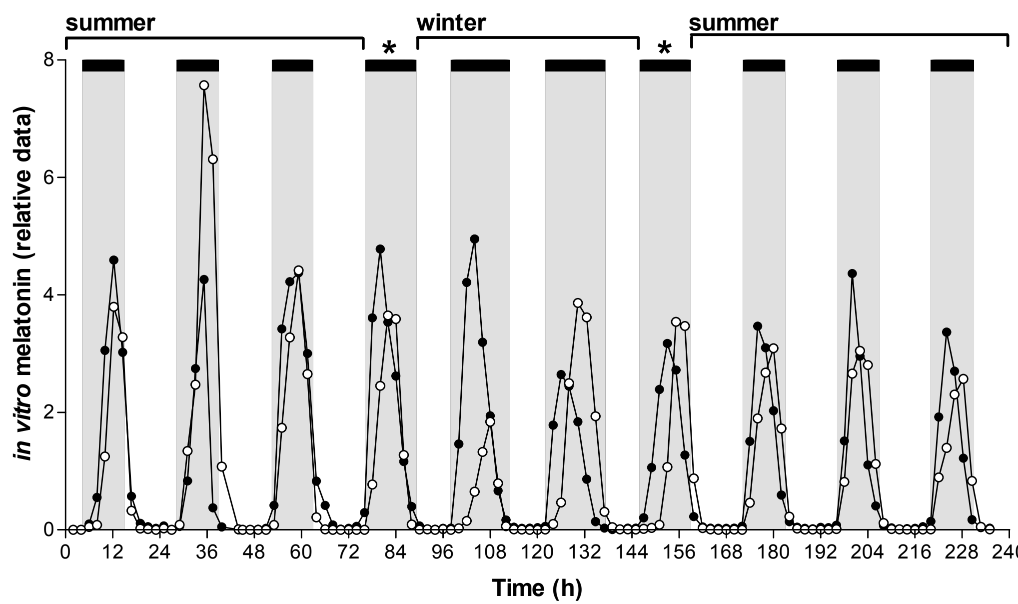
Flow through culture system



Bertolucci *et al.*, 2003 JBR







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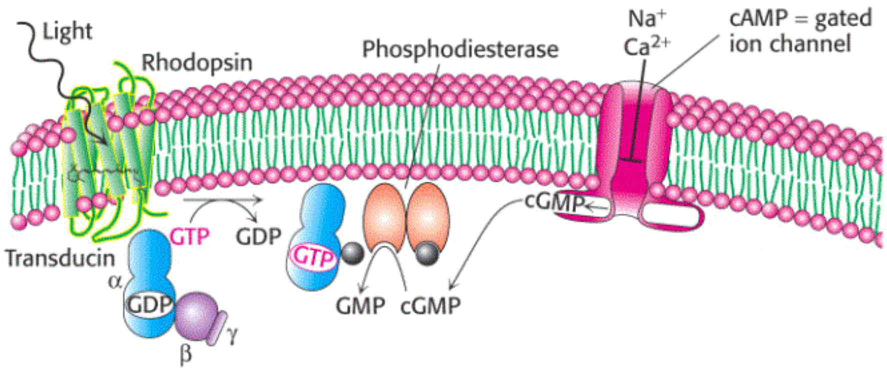

Identification of non visual opsin in *Podarcis sicula*

PINOPSIN

MELANOPSIN

-RT-PCR with primers designed on the conserved regions of orthologs
- 5'-3' RACE for full-length cloning

Phylogenetic analysis

Maximun parsimony analysis

Expression in photoreceptive organs

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Pinopsin

```
                 attgcttgctcagcctcacctgatctctgtccccaatagaagaa
atgcaagcttcaaacgcttcatgggttgaggtgagaaacaggaccccaggcccttttgag
 M  Q  A  S  N  A  S  W  V  E  V  R  N  R  T  P  G  P  F  E
gggccgcaatggccctacctagccccacagagcacctacatatcagtggctgtcctcatg
 G  P  Q  W  P  Y  L  A  P  Q  S  T  Y  I  S  V  A  V  L  M
gggctggtggtcatctcggccaccttggtgaacggcttggtgattgtggtctctgtccag
 G  L  V  V  I  S  A  T  L  V  N  G  L  V  I  V  V  S  V  Q
ttcaagaagctccgttctcctttgaactacgtcctggtgaacctggccgtggctgatctc
 F  K  K  L  R  S  P  L  N  Y  V  L  V  N  L  A  V  A  D  L
ttggtcaccttctttgggagcaccatcagcttcgtaaacaatgcccagggcttctttatc
 L  V  T  F  F  G  S  T  I  S  F  V  N  N  A  Q  G  F  F  I
tttggccaggcaacctgcgaatttgaaggcttcatggtctccttaacagggattgtgggt
 F  G  Q  A  T  C  E  F  E  G  F  M  V  S  L  T  G  I  V  G
ctttggtccttggcgatcctggcctttgagaggtatctcgtgatctgcaaaccggtgggt
 L  W  S  L  A  I  L  A  F  E  R  Y  L  V  I  C  K  P  V  G
gatttccgcttcccagcaaggcatgcggtgcttggttgtgctttcacttgggggtggtcg
 D  F  R  F  P  A  R  H  A  V  L  G  C  A  F  T  W  G  W  S
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 F  V  W  T  V  P  P  L  L  G  W  S  S  Y  V  P  E  G  L  R
acatcctgcggccccaactggtacagtggcggaagcagcaacaacagctatatcatgact
 T  S  C  G  P  N  W  Y  S  G  G  S  S  N  N  S  Y  I  M  T
ttgtttgtgacctgctttgcgatgcctctcagcacaatcctcttctcctacgccaacttg
 L  F  V  T  C  F  A  M  P  L  S  T  I  L  F  S  Y  A  N  L
ctcatgacgctgcgaacagtagcggctcagcagaaagagcaggagacaactcagagggcc
 L  M  T  L  R  T  V  A  A  Q  Q  K  E  Q  E  T  T  Q  R  A
gagagggaggtgacacgaatggttgtcgccatggtggctgccttcctcgtctgctggctg
 E  R  E  V  T  R  M  V  V  A  M  V  A  A  F  L  V  C  W  L
ccctacgccagcttcgccatggtggtcgccacccacaaggacctcgccatacggccagcg
 P  Y  A  S  F  A  M  V  V  A  T  H  K  D  L  A  I  R  P  A
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 L  A  S  L  P  S  Y  F  S  K  T  A  T  V  Y  N  P  I  I  Y
gtcttcatgaacaagcagttccgcagttgcctcctctacaagatgagctgtggccacaga
 V  F  M  N  K  Q  F  R  S  C  L  L  Y  K  M  S  C  G  H  R
gcattatcttcccaggacacgacaccagctgggatcagcctgccaggccgcctcaccacc
 A  L  S  S  Q  D  T  T  P  A  G  I  S  L  P  G  R  L  T  T
tcagcttcaaaaggaagcaggaatcaagtgtcgccttccttaacgatgc
 S  A  S  K  G  S  R  N  Q  V  S  P  S
```

Gallus gallus	69%
Columba livia	72%
Anolis carolinensis	77%
Phelsuma madagascariensis	72%
Bufo japonicus	68%

N: potential site for N-glycosylation (Asn5)

C: cysteines for an intramolecular disulfide bond (Cys106, Cys183)

E: glutamic acid for retinylidene Schiff-base counterion (Glu109)

K: lysine for Schiff-base linkage with a chromophore 11-cis-retinal (Lys273)

C: cysteines for palmitoylation (Cys316)

ERY motif at position 130–132 necessary to bind the α-subunit of transducin

C.Bertolucci
Dip. Biologia

Melanopsin

```
                acgcgggggcgggaagcatggcagaggcgcacgagcagacacagaat
atgggaactcagcaccgaataaaagtagatgttcctgatcgtgttctttacactgtaggc
 M   G   T   Q   H   R   I   K   V   D   V   P   D   R   V   L   Y   T   V   G
tcctgtgttcttgtcattggttctattggaatcacaggaaatcttcttgtcctctatgca
 S   C   V   L   V   I   G   S   I   G   I   T   G   N   L   L   V   L   Y   A
ttttacagcaacaagcgtctgaggacaccggcaaactatttcataatgaatttagcggca
 F   Y   S   N   K   R   L   R   T   P   A   N   Y   F   I   M   N   L   A   A
agtgatttctgatgtctgcaactcaagctccaatctgctttctcaacagcatgcacaca
 S   D   F   L   M   S   A   T   Q   A   P   I   C   F   L   N   S   M   H   T
gaatggatactcggagacataggttgtaacttttatgtattttgtggggcactctttgga
 E   W   I   L   G   D   I   G   C   N   F   Y   V   F   C   G   A   L   F   G
ataacttcaatgatgactttattagctatttcagttgatcgctactgtgtgattactaag
 I   T   S   M   M   T   L   L   A   I   S   V   D   R   Y   C   V   I   T   K
cctctgcagtctataaaaaggtcttcaaagaaacgctcatgcatcatcattgcctttgtc
 P   L   Q   S   I   K   R   S   S   K   K   R   S   C   I   I   I   A   F   V
tggctctactcgctgggatggagtgtatgtcctctctttggatggagttcctatatacct
 W   L   Y   S   L   G   W   S   V   C   P   L   F   G   W   S   S   Y   I   P
gaaggtttgatgatatcttgtacatgggactacgtatcctattctccagcaaacagaagt
 E   G   L   M   I   S   C   T   W   D   Y   V   S   Y   S   P   A   N   R   S
tataccatgatgttatgttgctttgtgttctttatccctctgataataatcttccactgc
 Y   T   M   M   L   C   C   F   V   F   F   I   P   L   I   I   I   F   H   C
tatctattcatgtttctggccattagaagtactggcaggaacgttcagaagttaggatca
 Y   L   F   M   F   L   A   I   R   S   T   G   R   N   V   Q   K   L   G   S
acctataaccggaagtcgaatgtttcacagtcagtgaagagtgaatggaagttagcaaaa
 T   Y   N   R   K   S   N   V   S   Q   S   V   K   S   E   W   K   L   A   K
attgcctttgtggctattgtggtgtttgttctgtcctggtctccctatgctta
 I   A   F   V   A   I   V   V   F   V   L   S   W   S   P   Y   A
```

Homo sapiens	53%
Mus musculus	54%
Rattus norvegicus	53%
Gallus gallus	80%
Xenopus laevis	75%
Gadus morhua	64%
Danio rerio	48%
Branchiostoma floridae	40%

C: Conserved sites disulfide bridge formation (Cys89, Cys167)

Y: aromatic residue conserved in all invertebrates (Tyr92)

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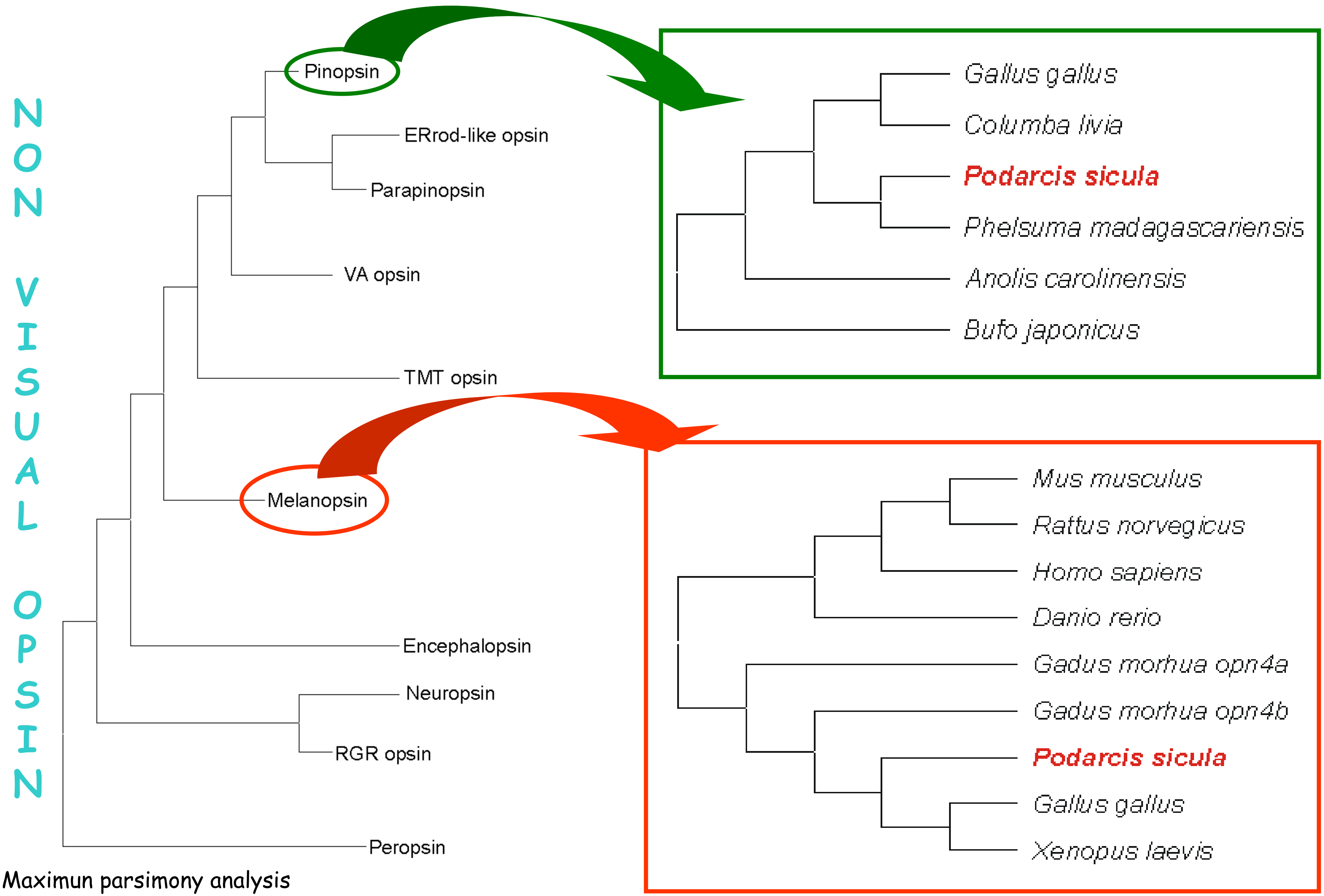

Maximun parsimony analysis

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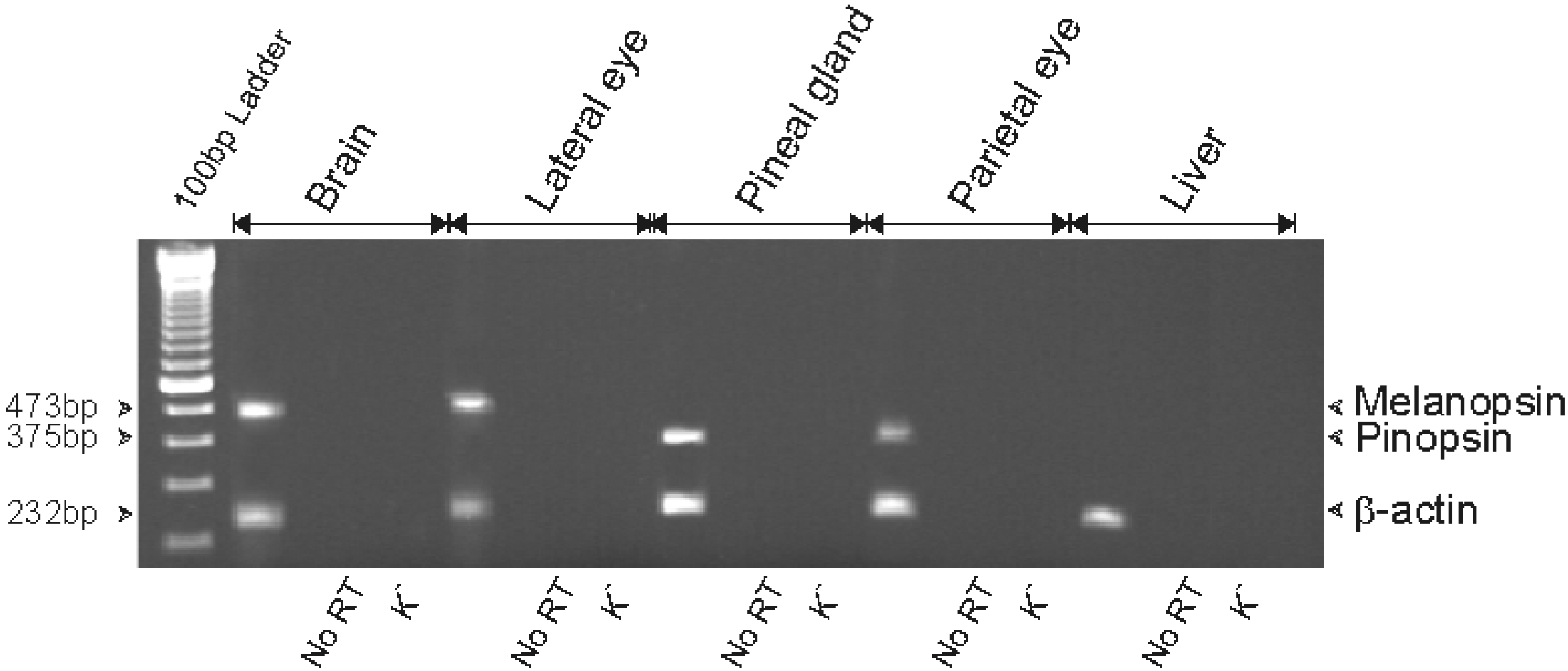

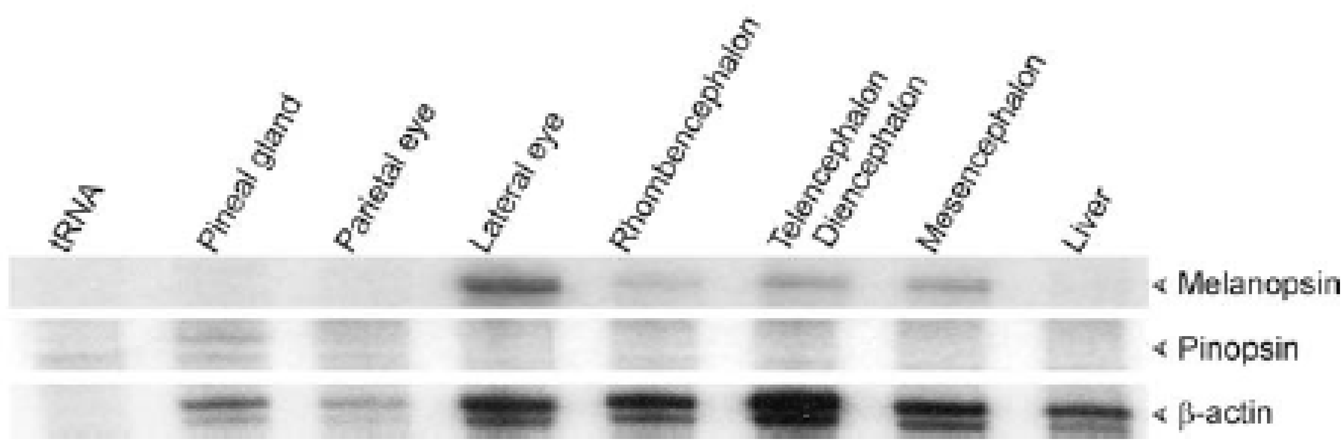

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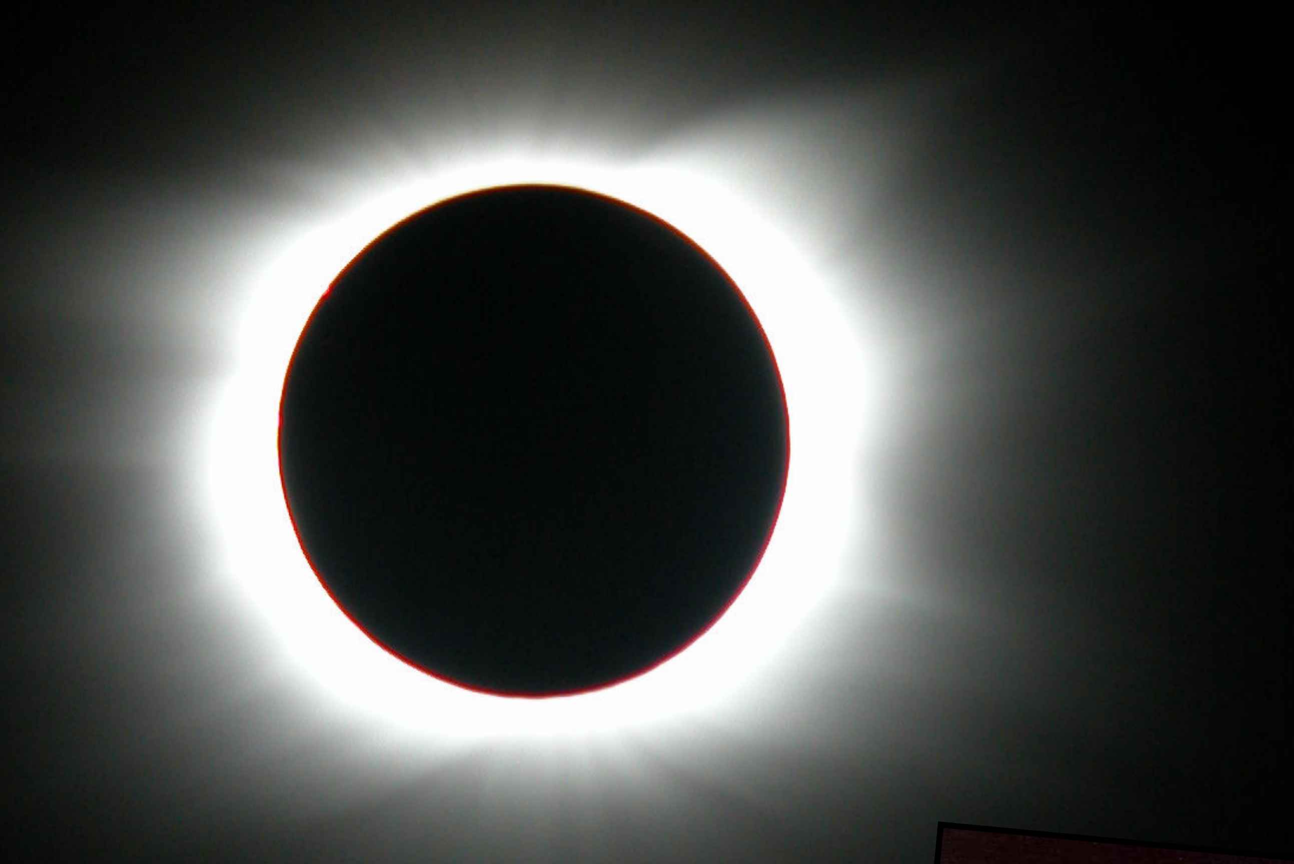
EVOLUTION IN THE DARK



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