VII Figures and legends

Figure 1. Diagrammatic representation of the development of the eye in a generalized vertebrate. A: The brain vesicles. The optic vesicle evaginates from the wall of the diencephalon and contacts the overlying ectoderm. B: The optic vesicle. The invagination of the optic vesicle leads to the development of an optic cup consisting of two layers. C: The optic cup becomes the multi-layered neural and a single-layered pigmented retinas as the lens is internalized. D: The structure of the adult eye.

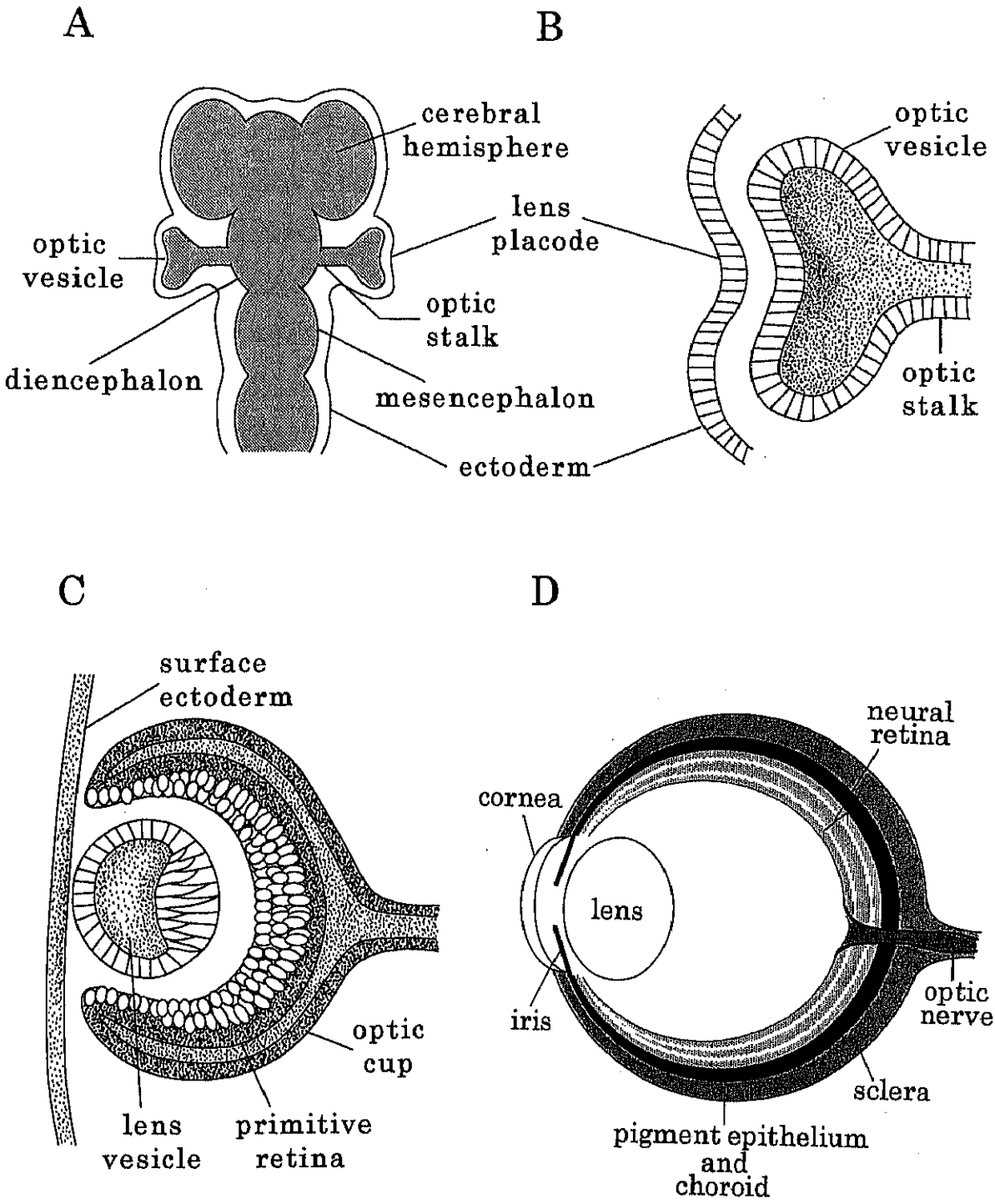

A
B
cerebral hemisphere
optic vesicle
lens placode
optic stalk
diencephalon
mesencephalon
ectoderm
optic vesicle
optic stalk
C
D
surface ectoderm
optic cup
lens vesicle
primitive retina
neural retina
cornea
lens
iris
optic nerve
sclera
pigment epithelium and choroid

Figure 2. Schematic diagram showing common retinal layers and synaptic relationships in the vertebrate retina. PCL, pigment cell layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; R, rods; C, cones; H, horizontal cells; B, bipolar cells; A, amacrine cells; G, ganglion cells; M, Müller cells.

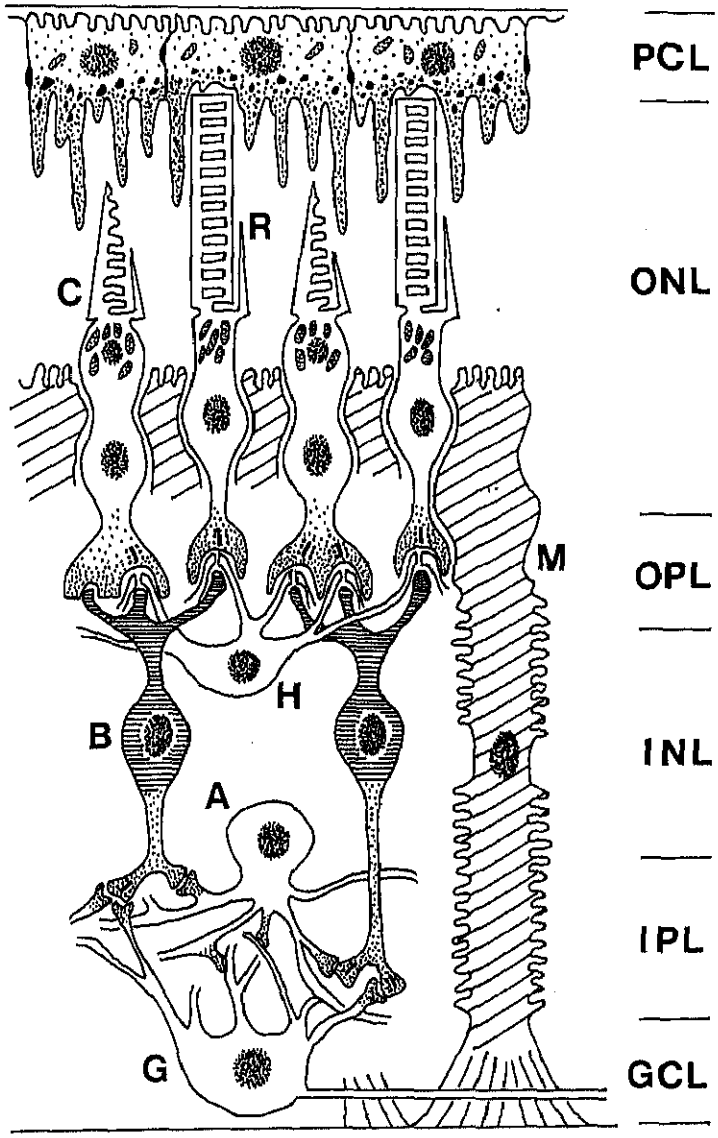

PCL
ONL
OPL
INL
IPL
GCL
R
C
M
H
B
A
G

Figure 3. Schematic diagram of a generalized synaptic neurotransmitter/receptor system. The neurotransmitter, stored in synaptic vesicles, is secreted by exocytosis in response to depolarizing electrical signals at a specialized region of the nerve terminal. The released neurotransmitter interacts with a membrane-bound receptor in the post-synaptic cell, resulting in a brief transmembrane current.

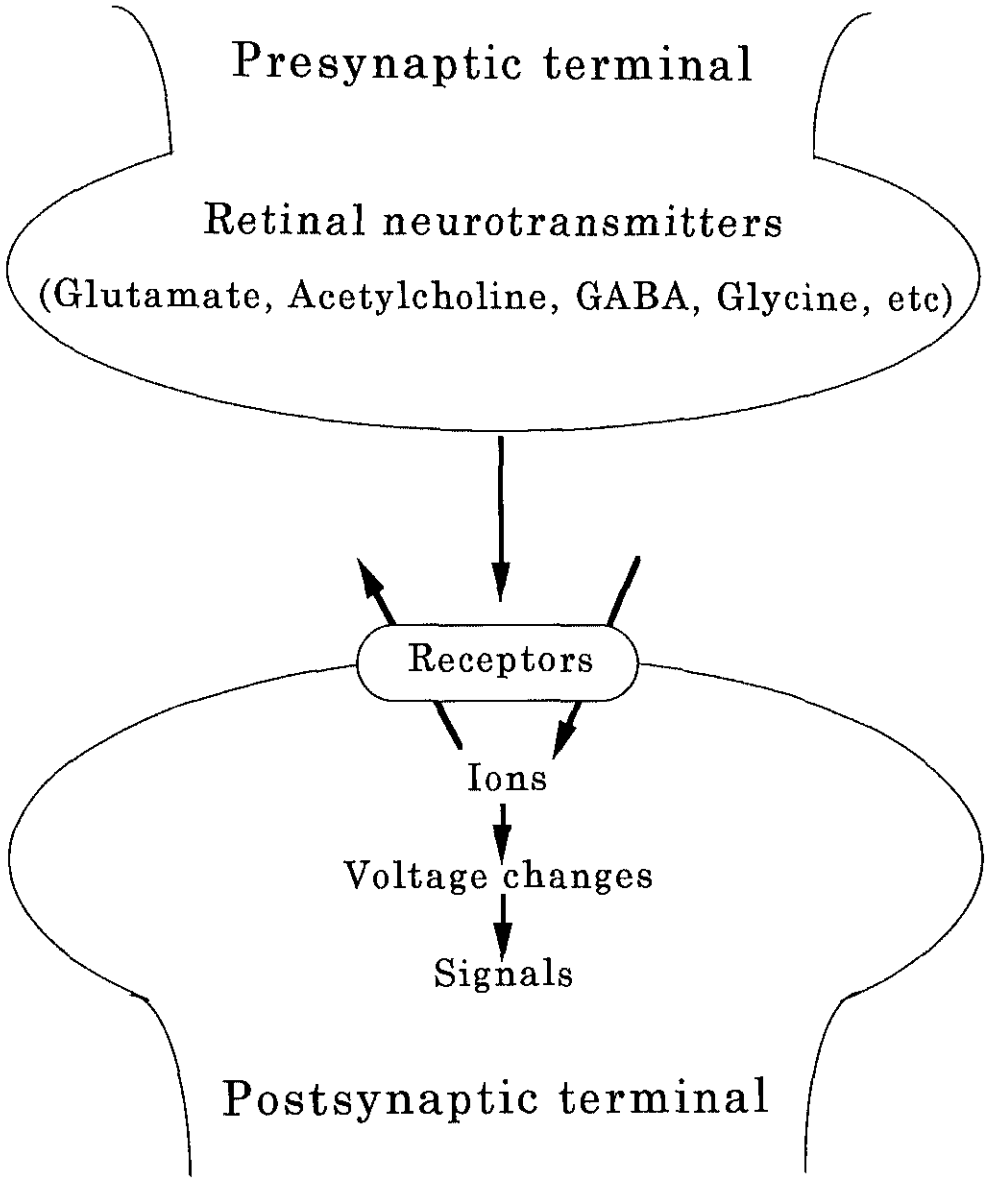

Presynaptic terminal
Retinal neurotransmitters
(Glutamate, Acetylcholine, GABA, Glycine, etc)
Receptors
Ions
Voltage changes
Signals
Postsynaptic terminal

Figure 4. Schematic diagram of the cholinergic synapse. ACh is synthesized in the pre-synaptic terminal by ChAT and packaged in membrane-bound vesicles. ACh is released in response to depolarization of the terminal and diffuses across the synaptic cleft to interact with receptor molecules on the surface of the post-synaptic cell. ACh is broken down by the hydrolyzing enzyme AChE, which is also localized on the external surface of the post-synaptic cell. ACh, acetylcholine; ChAT, choline acetyltransferase; AChRs, acetylcholine receptor; AChE, acetylcholinesterase.

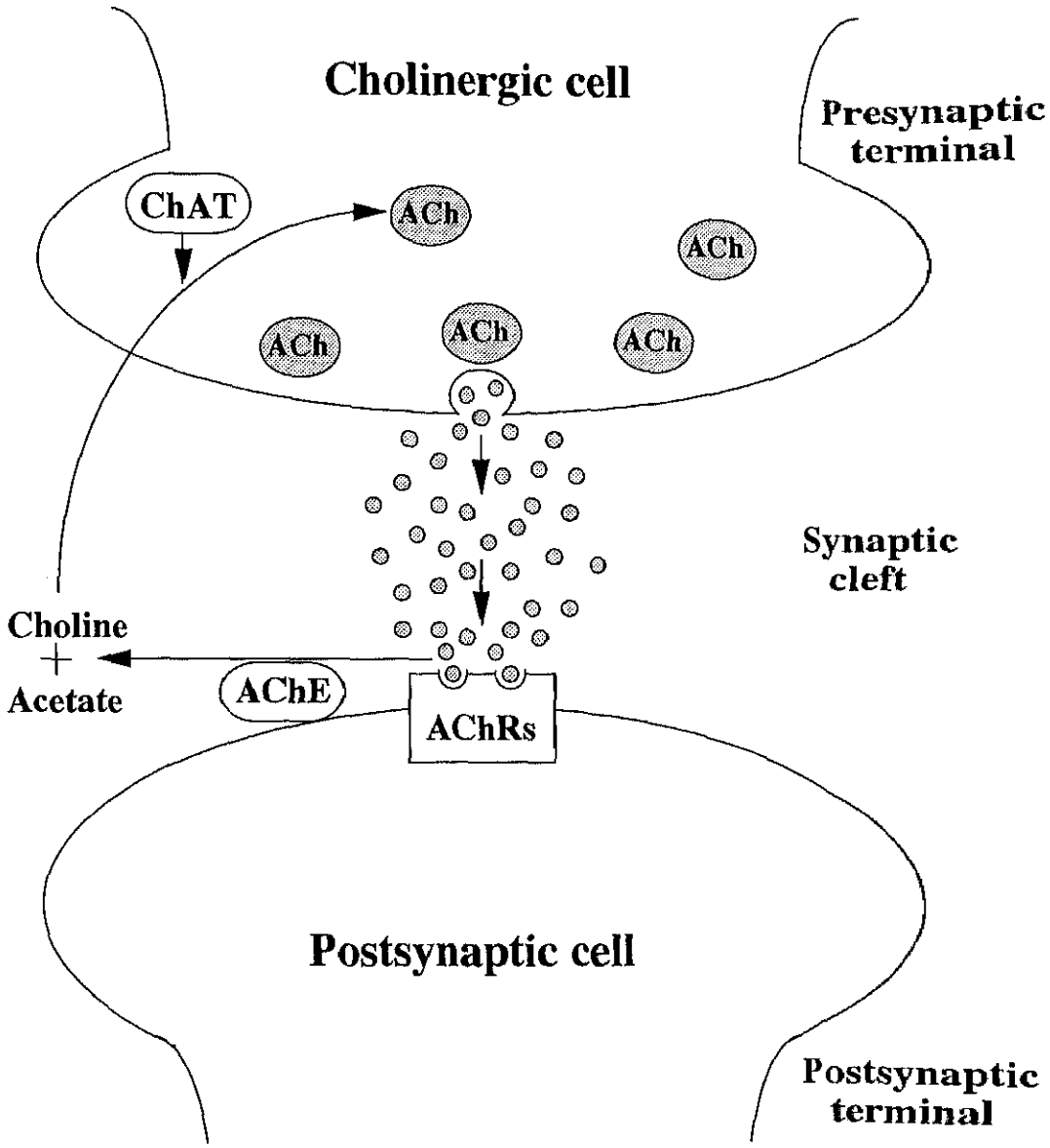

Cholinergic cell
Presynaptic terminal
ChAT
ACh
ACh
ACh
ACh
ACh
Synaptic cleft
Choline
Acetate
AChE
AChRs
Postsynaptic cell
Postsynaptic terminal

Figure 5. Sequence of steps in the immunocytochemical localization of ChAT, mAChRs and nAChRs in the adult newt retina.

Newt eyeball was removed from the orbit

⬇

Fixed in 4% paraformaldehyde in 0.1 M PB at pH 7.4
12 h/ 4℃

⬇

Equilibrated in 30% sucrose in 0.1 M PB at pH 7.4
12 h/ 4℃

⬋ ⬊

Wholemount preparation or Microtome cryosection (17 μm)

⬊ ⬋

Incubations in the primary antibody
[anti-ChAT (1:100), mAChRs (1:200), nAChRs (1:200) in a mixture of 3% normal rabbit serum or normal goat serum, 5% bovine serum albumin, and 0.3% TritonX-100 in 0.02 M PBS]
4 days (wholemount), 2 days (cryosection)/ 4℃

⬇

Incubations in the secondary antibody
[affinity purified rabbit biotinylated anti-goat IgG (H+L), affinity purified goat biotinylated anti-rat IgG (H+L)]
2 days (wholemount), 1.5 h (cryosection)/ R.T

⬇

Incubated in the Avidin and Biotin Complex
2 days (wholemount), 1.5 h (cryosection)/ R.T

⬇

Incubated in the DAB substrate Kit
3~10 min/ R.T

⬇

Terminated by immersing the tissue in ice-cold PBS

⬇

Mounted in glycerine-PBS solution

⬇

Examined under light microscopy

Figure 6. Sequence of steps in the histochemical localization of α-BTX binding sites in the adult newt retina.

Newt eyeball was removed from the orbit

⬇

Fixed in 4% paraformaldehyde in 0.1 M PB at pH 7.4
12 h/ 4℃

⬇

Equilibrated in 30% sucrose in 0.1 M PB at pH 7.4
12 h/ 4℃

⬇

Microtome cryosection (17 μm)

⬇

Washed in 0.02 M PBS, 1 % Triton X-100 in 0.02 M PBS,
and 0.02 M PBS / 15 min each

⬇

Incubations in the α-BTX binding solution
(10 μM biotin-conjugated α-BTX, 2 mg/ml
bovine serum albumin in 0.1 M PB
12h/R.T

⬇

Incubated in the Avidin and Biotin Complex
1.5 h/ R.T

⬇

Incubated in the DAB substrate Kit
3~10 min/ R.T

⬇

Terminated by immersing the tissue in ice-cold PBS

⬇

Mounted in glycerine-PBS solution

⬇

Examined under light microscopy

Figure 7. Sequence of steps in the histochemical localization of AChE in the adult newt retina.

Newt eyeball was removed from the orbit

⬇

Fixed in 4% paraformaldehyde in 0.1 M PB at pH 7.4
12 h/ 4℃

⬇

Equilibrated in 30% sucrose in 0.1 M PB at pH 7.4
12 h/ 4℃

⬇

Microtome cryosection (17 μm)

⬇

Washed in 0.1 M sodium acetate buffer (pH 6.0)
three washed/ 15 min each

⬇

Inhibit nonspecific cholinergic (butyrylcholinesterase, BChE)
activities[10^{-4} M tetraisopropylpyrophosphoramide (iso-OMPA)]
1 h/ R.T

⬇

Incubations in the AChE substrate solution
(2.56 mM acetylthiocholine iodide, 10^{-4} M iso-OMPA, 3.25 ml of 0.1 M sodium acetate buffer (pH 6.0), 0.25 ml of 0.1 M sodium citrate, 0.5 ml of 30 mM $CuSO_4$, 0.5 ml D.W, and 0.5 ml of 5 mM potassium ferricyanide)
2-3 h/ 37℃

⬇

Terminated by rinsing the tissue in 71 mM Na_2 SO_4
in 0.1 M sodium acetate buffer at pH 6.0
20 min/ 25℃

⬇

Mounted in glycerine-PBS solution

⬇

Examined under light microscopy

Figure 8. Cellular structure of the adult newt retina. A: Differential interference contrast photomicrograph of the retina in tangential section. B: Schematic drawing of the main neuronal layers. This drawing is based on observations of cells found in the newt retina by intracellular staining (modified from Niino 1993). PCL, pigment cell layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; P, photoreceptor cell; H, horizontal cell; B, bipolar cell; A, amacrine cell; G, ganglion cell. Scale bar=50 μm.

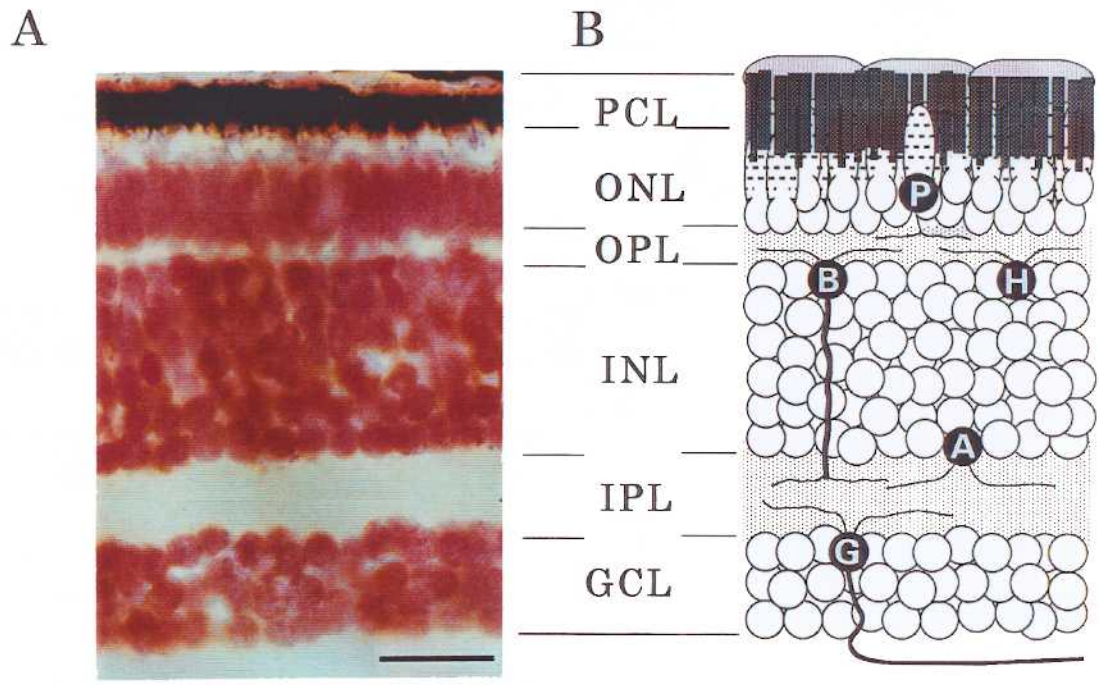

A
B
PCL
ONL
OPL
INL
IPL
GCL
P
B
H
A
G

Figure 9. Immunohistochemical localization of ChAT in the adult newt retina. Immunoreactivity is seen in conventional amacrine cells with somata at the INL/IPL border and in displaced amacrine cells with somata at the IPL/GCL border. A line indicate the IPL boundaries. Two ChAT-immunoreactive bands are seen within the IPL, at relative the depths of about 0-15% and 45-60% (defining the INL/IPL border as 0% and the IPL/GCL border as 100%). Non-specific staining can be seen in the inner segment of photoreceptors and in the OPL. PCL, pigment cell layer; RCL, receptor cell layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar=50 μm.

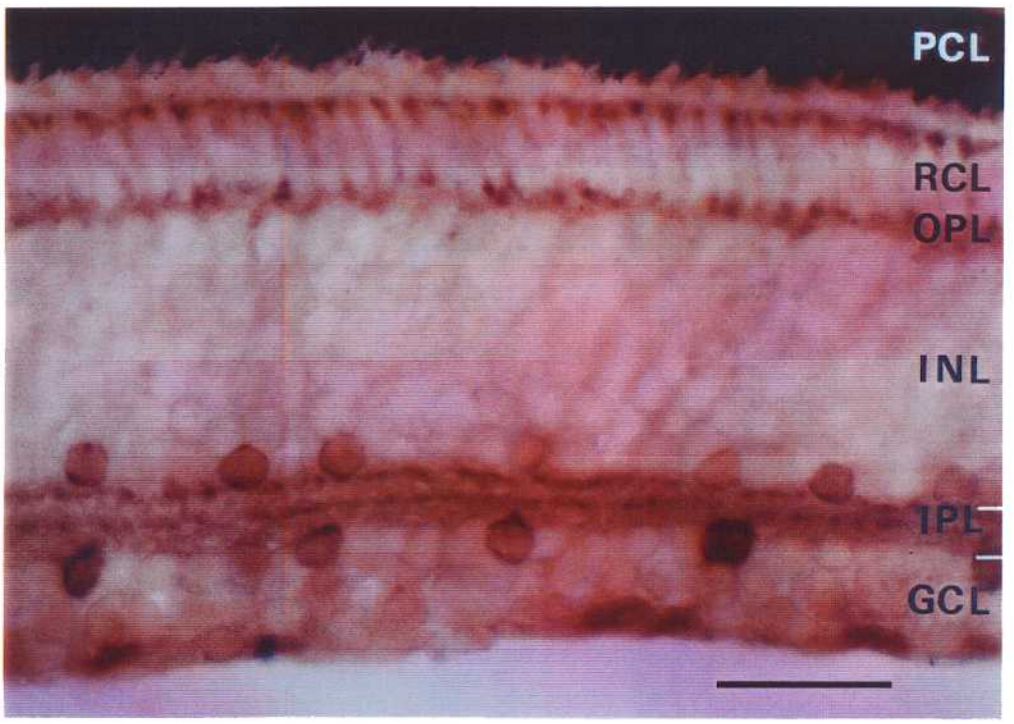

PCL
RCL
OPL
INL
IPL
GCL

Figure 10. Wholemount preparation of the adult newt retina showing immunohistochemical localization of ChAT. The same field is shown in each panel. A: Focus at the level of the GCL. B: Focus at the level of the INL. In each panel, arrowheads show examples of displaced amacrine cells in the GCL and arrows show conventional amacrine cells in the INL. Scale bar=50 μm.

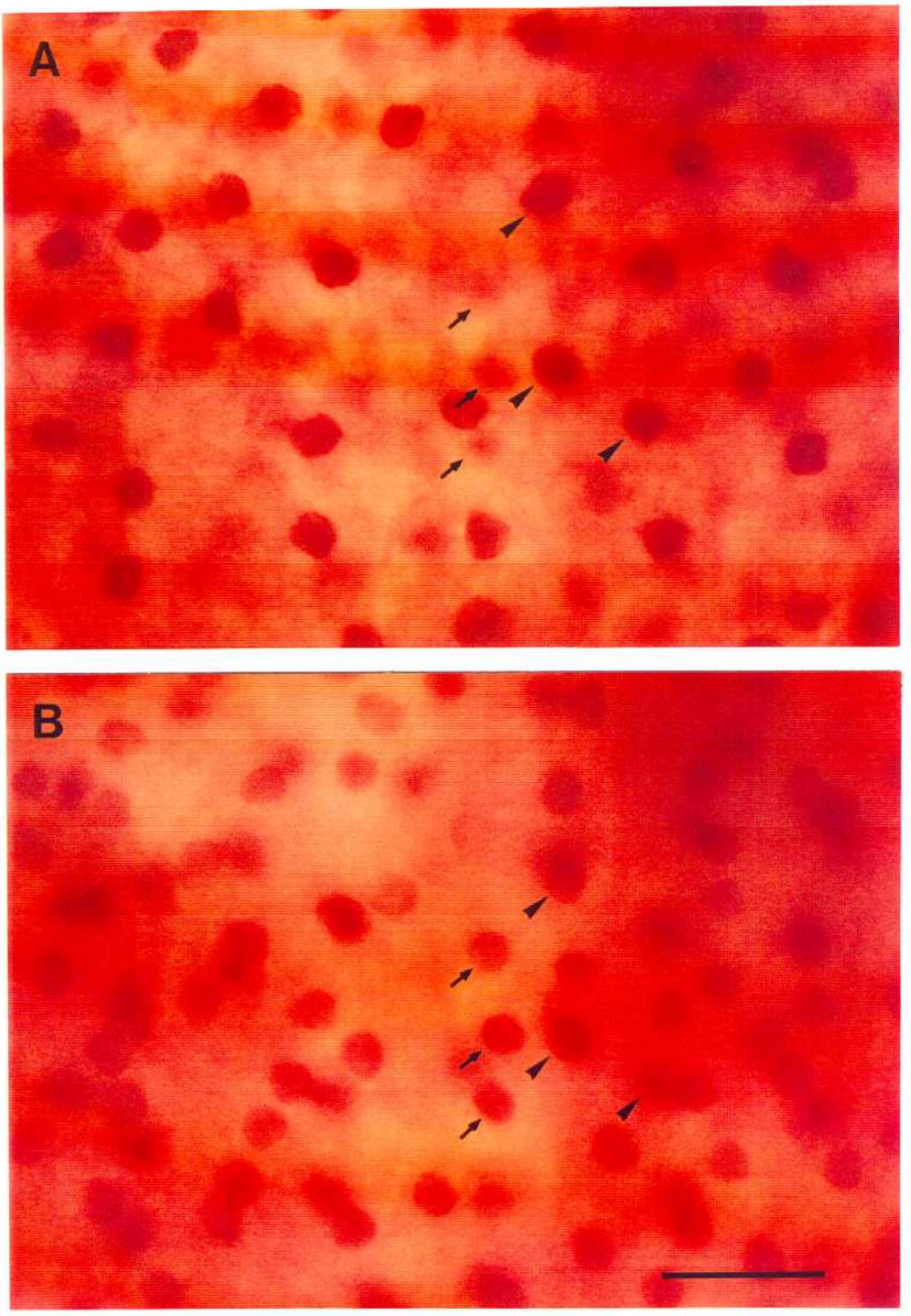

A
B

Figure 11. Histochemical staining for AChE in adult newt retina. A and B were obtained after different incubation times in substrate staining solution. A: AChE reaction product is first detected in a single band extending from 20-40% of the total thickness (100%) of the IPL. B: With increasing incubation time AChE reaction product gradually became thicker in width and finally occupies one half of the IPL width. Two types of AChE-positive somata are distinguished in the INL; somata closely apposed to the INL/IPL border (arrow) and somata lying one or one-half cell body away from the INL/IPL border(arrowheads). Dotted lines indicate the IPL boundaries. Abbreviations same as in Fig. 9. Scale bar=50 μm.

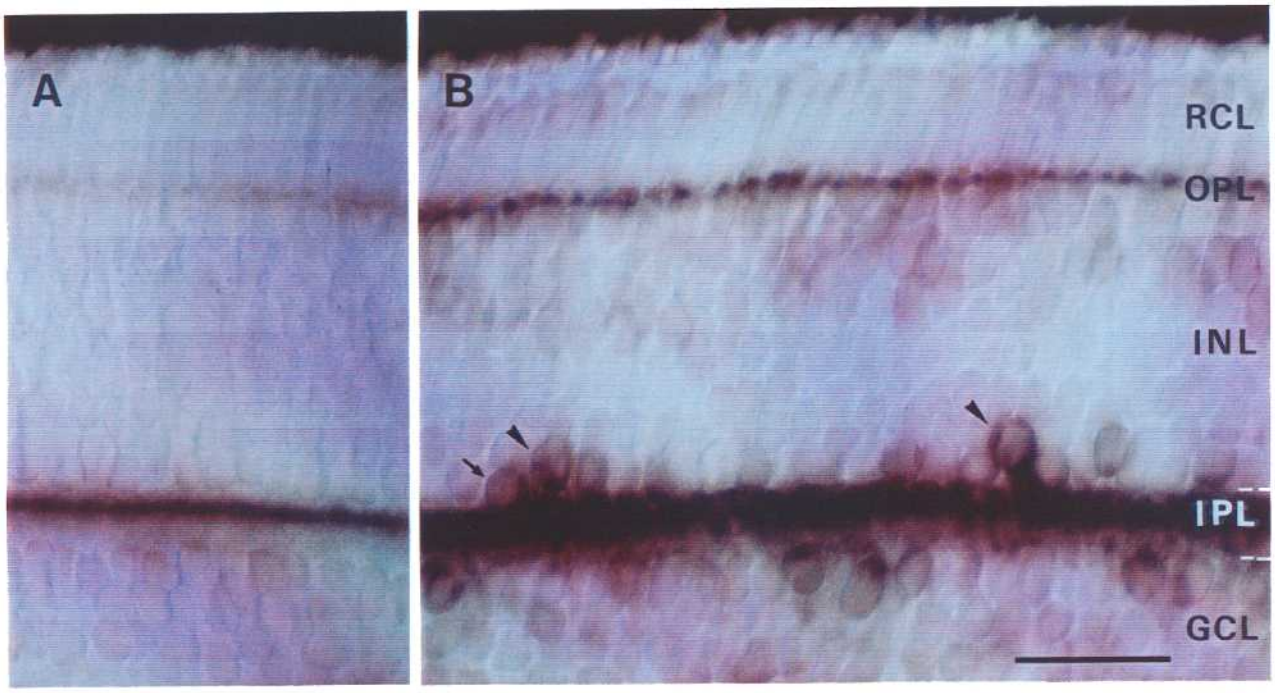

A
B
RCL
OPL
INL
IPL
GCL

Figure 12. Immunohistochemical localization of the mAChR subtype m2 in the adult newt retina. Intense mAChR immunoreactivity is detectable in ganglion cells. Two types of mAChR-immunoreactive somata are distinguished in the INL with somata closely apposed to the INL/IPL border (arrow) and somata lying one or two cell bodies away from the INL/IPL border (arrowhead). A line indicate the IPL boundaries. Two mAChR immunoreactive bands are seen within the IPL, at depths of 0-15% and 85-100%. The OPL and horizontal cells (double arrow) are very weakly stained. Abbreviations same as in Fig. 9. Scale bar=50 μm.

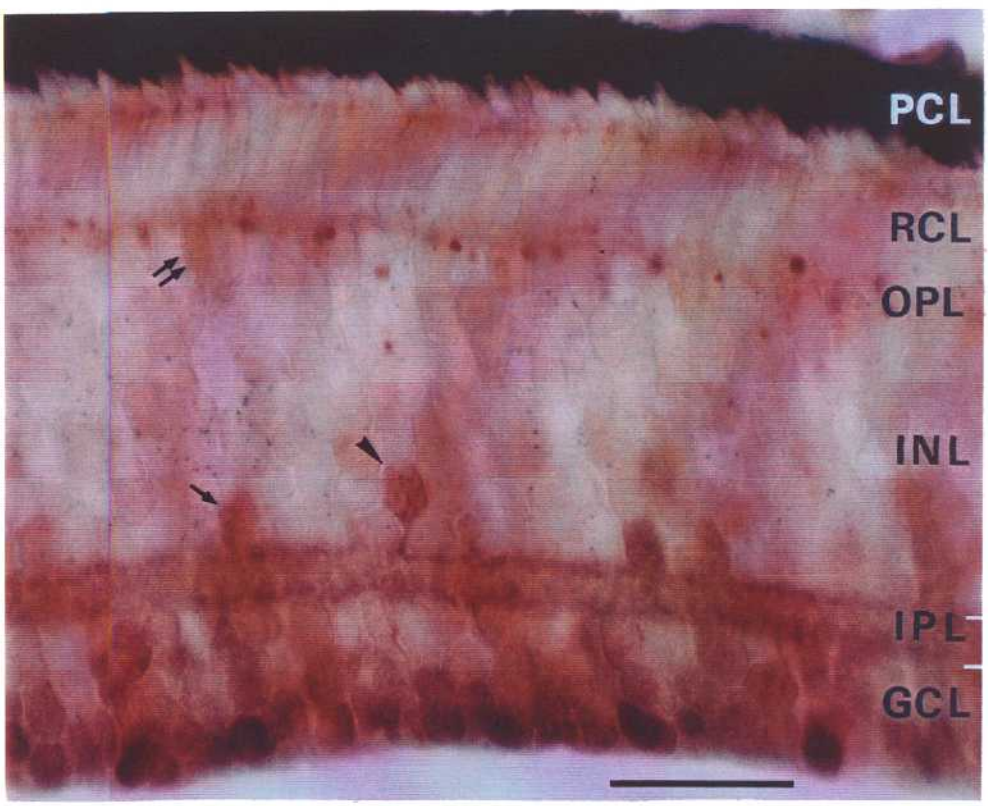

PCL
RCL
OPL
INL
IPL
GCL

Figure 13. Localizations of three nAChR markers in the adult newt retina. A: α-BTX binding sites. α-BTX binding sites are detectable in the OPL and the whole thickness of the IPL. B: Immunohistochemical localization of the α3 subunit of nAChRs. α3 subunit immunoreactivity is detectable in both of the OPL and IPL, and Müller cell-like endfeet (arrow). C: Immunohistochemical localization of the α8 subunit of nAChRs. α8 subunit immunoreactivity is detectable in both of the OPL and IPL, and radial processes (arrow) and endfeet of Müller cells (arrowhead). Abbreviations same as in Fig. 9. Scale bar=50 μm.

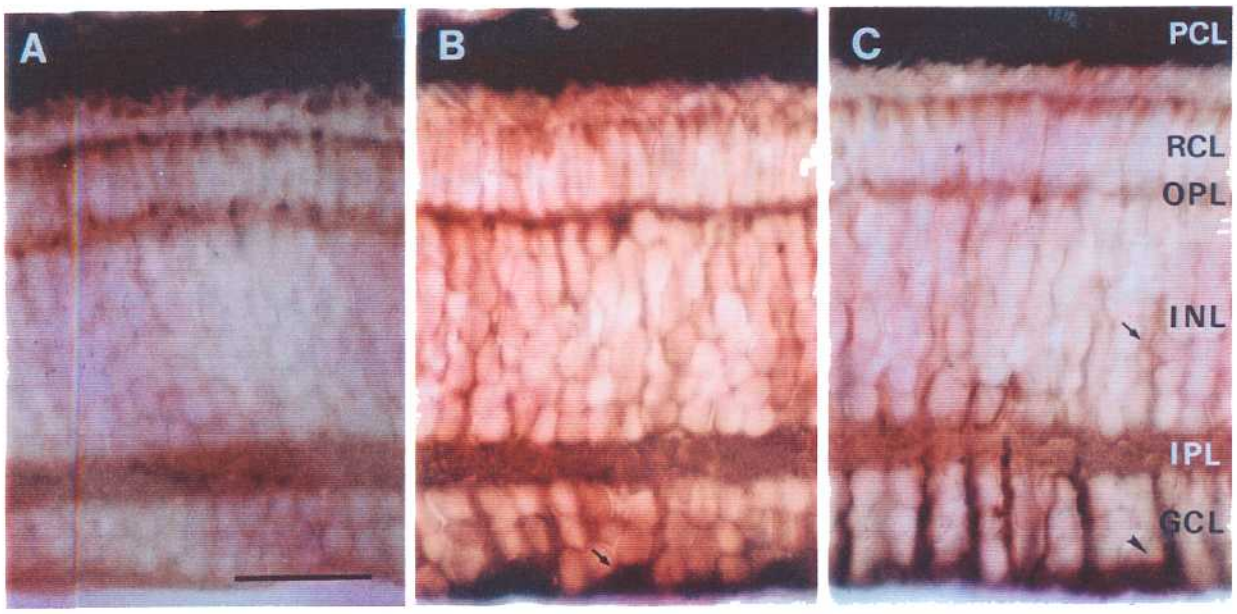

A
B
C
PCL
RCL
OPL
INL
IPL
GCL

Figure 14. Schematic diagram showing the relative distributions and relative concentrations of ChAT, AChE, mAChR, and nAChR in the IPL of the adult newt retina. The filled areas represent qualitatively increasing intensity of immunoreactivity or AChE activity. The numbers on the left indicate the depth of the IPL defining the INL/IPL border as 0% and the IPL/GCL border as 100%.

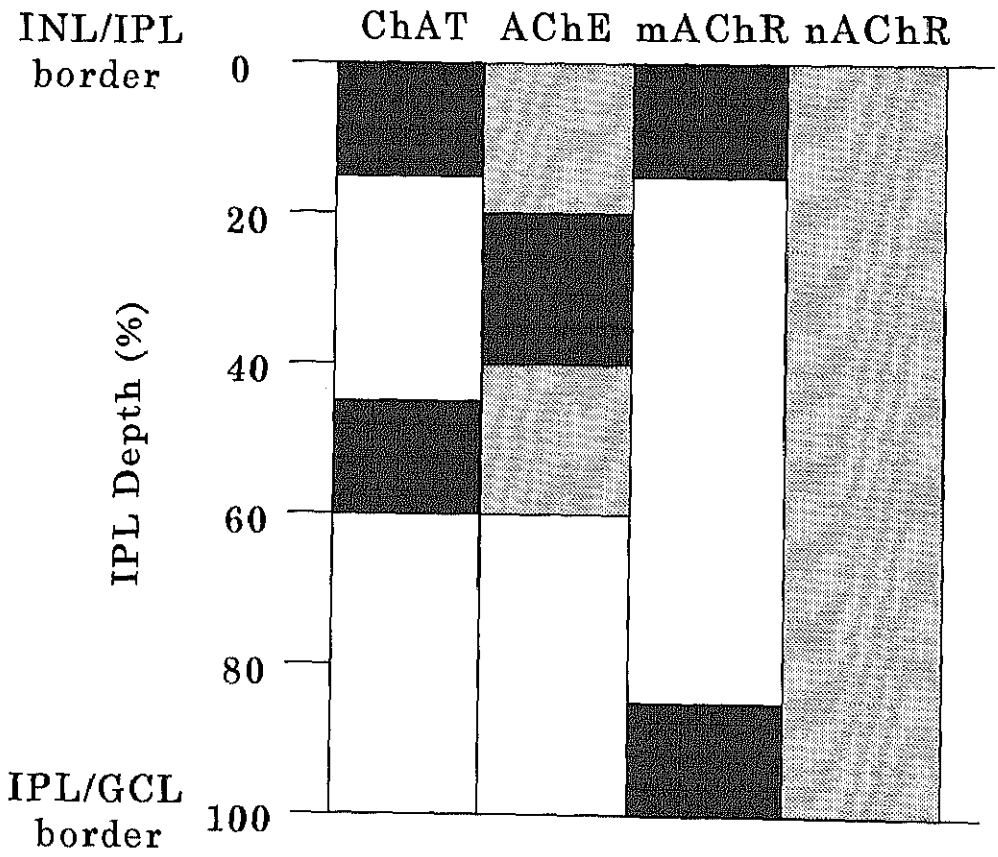

INL/IPL
border
ChAT
AChE
mAChR
nAChR
0
20
40
60
80
100
IPL Depth (%)
IPL/GCL
border

Figure 15. Histological sections of normal newt embryo at the level of the eye region. A: Transverse section of embryonic stage 26. A single-walled primary optic vesicle becomes transfered into the double-walled optic cup. B: Transverse section of embryonic stage 32. The lens is formed C: Transverse section of embryonic stage 39. D: Transverse section of embryonic stage 42. E, eye; L, lens; NP, neural plate. Scale bar=100 μm.

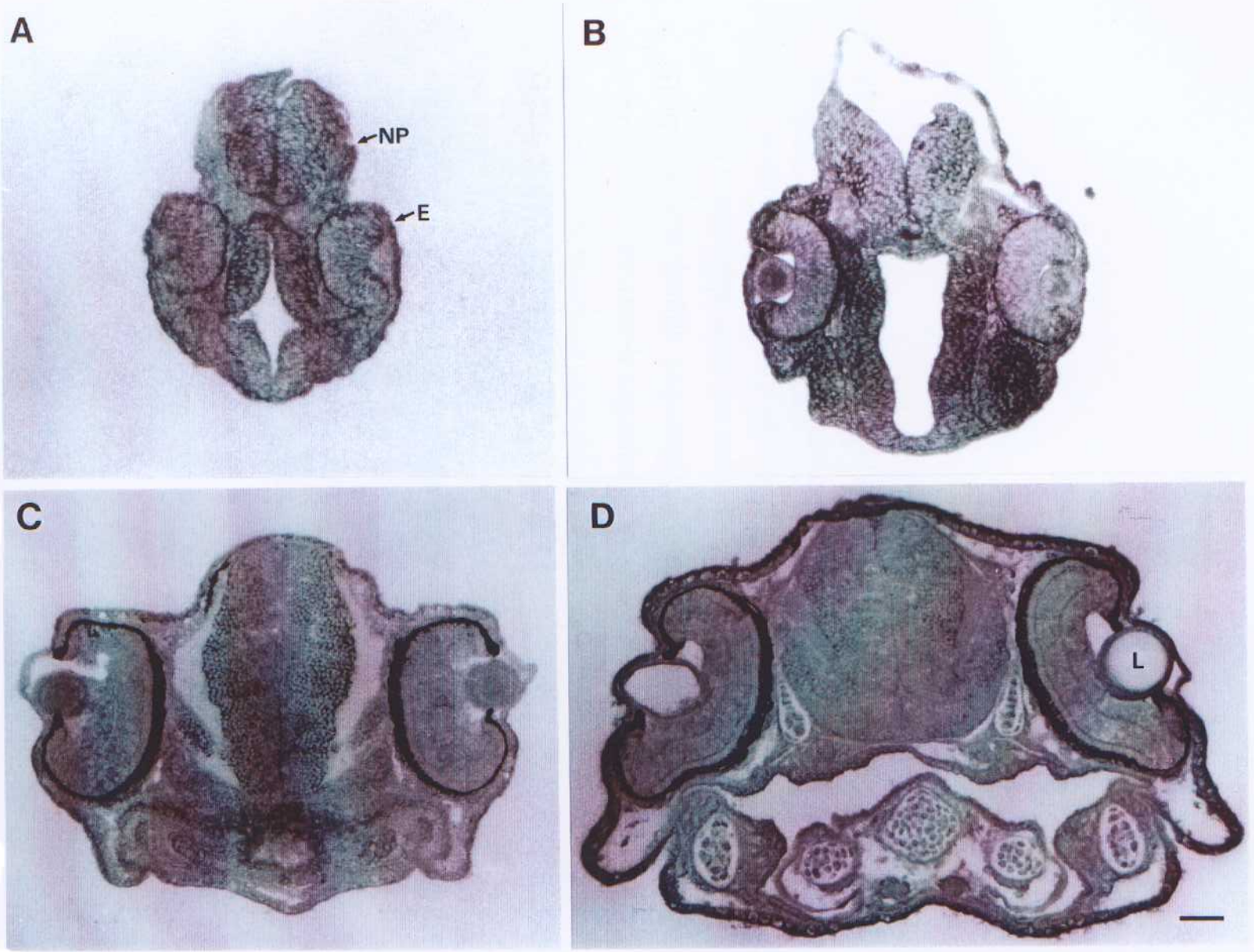

A
NP
E
B
C
D
L

Figure 16. Schematic diagram showing the stage of development of the newt retina. Developing retinas were divided into four stages on the basis of their morphological appearance. The neural retina at embryonic stages between 25 and 26 is a few cell thick, predominantly consisting of ovoidprogenitor cells. The retina at embryonic stages between 30 and 32, consists of progenitor cells and a thin row of round presumptive ganglion cells near the vitreal surface. The retina at embryonic stages between 36 and 39 is characterized by multilayered cells with no segregation of the synaptic layers. The retina at embryonic stages above 40 is a penta-laminar, having three nuclear layers and two synaptic layers. Abbreviations same as in Fig. 9.

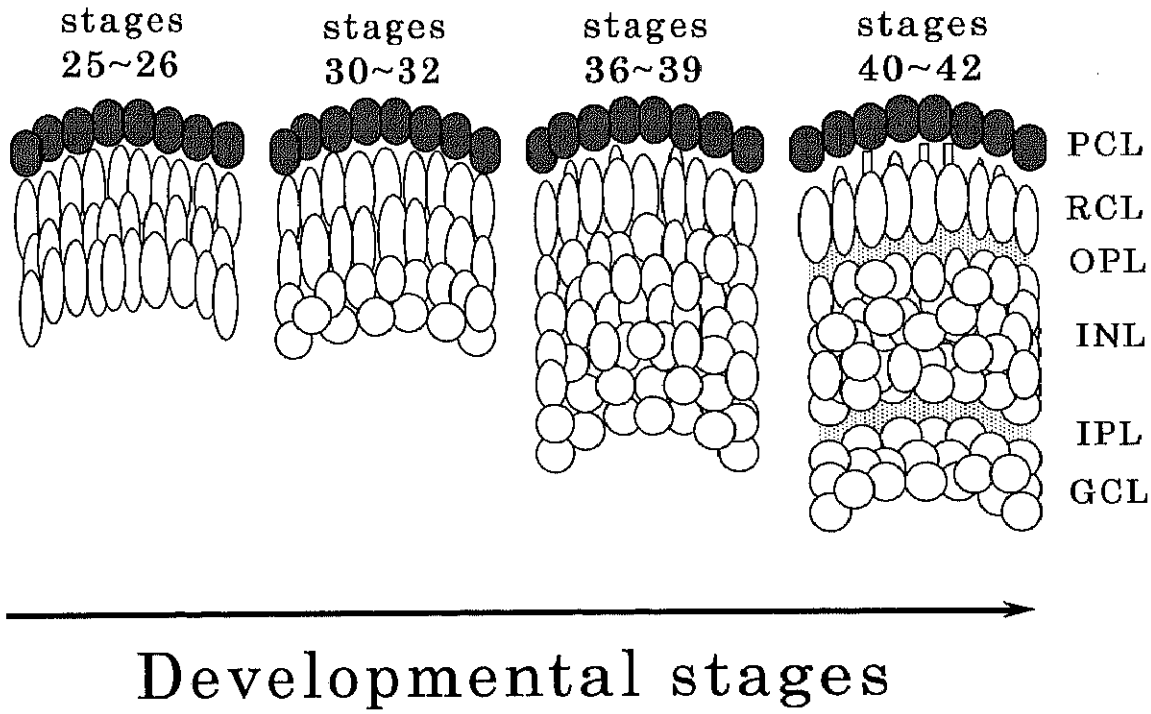

stages
25~26
stages
30~32
stages
36~39
stages
40~42
PCL
RCL
OPL
INL
IPL
GCL
Developmental stages

Figure 17. The time course of appearance of ChAT immunoreactivity in developing newt retina. A: An eye at embryonic stage 36. No ChAT immunoreactivity is seen. B: Stage 42. ChAT immunoreactivity is seen in the IPL, conventional amacrine cells (arrowhead), and displaced amacrine cells (arrow). C: Stage 46. D: Stage 49. Two ChAT immunoreactive bands are indicated by arrows. L, lens. Other abbreviations are the same as those in Fig. 9. Scale bar=50 μm.

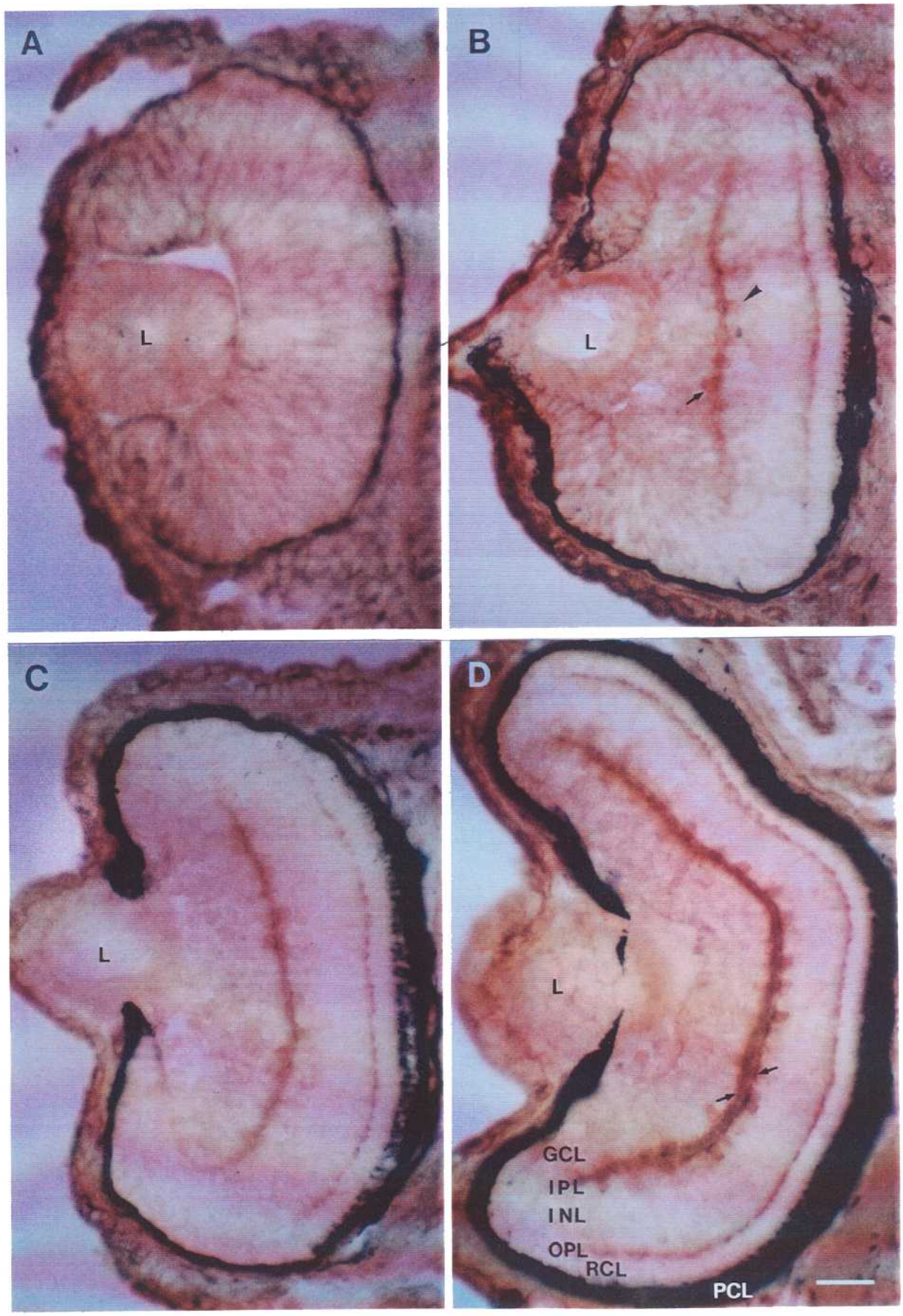

A
L
B
L
C
L
D
L
GCL
IPL
INL
OPL
RCL
PCL

Figure 18. The time course of appearance of AChE reaction product in developing newt retina. A: An eye at embryonic stage 31. The AChE-positive cells are seen in the most proximal region of the retina (arrowheads). B: Stage 36. AChE-positive cells are found at more distal levels of the retina.C: Stage 42. Two types of AChE-positive somata can be seen at the INL/IPL border (arrowhead) and the IPL/GCL border (arrow). At the same time, the OPL becomes weakly AChE-positive. D: Stage 49. The AChE staining pattern is almost identical to that in the mature retina. L, lens. Other abbreviations are the same as those in Fig. 9. Scale bar=50 μm.

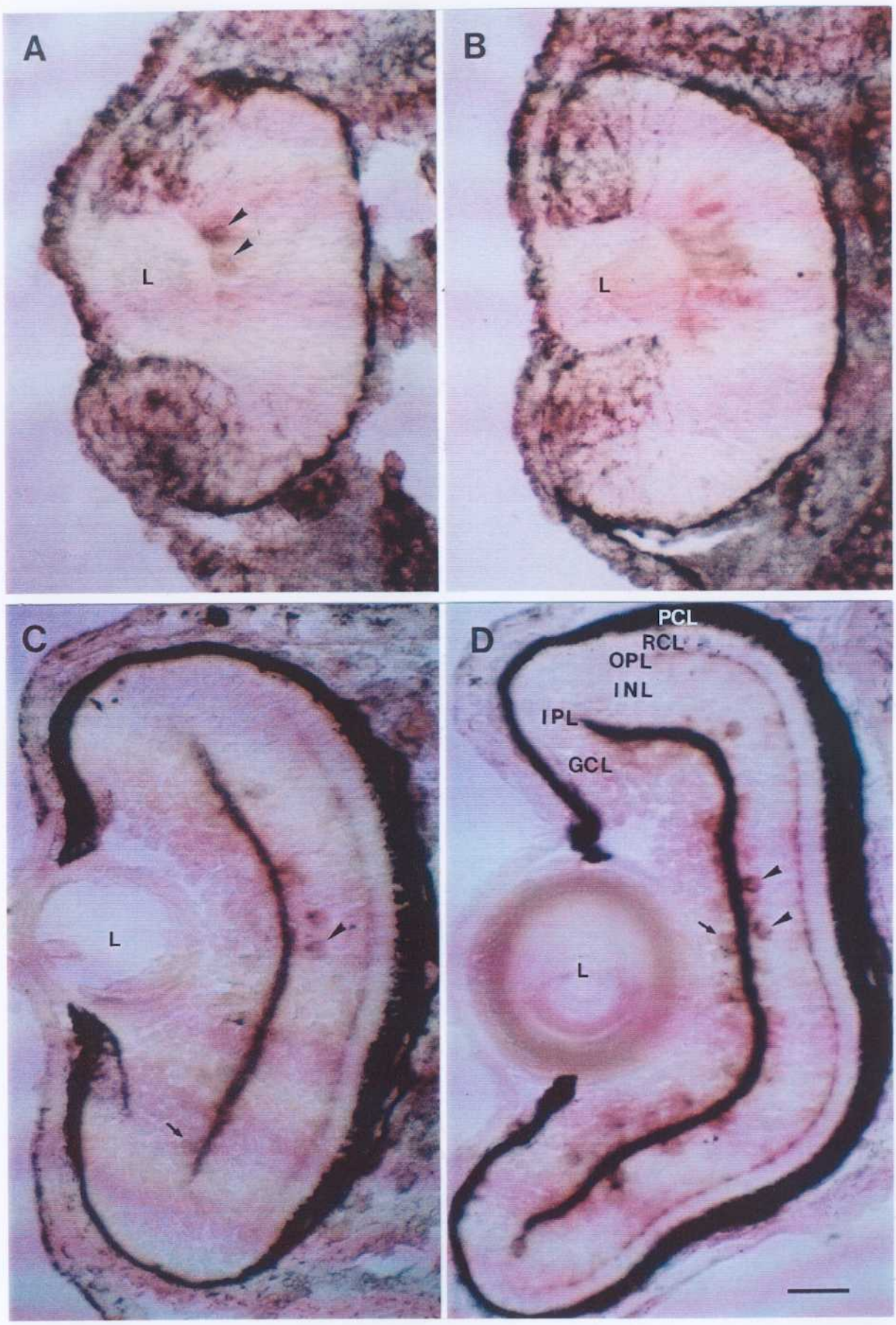

A
L
B
L
C
L
D
PCL
RCL
OPL
INL
IPL
GCL
L

Figure 19. The time course of appearance of mAChR (m2)-immunoreactivity in developing newt retina. A: An eye at embryonic stage 31. mAChR immunoreactivity is seen in the most proximal region of the central retina (arrowheads). B: Stage 39. The mAChR immunoreactive round somata increase in number. C: Stage 42. Some somata in amacrine cell layer (arrowhead) and horizontal cell layer (arrows) are immunoreactive. D: Stage 49. The immunoreactivity of horizontal cell-like somata becomes very weak. L, lens. Other abbreviations are the same as those in Fig. 9. Scale bar=50 μm.

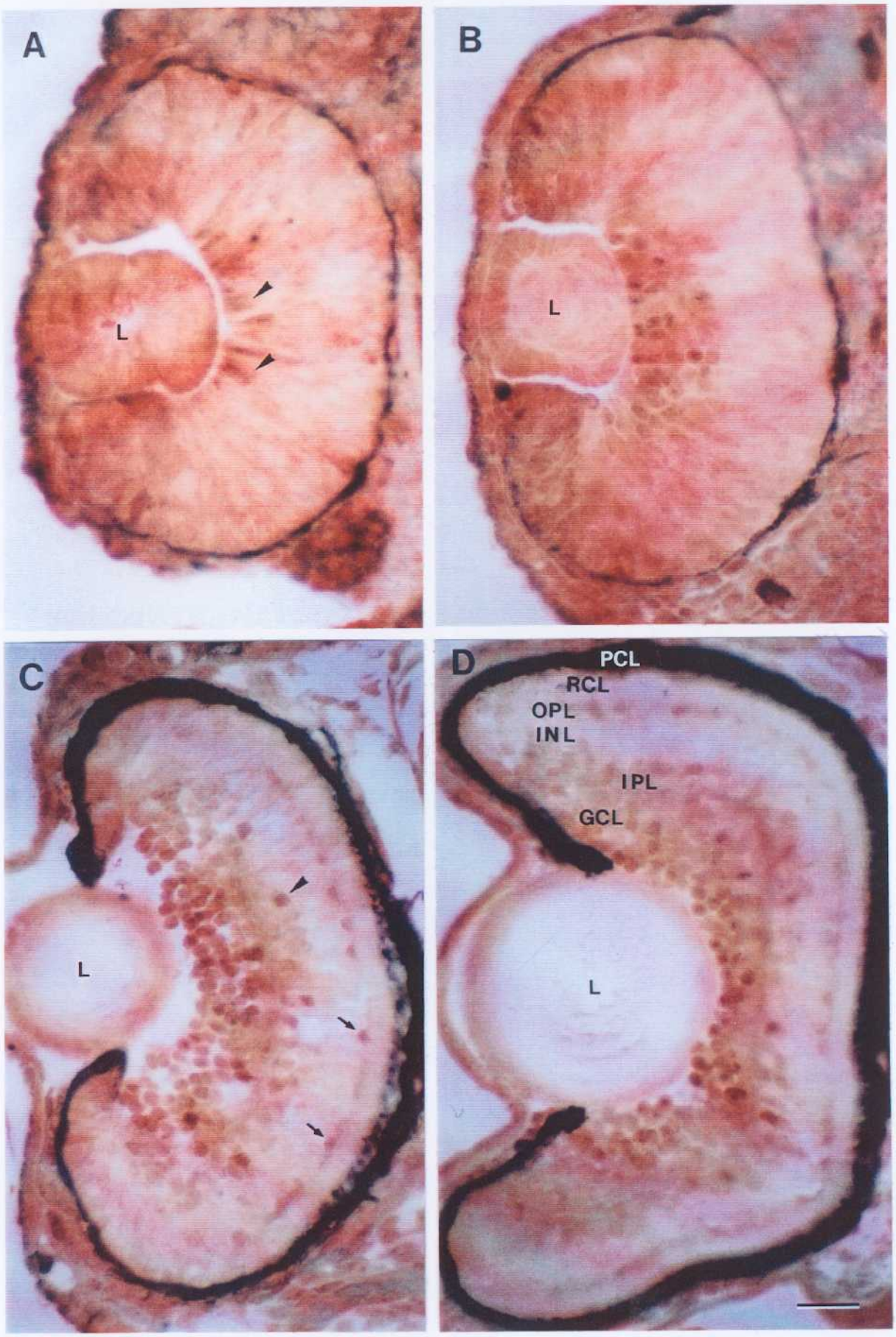

A
L
B
L
C
L
D
PCL
RCL
OPL
INL
IPL
GCL
L

Figure 20. The time course of appearance of α-BTX binding sites in developing newt retina. A: An eye at embryonic stage 31. No α-BTX binding sites are seen. B : An eye at embryonic stage 42. The α-BTX binding sites were seen in the IPL (arrowhead) and the OPL (arrow). C: An eye at embryonic stage 46. D : An eye at embryonic stage 49. The α-BTX binding pattern is almost identical to that in the adult retina. L, lens. Other abbreviations are the same as those in Fig. 9. Scale bar= 50 μm.

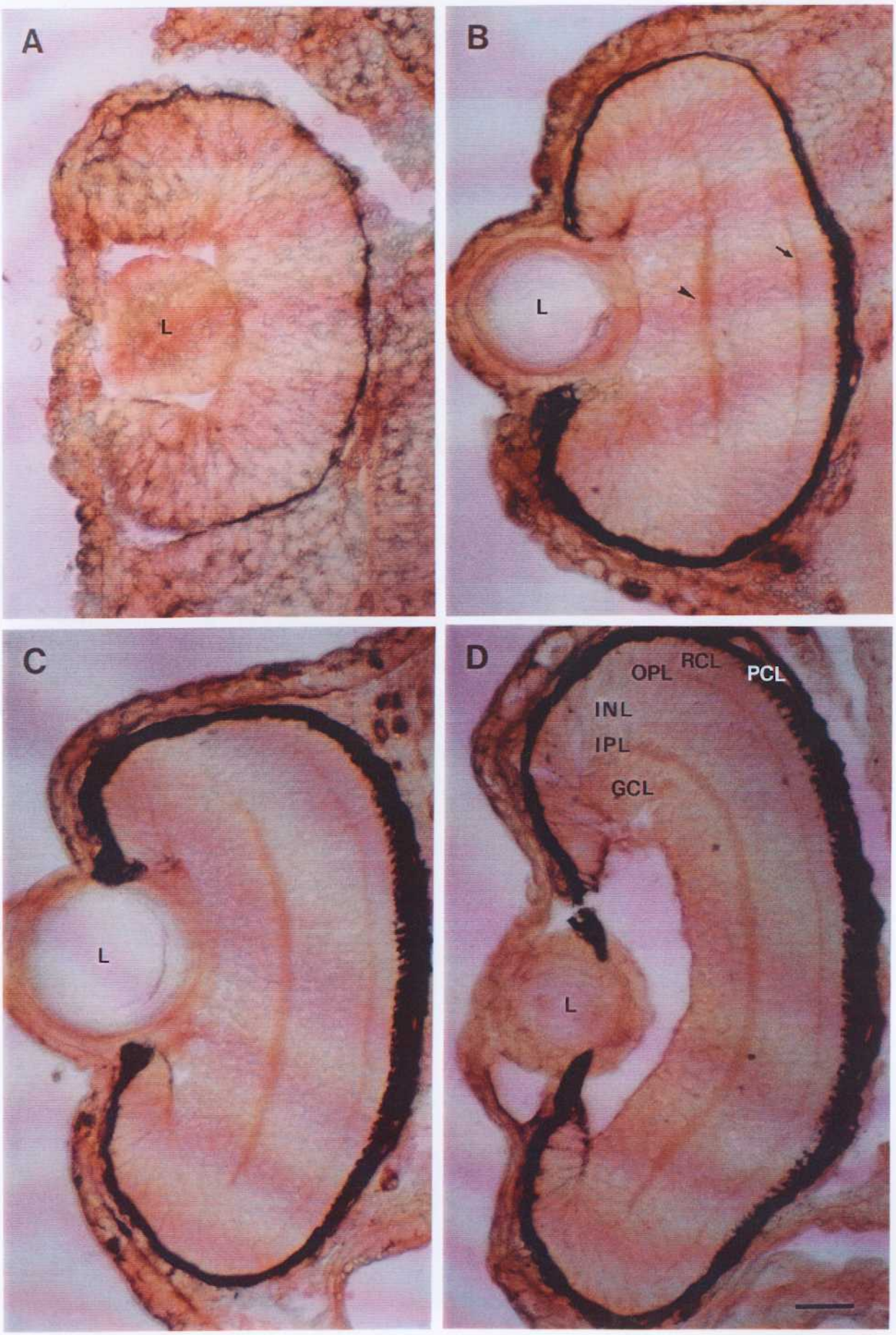

A
L
B
L
C
L
D
OPL
RCL
PCL
INL
IPL
GCL
L

Figure 21. The time course of appearance of α3 nAChR immunoreactivity in developing newt retina. A: An eye at embryonic stage 31. No α3 subunit immunoreactivity was detectable. B: An eye at embryonic stage 42. The presumptive IPL (arrowhead) and the OPL (arrow) became weakly α3-ir. C: An eye at embryonic stage 46. D: An eye at embryonic stage 49. α3-ir pattern is almost identical to that in the adult retina. An arrow indicates α3-ir Müller cell-like endfeet. L, lens. Other abbreviations are the same as those in Fig. 9. Scale bar=50 μm.

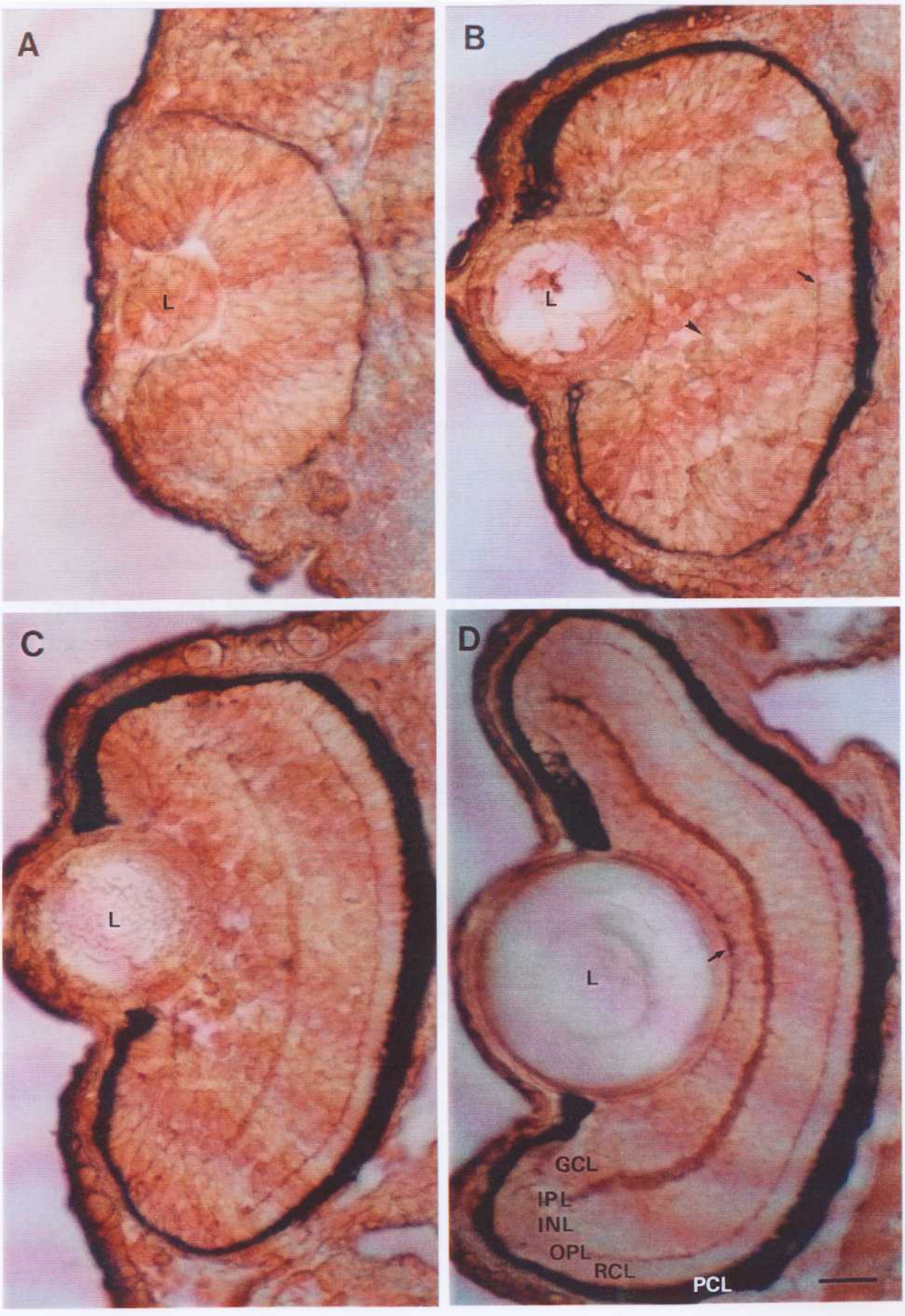
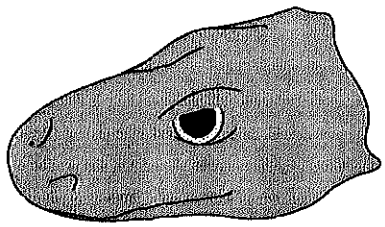
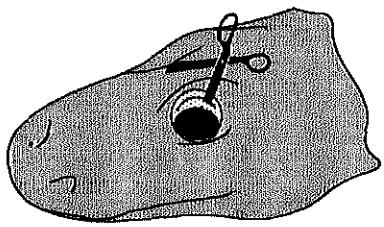

A
L
B
L
C
L
D
L
GCL
IPL
INL
OPL
RCL
PCL

Figure 22. Outline of operative procedures to surgical removal of the normal retina from the adult newt eye. After a deeply anesthetized animal was laid on a chamber (A), the dorsal half of the eye was cut open along the corneo-scleral junction (B). The retina and lens were removed (C), and then the eye flap consisting of iris and cornea was gently replaced at its original position (D).

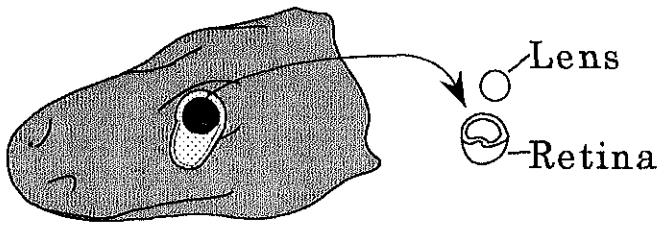
A



B



C



D

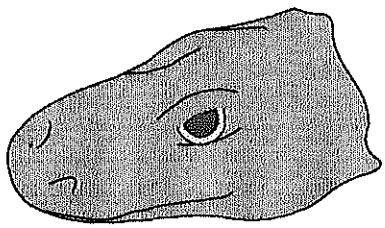


Figure 23. Schematic diagram showing retinal regeneration in newts following complete removal of the original retina. n 'early'-regenerating retina is 1-2 cells thick, and comprises pigmented cells and progenitor cells with nonpigmented cytoplasm. An 'intermediate-I'-regenerating retina is 3-4 cells thick, consisting predominantly of progenitor cells. An 'intermediate-II'-regenerating retina is 4-5 cells thick, consisting of a progenitor cell layer with only one row of rounded presumptive ganglion cells near the vitreal surface of the retina. An 'intermediate-III'-regenerating retina is 5-7 cells thick and corresponds to the stage just before or at the beginning of formation of the synaptic layers. A 'late'-regenerating retina has a penta-laminar structure, having three nuclear layers and two synaptic layers. Abbreviations same as in Fig. 9.

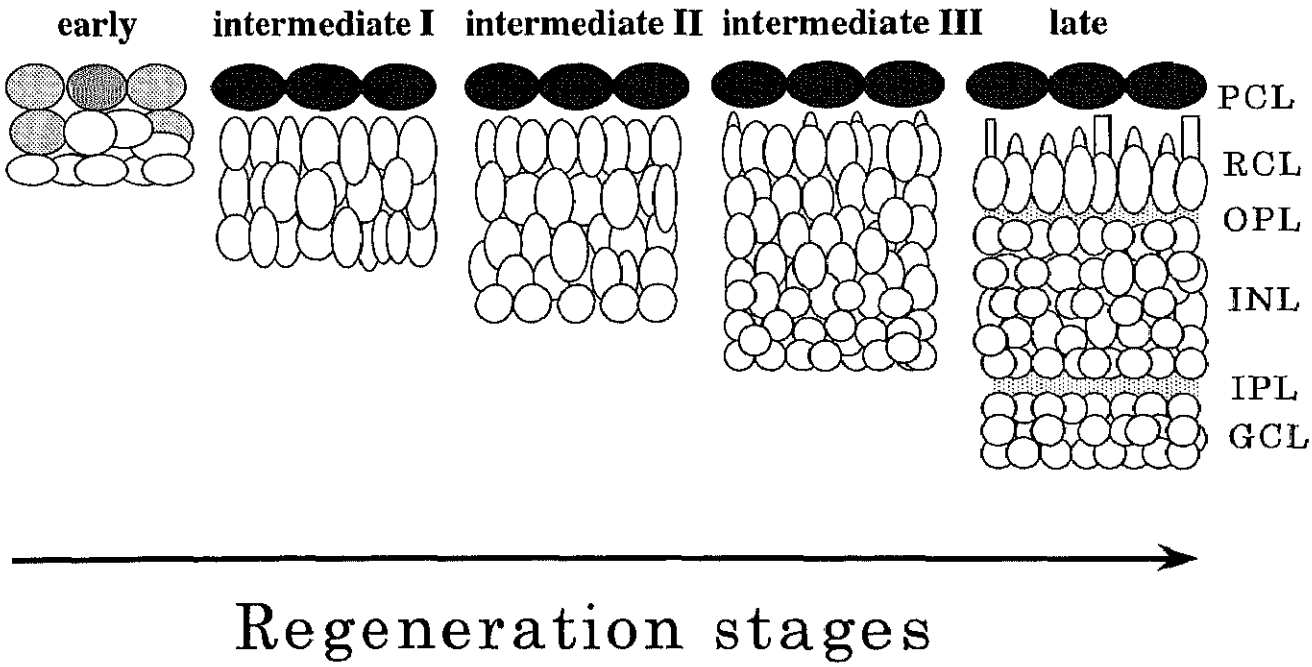

early
intermediate I
intermediate II
intermediate III
late
PCL
RCL
OPL
INL
IPL
GCL
Regeneration stages

Figure 24. The time course of appearance of ChAT immunoreactivity in regenerating newt retina. A: A retina at the intermediate-II stage. No ChAT immunoreactivity is seen. B: A retina at transition period between the intermediate-III stage and the late stage. ChAT-immunoreactive conventional amacrine cells and displaced amacrine cells are indicated by arrowheads and an arrow, respectively. C: A retina at early period of the late stage. D: A retina at later period of the late stage. Dotted lines indicate the IPL boundaries. Two ChAT-immunoreactive bands start to segregate (arrowheads). ChAT immunoreactive pattern is identical to that in the mature retina. Abbreviations same as in Fig. 9. Scale bar=50 μm.

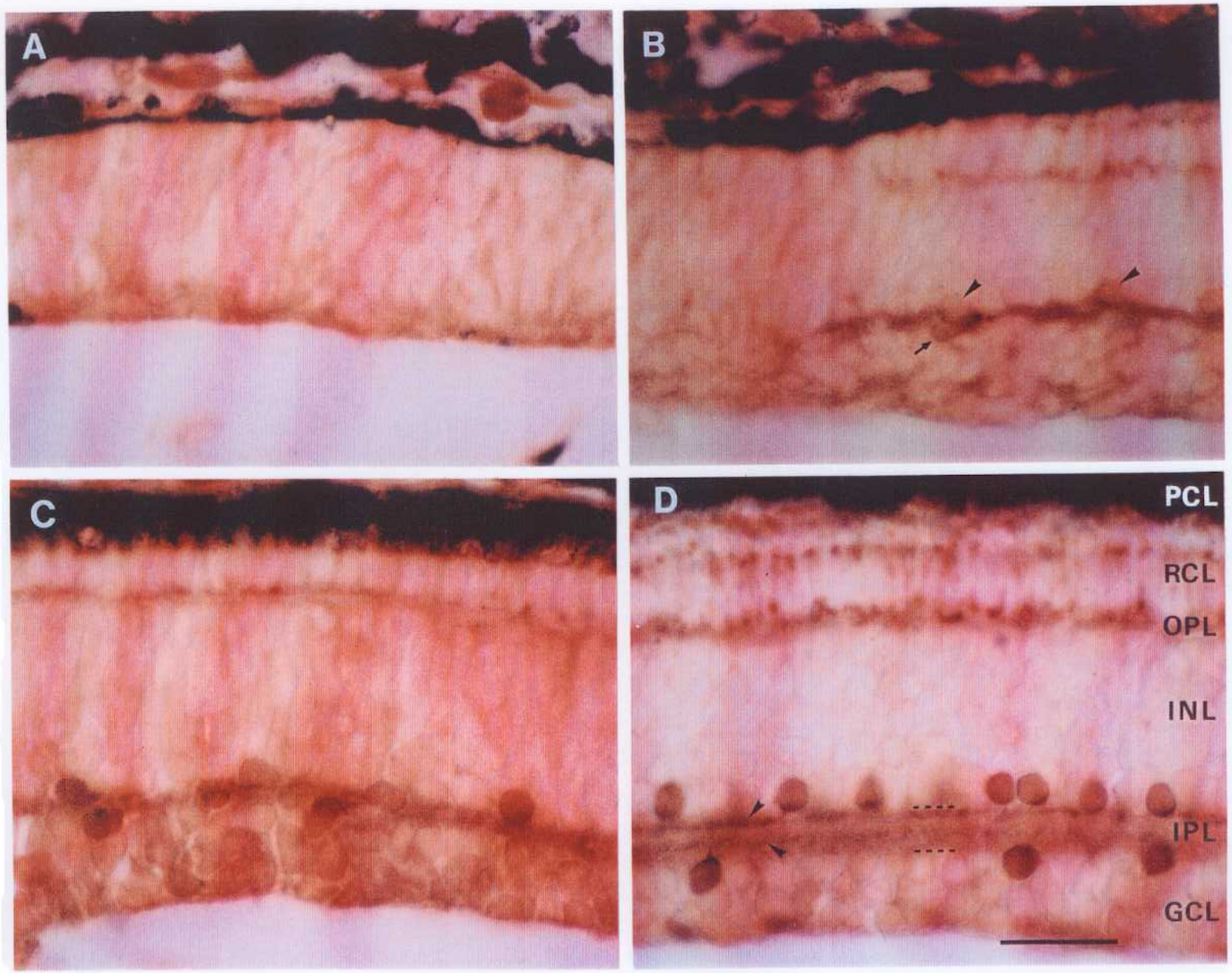

A
B
C
D
PCL
RCL
OPL
INL
IPL
GCL

Figure 25. The time course of appearance of AChE reaction product in regenerating newt retina. A: A retina at the intermediate-II stage. The AChE-positive cell in the most proximal region of the retina is indicated by an arrowhead. B: A retina at the intermediate-III stage. The AChE-positive cells are seen in the most proximal region of the retina (arrow) and at more distal levels (arrowhead). C: A retina at transition period between the intermediate-III stage and late stage. The presumptive IPL is indicated by an arrowhead. D: A retina at early period of the late stage. The AChE-positive IPL and OPL (arrow) are detectable. E: A retina at later period of the late stage. Dotted lines indicate the IPL boundaries. The AChE staining pattern is almost identical to that in the mature retina. Abbreviations same as in Fig. 9. Scale bar=50 μm.

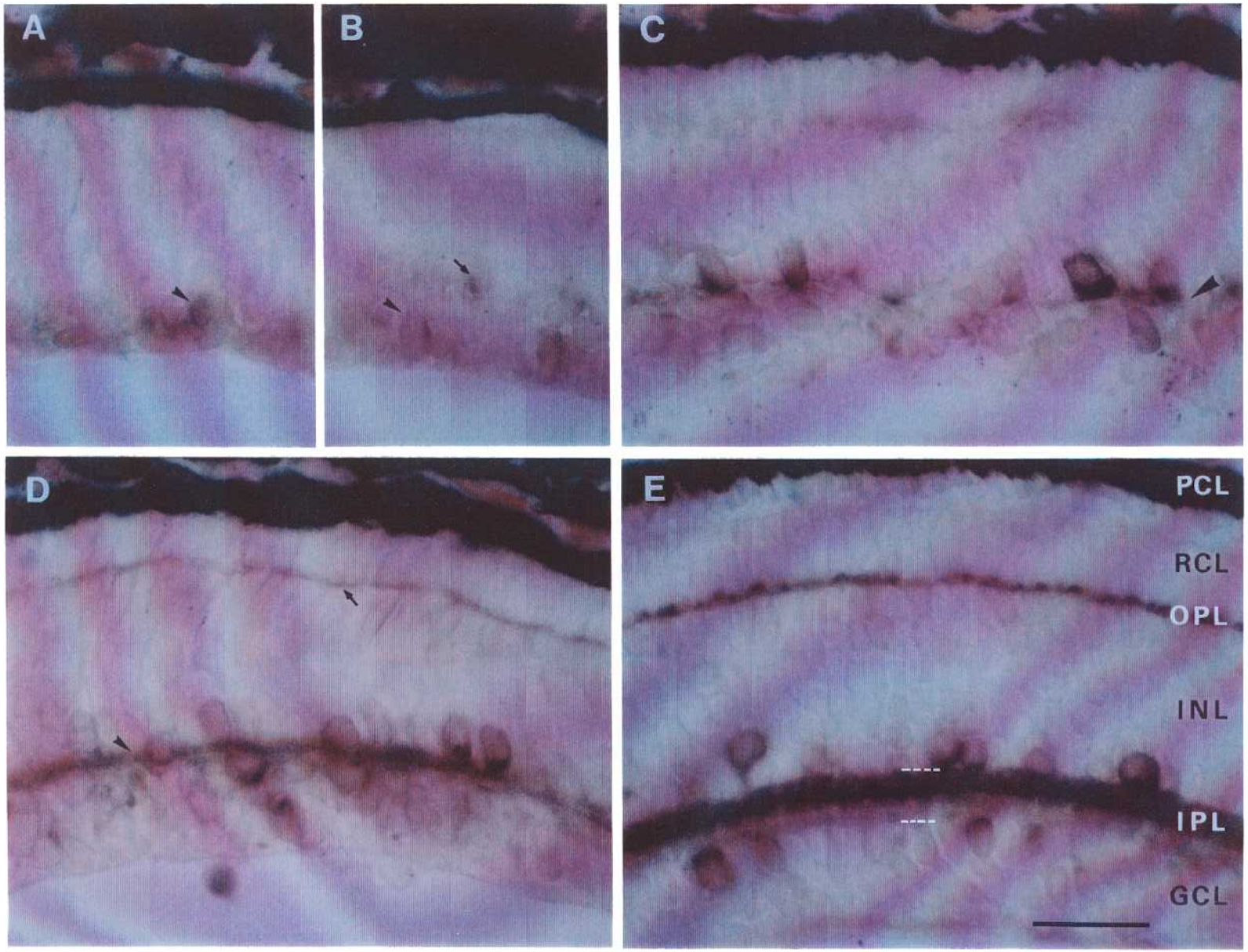

A
B
C
D
E
PCL
RCL
OPL
INL
IPL
GCL

Figure 26. The time course of appearance of mAChR (m2) immunoreactivity in regenerating newt retina. A: A retina at the intermediate-II stage. The mAChR-immunoreactive somata are seen in the most proximal region of the retina (arrowheads). B: A retina at the intermediate-III stage. An arrowhead indicates the IPL which is about to segregate. C: A retina at early period of the late stage. Some somata in horizontal cell layer (arrows) are immunoreactive. D: A retina at later period of the late stage. Dotted lines indicate the IPL boundaries. The mAChR staining pattern is almost identical to that in the mature retina. Note that horizontal cell-like somata becomes less immunoreactive. Abbreviations same as in Fig. 9. Scale bar=50 μm.

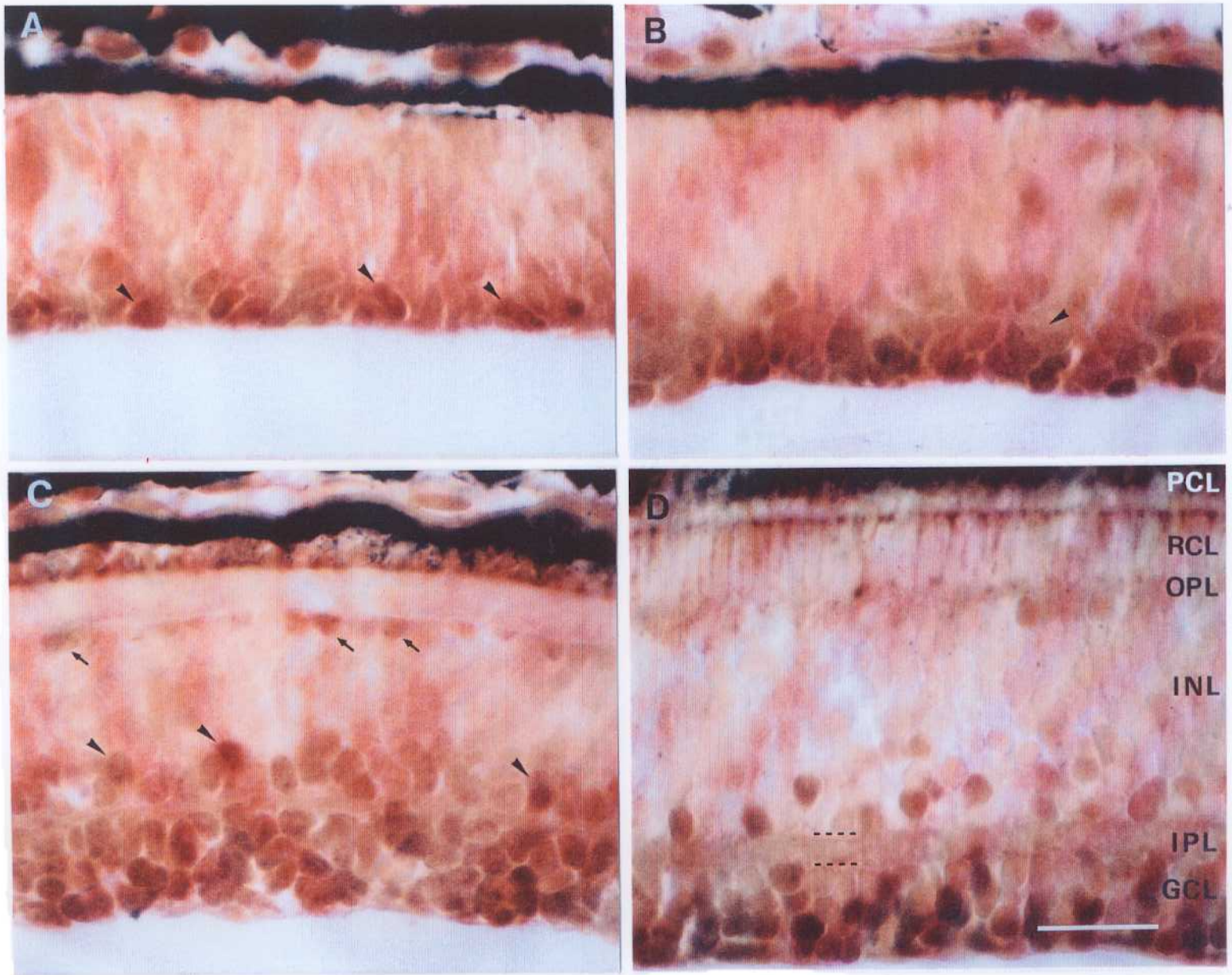

A
B
C
D
PCL
RCL
OPL
INL
IPL
GCL

Figure 27. The time course of appearance of α-BTX binding sites in regenerating newt retina. A: A retina at the intermediate-II stage. No α-BTX binding sites were detectable. B: A retina at transition period between the intermediate-III stage and the late stage. The α-BTX binding sites became detectable in the presumptive IPL (arrowhead). C: A retina at early period of the late stage. Both the IPL and OPL (arrow) were stained by α-BTX. D: A retina at later period of the late stage. The α-BTX binding pattern is almost identical to that in the mature retina. Abbreviations same as in Fig. 9. Scale bar=50 μm.

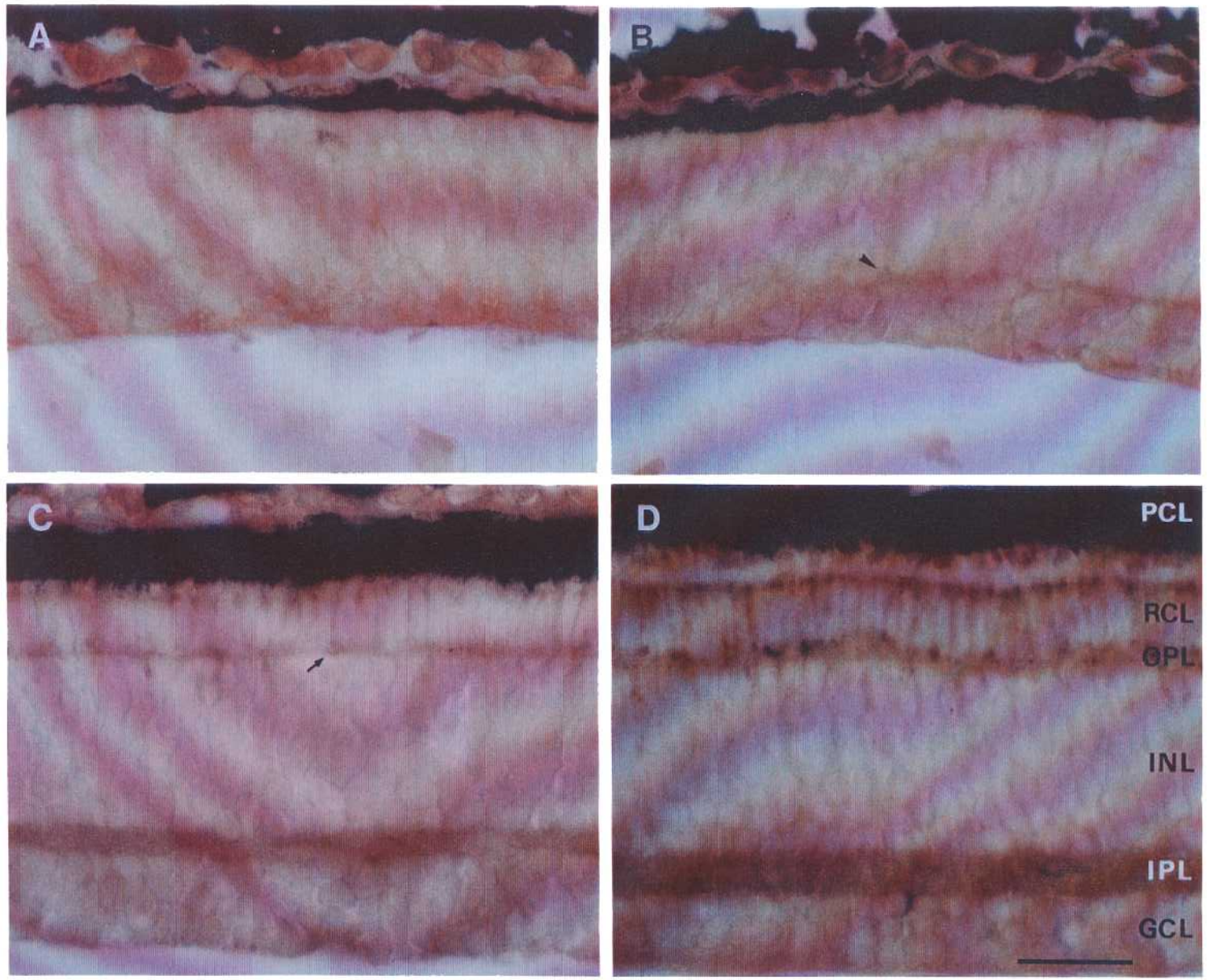

A
B
C
D
PCL
RCL
OPL
INL
IPL
GCL

Figure 28. The time course of appearance of α3 nAChR immunoreactivity in regenerating newt retina. A: A retina at the intermediate-II stage. No α3 subunit-immunoreactivity was detectable. B: A retina at transition period between the intermediate-III stage and the late stage. α3 subunit-immunoreactivity became detectable in the presumptive IPL (arrowhead). C: A retina at early period of the late stage. Both the IPL and the OPL (arrow) were α3-ir. D: A retina at later period of the late stage. α3-ir pattern is almost identical to that in the mature retina. An arrowheads indicates α3-ir Müller cell-like endfeet. Abbreviations same as in Fig. 9. Scale bar=50 μm.

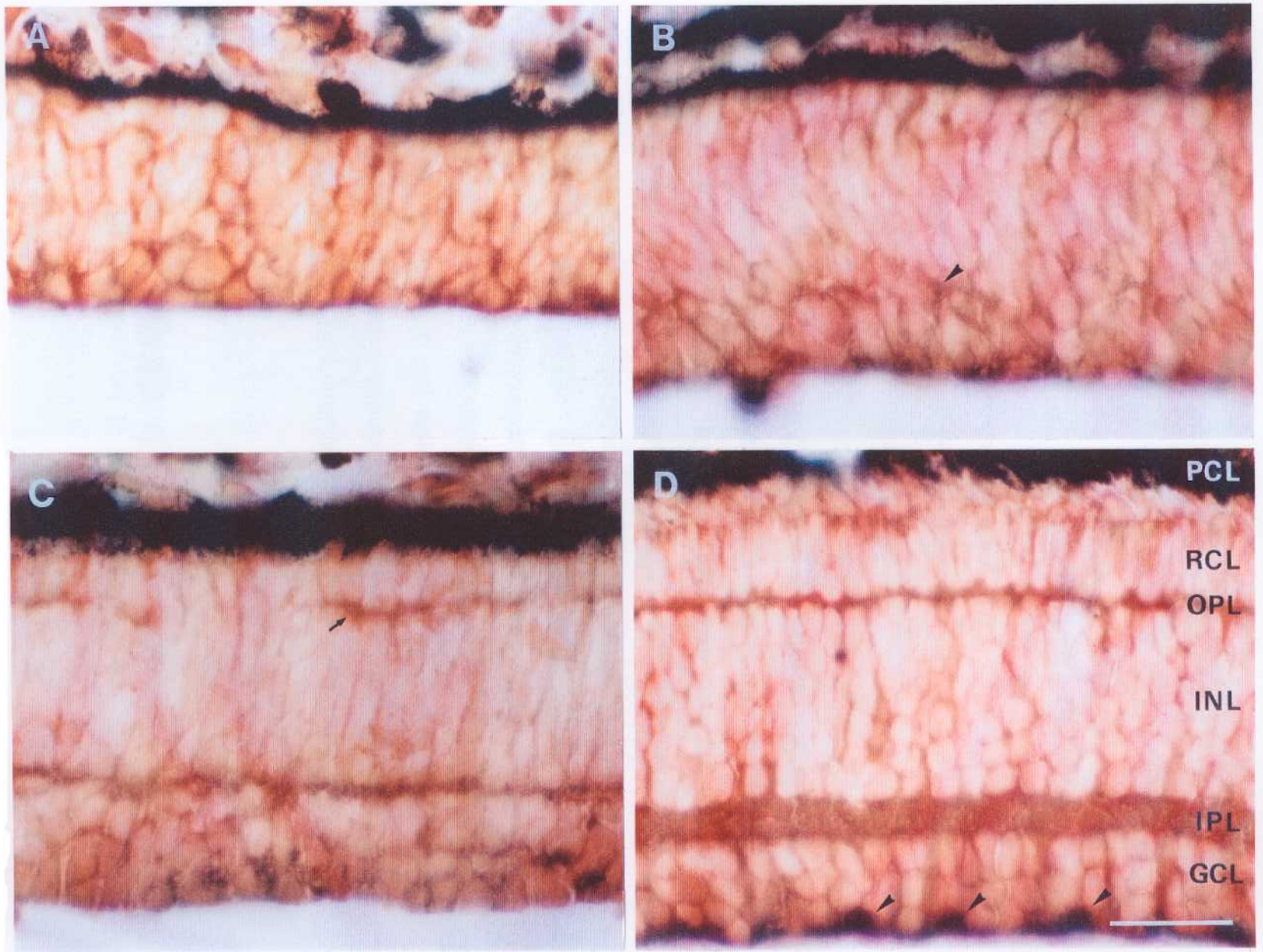
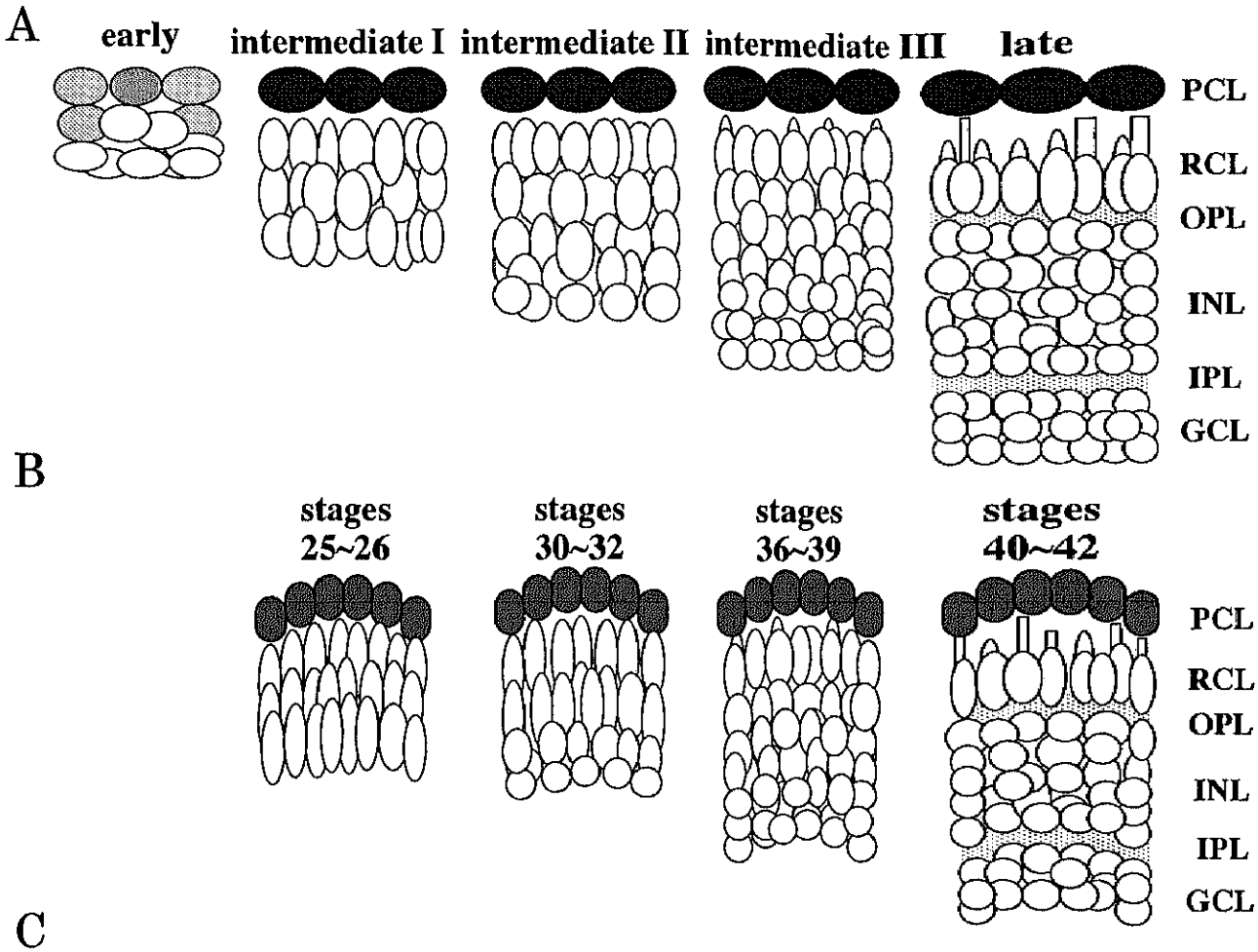

A
B
C
D
PCL
RCL
OPL
INL
IPL
GCL

Figure 29. Comparison of appearance and maturation of cholinergic system components during retinal development and regeneration in the newt. A: Schematic diagram showing morphological characteristics of regenerating retinas at five progressive stages following complete removal of the original retina of the adult newt.. B: Schematic diagram showing the morphological characteristic of the developing retinas at four progressive stages. C: The time course of appearance of ChAT, AChE, mAChR, and nAChR markers during retinal development and regeneration. Each dotted line indicates the onset and maintenance of the above molecules. Abbreviations same as in Fig. 9.


A
early
intermediate I
intermediate II
intermediate III
late
PCL
RCL
OPL
INL
IPL
GCL
B
stages 25~26
stages 30~32
stages 36~39
stages 40~42
PCL
RCL
OPL
INL
IPL
GCL
C
ChAT
AChE
mAChR
nAChR