Developmental Expression of Muscarinic Acetylcholine Receptors in Chick Retina: Selective Induction of M_2 Muscarinic Receptor Expression *In Ovo* by a Factor Secreted by Müller Glial Cells

Kristen E. Belmonte, Lise A. McKinnon, and Neil M. Nathanson

Department of Pharmacology, University of Washington School of Medicine, Seattle, Washington 98195-7750

Muscarinic acetylcholine receptors (mAChRs) play an important role in signal processing in the retina. We have used subtype-specific antibodies to identify the changes in the localization of mAChR expression during embryonic development of the retina *in vivo* and their relationship to the changes in mAChRs in retinal cells in culture. We have demonstrated previously that treatment of fresh retinal cultures with conditioned media from mature retinal cultures specifically induces expression of the M_2 mAChR (McKinnon et al., 1998). We show that the M_2-inducing activity, which we tentatively have called MARIA (muscarinic acetylcholine receptor-inducing activity) is produced by Müller glial cells in culture, because significant activity can be found in media conditioned by essentially neuron-free cultures of Müller glia, as well as by a Müller glial cell line but not several neuroblastoma cell lines. We also demonstrate that the appearance of the M_2 receptor *in vivo* occurs concomitantly with the appearance of significant numbers of Müller glial cells in the developing retina. Furthermore, the administration of crude or partially purified preparations of MARIA to developing chick embryos *in ovo* induces precocious expression of M_2 mAChRs in the appropriate cell types in the retina. These results show that a factor secreted by cultured retinal Müller glia can regulate M_2 mAChR expression *in vivo* and *in vitro* and suggest that the secretion of MARIA by Müller glia *in vivo* may be responsible for the normal induction of M_2 mAChR expression during embryonic development.

Key words: muscarinic; Müller glia; reporter gene; retina; chick; development; neurotrophic factor

The five subtypes of muscarinic acetylcholine receptors (mAChRs) are the products of distinct genes and belong to the superfamily of G-protein-coupled receptors (Nathanson, 1987; Wess, 1996). Many studies have investigated their role in signaling in the retina. In the adult cat retina, mAChRs modulate the activity of brisk ganglion cells (Schmidt et al., 1987). In frog and rabbit, mAChRs on glycinergic amacrine cells are involved in inhibition of the OFF channel pathway by the ON channel pathway (Bonaventure et al., 1989; Jardon et al., 1992). Retinal ganglion cells exhibit spontaneous activity that is involved in the maturation of retinogeniculate connections (Feller et al., 1996; Sernagor and Grzywacz, 1996; Catsicas et al., 1997; Penn et al., 1998; Wong et al., 1998); cholinergic starburst amacrine cells directly participate in these bursts (Zhou, 1998). Cholinergic regulation of this activity in rabbits switches from nicotinic to muscarinic after birth (Zhao et al., 1999; Zhou and Zhao, 1999). Thus, mAChRs are physiologically important in retinal function.

Autoradiographic studies localized most mAChRs to two bands within the inner plexiform layer (IPL) of the embryonic chick retina (Sugiyama et al., 1977), which consists of ganglion cell dendrites and amacrine and bipolar cell neurites (Mey and Thanos, 1992) and three bands within the IPL after hatching. Our laboratory used subtype-specific antibodies to demonstrate that the M_2, M_3, and M_4 subtypes of mAChRs each localized to distinct bands within the IPL of the adult chicken (Fischer et al., 1998).

Skorupa and Klein (1993) showed that there was a decrease in the apparent molecular weight of the mAChR during development of the chick embryonic retina *in vivo* and in culture. This shift in apparent weight could be accelerated in cultured retinal cells by the addition of conditioned medium from "mature" cultures (Skorupa and Klein, 1993). We have demonstrated previously that these changes result from the induction of expression of the M_2 mAChR gene both during embryonic development *in vivo* and in cultured retinal cells. The induction of expression in culture was attributable to the action of a developmentally regulated secreted factor (McKinnon and Nathanson, 1995; McKinnon et al., 1998). A large number of neurotrophic and growth factors were unable to induce M_2 gene transcription in retinal cells, suggesting that this secreted factor may represent a previously unidentified factor (McKinnon et al., 1998).

This work uses subtype-specific antibodies to determine the localization of mAChRs during embryonic development of the retina *in vivo* and its relationship to changes in mAChR expression *in vitro*. We show that this muscarinic acetylcholine receptor-inducing activity, tentatively named MARIA, is produced by Müller glial cells in culture, and that the administration of partially purified preparations of MARIA *in ovo* induces precocious expression of M_2 in the appropriate cell types. These results show that MARIA secreted by cultured retinal Müller glia can regulate M_2 mAChR expression *in vivo* and suggest that secretion of MARIA by Müller glia may be responsible for the normal induction of M_2 mAChR expression during embryonic development.

MATERIALS AND METHODS

Immunocytochemistry on embryonic chick retinal sections. Eyes (vitreous humor removed) from developing white Leghorn chick embryos (H&N International, Redmond, WA) were fixed in 2% paraformaldehyde–6% sucrose at room temperature for 45 min. After fixation, the retinas were cryoprotected in 30% sucrose in PBS at room temperature for 5 hr, embedded in OCT (Miles, Elkhart, IN), and frozen in liquid nitrogen. Sections of 20 μm each were collected on gel-coated slides and ringed with rubber cement. The slides were incubated with blocking buffer [PBS plus 0.3% Triton X-100 (PBST) and 5% donor goat serum] at room temperature for 1 hr. Primary antibodies were diluted in PBST and incubated on the slides for 24 hr in a humidified chamber at room temperature. All muscarinic receptor antibodies were generated in our laboratory (McKinnon and Nathanson, 1995; McKinnon et al., 1998) and were diluted as follows: anti-M_2, 1:200; anti-M_3, 1:1000; anti-M_4, 1:500. Dilutions for the cell markers were as follows: anti-neurofilament RMO 270 [Dr. Virginia Lee (University of Pennsylvania, Philadelphia, PA)], 1:500; anti-vimentin H5 [developed by Dr. J. R. Sanes (Washington University School of Medicine, St. Louis, MO), obtained from the Developmental Studies

Received June 6, 2000; revised Aug. 22, 2000; accepted Sept. 5, 2000.

This research was supported by National Institutes of Health Grants R01-HL30639, (N.M.N.), T32-NS07332 (K.E.B.), and F32-NS10944–01 (K.E.B.).

Correspondence should be addressed to Neil M. Nathanson, Department of Pharmacology, Box 357750, University of Washington, Seattle, WA 98195-7750. E-mail: nathanso@u.washington.edu.

Dr. McKinnon's present address: National Institute of Neurological Disorders and Stroke, National Institutes of Health, MSC 4064, Bethesda, MD 20892-4064.

 0270-6474/00/208417-09$15.00/0

Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa, Department of Biological Sciences (Iowa City, IA)] 1:10; anti-tenascin M1-B4 [Developmental Studies Hybridoma Bank, developed by Dr. D. M. Fambrough (Johns Hopkins University, Baltimore, MD)], 1:10; and anti-acetylcholinesterase [developed by Dr. Richard L. Rotundo (University of Miami, Miami, FL)] (Rotundo, 1984), 1:500. After being washed with PBS, the slides were incubated with biotinylated goat anti-rabbit (1:500; Vector Laboratories, Burlingame, CA) and Alexa 568 goat anti-mouse (1:100; Molecular Probes, Eugene, OR) diluted in PBST for 2 hr at room temperature, followed by FITC ExtrAvidin (1:50; Sigma, St. Louis, MO) diluted in PBST for 2 hr at room temperature. The slides were washed extensively with PBS and then coverslipped with Vectashield and visualized with a Bio-Rad (Richmond, CA) MRC600 confocal microscope.

Preparation of primary retinal cultures. Fertilized eggs from white Leghorn chickens were incubated in a humidified environment at 38°C until days 8 or 9 of incubation. Retinas were dissected free of pigmented epithelium and dissociated as described by Reh (1992). Retinas were trypsinized in 0.25% trypsin (Worthington, Freehold, NJ) for 13 min at room temperature. The trypsin was inactivated by the addition of fetal bovine serum (final concentration, 15%). Retinas were centrifuged at 1000 rpm for 10 min and triturated in DMEM–F12 medium containing 1% penicillin–streptomycin, 330 mM glucose, 5 mM HEPES, pH 7.4, 30 μg/ml transferrin, 6 ng/ml putrecine, 5.2 ng/ml sodium selenite, and 6 ng/ml progesterone (Sigma). Cells dissociated from embryonic day 9 (E9) retinas were plated on 150 mm plates at a density of $4–5 \times 10^7$ cells per plate and were used for the collection of conditioned media. Cells dissociated from E8 retinas were plated on 24-well plates coated with poly-D-lysine at a density of $2–2.5 \times 10^6$ cells per well. Cultures were incubated in a 5% CO_2 environment. The day of plating was considered to be culture day 0. Conditioned medium was collected from the E9 cultures every 24 hr, beginning on culture day 6 continuing for up to a month, and stored at −80°C.

Immunocytochemistry on cultured cells. Retinal cultures were prepared as described from E9 retina and plated on either glass slides or 150 mm plates coated with poly-D-lysine. On culture days 6, 10, 20, and 27, cells were fixed with 4% paraformaldehyde–6% sucrose in PBS. For those cells that were grown on 150 mm plates, 25 mm circles were excised from the plate with a metal stamp, and the circles were fixed to glass slides with enamel. After fixation, cells were incubated with primary antibody diluted in PBST for 24 hr. After being washed with PBS, the slides were incubated with Alexa 568 goat anti-mouse (Molecular Probes) diluted 1:100 in PBST for 2 hr at room temperature. The slides were washed extensively with PBS and then coverslipped with Vectashield and visualized with a Bio-Rad MRC600 confocal microscope at low power.

Partial purification of MARIA. Conditioned medium (collected from the E9 cultures every 24 hr, beginning on culture day 6 continuing for up to a month) was concentrated threefold at 4°C with an Amicon Ultrafiltration cell using a PM30 Diaflo Ultrafilter (retains proteins at ≥30 kDa; Amicon, Beverly, MA). The concentrated serum-free conditioned medium (SFCM) was dialyzed over night in 20 mM HEPES and 10 mM NaCl, pH 7.4, at 4°C. The dialyzed SFCM was passed over a DEAE Sephacel (Amersham Pharmacia Biotech, Piscataway, NJ) column. Flow through was collected and dialyzed overnight against PBS at 4°C.

Collection of conditioned medium from glial and neuroblastoma cell lines. SN56 cells (Blusztajn et al., 1992) and IMR32 cells (American Type Culture Collection, Manassas, VA) were grown in DMEM supplemented with 10% FBS. The immortalized rat Müller glial cell line (Sarthy et al., 1998) was grown in DMEM–F12 supplemented with 10% FBS, 1% penicillin–streptomycin, 330 mM glucose, and 5 mM HEPES, pH 7.4. Conditioned media was collected from each of the cell lines and concentrated fivefold at 4°C with an Amicon Ultrafiltration cell using a PM30 Diaflo Ultrafilter.

Testing for MARIA activity in transiently transfected primary retinal cultures. To test for MARIA activity *in vitro*, cultured retinal cells were prepared from E8 retina and plated on 24-well plates as described above. A 2 kb *Eco*RI/*Hin*dIII fragment of the M_2 promoter region ligated to the firefly luciferase reporter gene in the PGL3 expression vector, designated pNMR27 (Rosoff et al., 1996), was used to measure the effect of MARIA on M_2 gene transcription as described previously (McKinnon et al., 1998). Cells were transfected using the calcium phosphate precipitation method (Sambrook et al., 1989) with 600 ng/well pNMR27 or PGL3 and 100 ng/well pRSV-β-galactosidase. The cells were incubated with the DNA–calcium phosphate precipitate for 4 hr and then treated with 10% glycerol for 3 min. After glycerol shock, retinal cells were treated with one of the following: control media (SFCM) collected from primary retinal cultures (90% in fresh media), concentrated media collected from one of the various cell lines (90% 5× concentrate in fresh media), or partially purified MARIA (100 μl added to 500 μl of fresh media per well) for 24–36 hr. Cells were lysed and assayed for luciferase and β-galactosidase activities as described previously (Johnson and Nathanson, 1994).

Testing for in vivo induction of M_2 expression by MARIA. To test for MARIA activity *in vivo*, 1.5 ml of concentrated SFCM or partially purified MARIA was injected into E6–E8 eggs through a small hole in the shell (Halvorsen and Nathanson, 1981). The hole was then covered with tape, and the eggs were returned to the incubator for the indicated times. Eyes were removed at E9 and prepared for immunohistochemistry as described above.

RESULTS

Localization of mAChRs in the developing retina

Previous biochemical and molecular biological studies have shown that there are dramatic changes in the relative abundance of mAChR subtypes in the chick retina during embryonic development because of induction of expression of the M_2 receptor during the middle of the second week of embryonic development (McKinnon and Nathanson, 1995). The pattern of expression of M_2, M_3, and M_4 mAChRs in the retina has been described previously both late in development and after hatching, as well as in cultured retinal cells (Fischer et al., 1998; McKinnon et al., 1998). However, the patterns of expression of the different mAChR subtypes in the developing retina during the time of these maximal changes in subtype-specific expression *in vivo* have not been defined previously. Thus, subtype-specific antibodies (McKinnon and Nathanson, 1995) were used to localize the M_2, M_3, and M_4 mAChRs in embryonic chick retina, and antibodies against cell-specific markers were used to identify the retinal cell types that express these receptors.

Anti-M_4 antibodies labeled cells in the inner nuclear layer (INL), the IPL, and the ganglion cell layer (GCL) at E8 and expression in this area was maintained through E19 (Fig. 1). Expression within the INL and the GCL appeared to be in cell bodies, whereas expression within the IPL appeared as two distinct strata. The level of expression appeared to remain relatively constant as the retina developed and thickened. M_4 expression was seen on amacrine cells, because it colocalized with tenascin, a protein present in amacrine cell processes (Bartsch et al., 1995), from E9 to E19 within the IPL. Colocalization of M_4 with acetylcholinesterase in the IPL is not apparent at E9–E12 but then appears in the IPL at E15 and is maintained through E19. M_4 colocalization with acetylcholinesterase in the INL appears at E12 and is maintained through E19. M_4 expression detected on somata within the GCL appeared to colocalize with neurofilament, a protein found in ganglion cell axons (Bradshaw et al., 1995), and with displaced ganglion cells in the INL but not with M_4-labeled strata in the IPL.

Anti-M_3 antibodies labeled cells in the outer plexiform layer (OPL) (Fig. 2) and in the INL. Expression of M_3 within the OPL and the INL appeared weakly at E9 and increased through E19. Colocalization of M_3-labeled cells in the OPL was seen with tenascin, which labels horizontal cells. Anti-M_3 antibodies also labeled cells in the IPL beginning at E15. Expression of M_3 mAChRs in the inner portion of the INL appeared to be on amacrine cell bodies, a subset of which colocalized with acetylcholinesterase. Expression of M_3 mAChRs within the IPL appeared as three distinct strata. In contrast to the anti-M_4-labeled strata, these anti-M_3-labeled strata did not colocalize with acetylcholinesterase-labeled strata but were located either just distal or proximal to them.

The overall increase in expression detected by anti-M_2 antibodies in the developing retina was the most dramatic of the three subtypes investigated (Fig. 3). No cells were labeled with anti-M_2 antibodies at E9; this lack of expression of M_2 is consistent with the inability to detect M_2 receptor expression by immunoprecipitation and immunoblot analyses at E9 (McKinnon and Nathanson, 1995). The initial appearance of M_2 mAChRs occurred at E10 in amacrine cells of the INL and ganglion cells of the GCL, and this expression increased markedly through E19. This initial appearance of M_2 mAChRs at E10 appeared to coincide with the initial appearance of Müller glial cells in the retina, labeled with vimentin (Willbold et al., 1995), although there did not appear to be any colocalization of M_2 with vimentin. By E15, M_2 mAChRs appeared in the IPL as three distinct strata that were maintained through E19. The expression of M_2 mAChRs in the IPL colocalized with tenascin and, similar to that which was seen with M_3 mAChR-labeled strata in the IPL, the M_2 mAChR-labeled strata did not

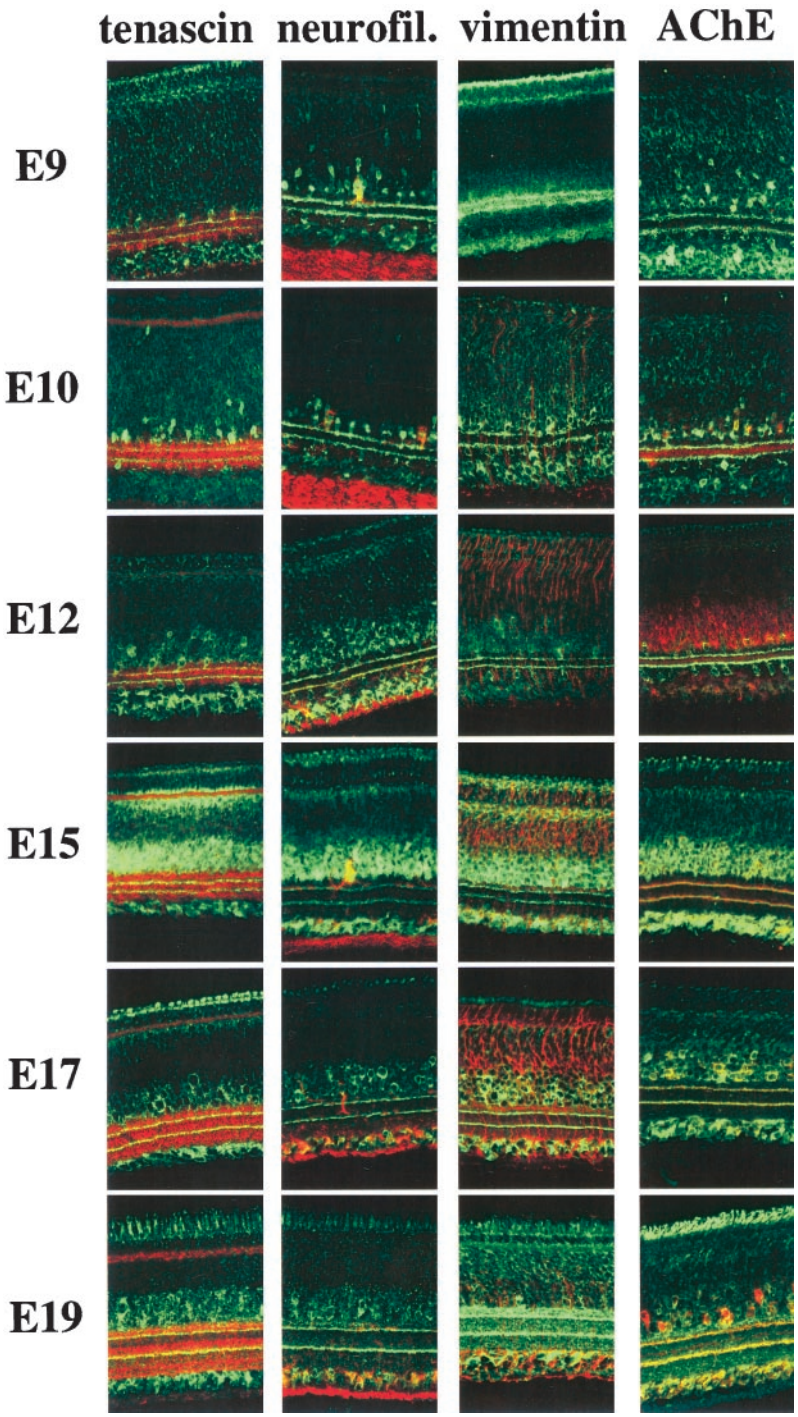

Figure 1. Colocalization of M_4 mAChRs with markers for retinal neurons and Müller glia. Embryonic retinas from E9, E10, E12, E15, E17, and E19 chicks were sectioned and prepared for immunocytochemistry as described in Materials and Methods. mAChRs were immunolabeled with FITC-conjugated secondary antibody (*green*), and retinal cell markers were immunolabeled with Alexa 568-conjugated secondary antibodies (*red*). See Results for specificity of marker antigens. Photographs were taken using confocal microscopy.

colocalize with acetylcholinesterase. Instead, M_2 mAChR-labeled strata was located just proximal to acetylcholinesterase-labeled strata.

Generation of partially purified preparations of MARIA from concentrated conditioned media

MARIA has been shown previously to induce precocious M_2 mAChR expression in cultured retinal neurons (McKinnon et al., 1998). We therefore wanted to determine whether MARIA could induce precocious expression of M_2 mAChRs *in vivo*. SFCM was collected from mature retinal cultures and was concentrated approximately threefold with a PM30 Diaflo Ultrafilter, which removes low-molecular weight polypeptides and retains molecules that are >30 kDa. The concentrated SFCM was applied to a DEAE Sephacel column. It has been shown previously that MARIA is not retained by a DEAE Sephacel column (McKinnon et al., 1998), whereas transferrin, the only protein component added to SFCM, is retained. Thus, this step provides a substantial degree of purification. The resulting material had a protein concentration of ~20–60 μg/ml compared with 1.2–2.0 mg/ml of the starting concentrated SFCM. The SFCM (nonconcentrated start material) and the DEAE Sephacel-purified preparation were then tested for the presence of MARIA using E8 cultured retina that had been transfected with a M_2 promoter–luciferase reporter gene construct.

E8 retinal cells were treated with control, SFCM, or DEAE-purified material for 24 hr. Cells were then lysed and assayed for luciferase activity. Figure 4 demonstrates that the SFCM induced a 7 ± 1-fold increase in M_2 mAChR gene transcription compared with controls, and the DEAE Sephacel-purified preparation increased M_2 mAChR gene transcription 18 ± 2-fold compared with

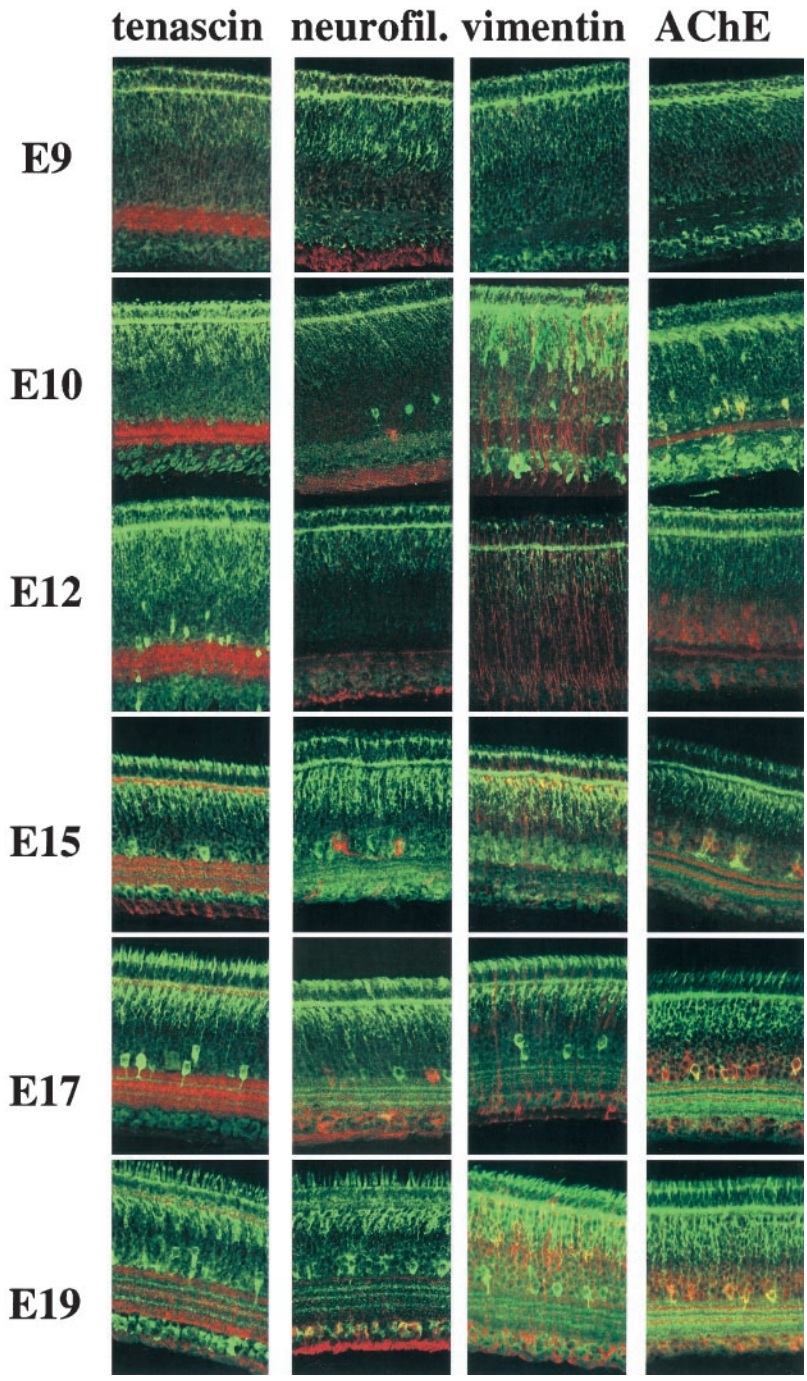

Figure 2. Colocalization of M_3 mAChRs with markers for retinal neurons and Müller glia. Embryonic retinas from E9, E10, E12, E15, E17, and E19 chicks were sectioned and prepared for immunocytochemistry as described in Materials and Methods. mAChRs were immunolabeled with FITC-conjugated secondary antibody (*green*), and retinal cell markers were immunolabeled with Alexa 568-conjugated secondary antibodies (*red*). See Results for specificity of marker antigens. Photographs were taken using confocal microscopy.

controls. This confirmed that the partially purified preparation retained significant amounts of MARIA after the ultrafiltration and ion exchange purification steps. This partially purified preparation of MARIA was then injected into developing eggs to test for *in ovo* induction of M_2 expression.

MARIA causes precocious expression of M_2 mAChRs *in ovo*

As an initial test to determine whether MARIA could induce M_2 mAChR receptor expression *in ovo*, crude concentrated SFCM was injected into developing eggs at E6, and the expression of M_2 was tested by immunocytochemistry at E9, a time when M_2 mAChR expression was normally absent. Control eggs were injected with the same volume of fresh serum-free media. As shown in Figure 5, addition of SFCM caused a dramatic induction of M_2 mAChR expression in the tenascin-positive cells of the INL.

We next tested whether the partially purified preparations of MARIA were also active *in ovo*. Because MARIA is able to induce M_2 mAChR gene transcription in cultured retinal cells within 24 hr, the partially purified preparation of MARIA was injected into developing eggs at E8, and M_2 mAChR expression was examined the next day. Expression of M_2, M_3, and M_4 mAChRs in the retina at E9 after administration of fresh serum-free media did not look different from controls. However, administration of the partially purified preparation of MARIA at E8 again caused significant expression of M_2 mAChRs in the E9 retina that colocalized with tenascin (Fig. 5*A*). This M_2 mAChR expression pattern, which resulted from application of either concentrated SFCM or partially purified MARIA, appeared to be similar to the pattern of expression that is normally seen in the retina by E15 or later. The expression of M_3 and M_4 mAChRs did not appear to be

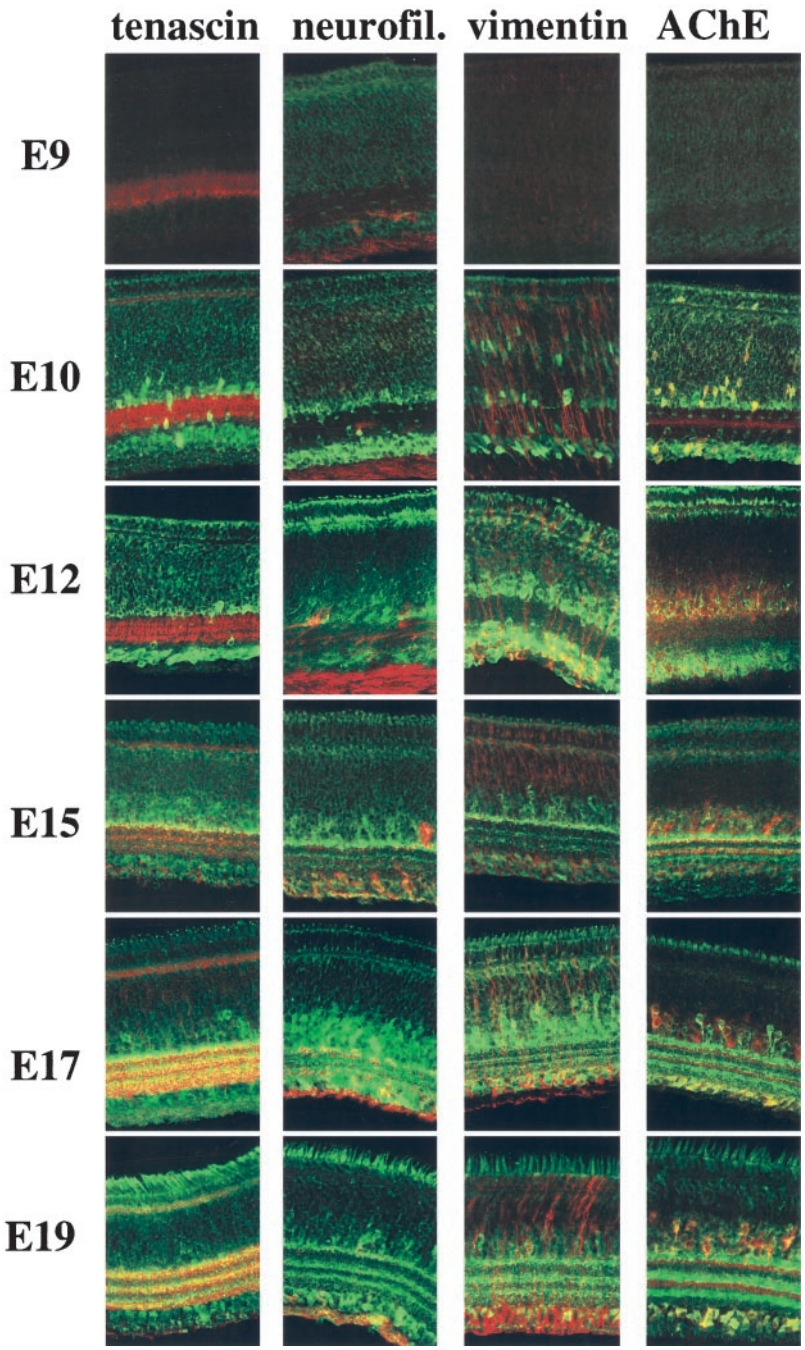

Figure 3. Colocalization of M_2 mAChRs with markers for retinal neurons and Müller glia. Embryonic retinas from E9, E10, E12, E15, E17, and E19 chicks were sectioned and prepared for immunocytochemistry as described in Materials and Methods. mAChRs were immunolabeled with FITC-conjugated secondary antibody (*green*), and retinal cell markers were immunolabeled with Alexa 568-conjugated secondary antibodies (*red*). See Results for specificity of marker antigens. Photographs were taken using confocal microscopy.

changed by the presence of MARIA, nor did the expression of cellular markers appear changed (Fig. 5*B*). Thus, the partially purified preparation of MARIA is able to specifically induce M_2 mAChR expression *in vivo* in the appropriate cell types in the retina without having major effects on the expression of the M_3 or M_4 mAChR or on the fates of the major cell types in the retina.

Retinal Müller glia are the cellular source of MARIA

Because we were able to demonstrate induction of M_2 mAChR expression both *in vivo* and *in vitro* by MARIA found in serum-free media conditioned by mature retinal cultures, we wanted to determine the cellular source of MARIA. MARIA could come from a neuronal cell type within the retina, or it could be secreted by the retinal Müller glia. To determine the cellular source of MARIA, we took advantage of the observation that dissociated retina grown on a nonadherent surface at high density are virtually neuron-free after 7 d *in vitro* (Hoffmann, 1988). Retinal cultures were fixed and stained for neurofilament (ganglion cells), tenascin (amacrine and horizontal cells), and vimentin (Müller glia) on days 6, 13, 20, and 27. Furthermore, conditioned media was collected from the cells each day and tested for MARIA on days 6, 13, 20, and 27 using E8 cultured retina that had been transfected with an M_2 promoter–luciferase reporter gene construct. After 24 hr, the cells were lysed and assayed for luciferase activity.

Cells that were fixed and stained on culture days 6 and 13 showed a mixture of cell types, containing both neurons and glia. E8 retinal cells treated for 24 hr with SFCM taken from retinal cells on culture days 6 and 13 showed a 3.2 ± 0.6-fold and a 7.8 ± 1.7-fold increase, respectively, in M_2 mAChR gene transcription compared with controls (Fig. 6*A*,*B*). Cells that were fixed and stained on culture day 20 contained only Müller glia and a few remaining amacrine cells. E8 retinal cells treated for 24 hr in SFCM taken from retinal cells on culture day 20 exhibited a 6.5 ± 0.5-fold increase in M_2 mAChR gene transcription compared with controls (Fig. 6*A*,*B*). By culture day 27, only Müller glia can be detected in

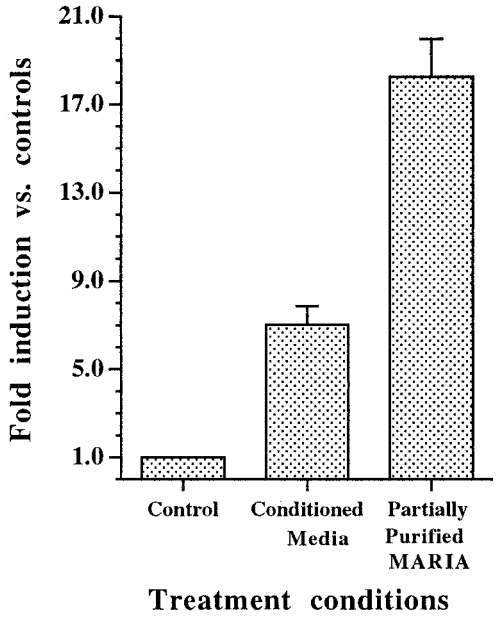

Figure 4. Application of conditioned medium containing MARIA stimulates transcription of the M_2 mAChR gene. Embryonic chick retinal cultures were prepared from E8 retina, plated in 24-well plates, and transfected on culture day 1 with the M_2 promoter–luciferase reporter gene construct, as described in Materials and Methods. After transfection, cells were treated for 24 hr with conditioned media, a partially purified preparation of MARIA, or control medium (see Materials and Methods). The data represent luciferase activity/β-galactosidase activity, expressed as fold-increase compared with controls. Values are the mean ± SEM; $n = 3$.

the cultures, and E8 retinal cells treated for 24 hr in SFCM taken from retinal cells on culture day 27 exhibited a 6.8 ± 0.8-fold increase in M_2 mAChR gene transcription compared with controls (Fig. 6*A*,*B*). Thus, conditioned medium taken from essentially pure cultures of Müller glia is able to induce retinal M_2 mAChR gene transcription.

To further confirm that retinal Müller glia are the cellular source of MARIA, we tested concentrated media conditioned by two different neuroblastoma cell lines, as well as media conditioned by an immortalized rat Müller glial cell line, for the presence of MARIA. Each of the concentrated conditioned media was tested using E8 cultured retina that had been transfected with an M_2 promoter–luciferase reporter gene construct. After 48 hr, the cells were lysed and assayed for luciferase activity as described. Retinal cells that were treated with concentrated media conditioned by either SN56 or IMR32 neuroblastoma cell lines did not show any significant difference in M_2 mAChR gene transcription compared with controls (Fig. 6*C*). However, retinal cells that were treated with concentrated media collected from the rat Müller glial cell line showed a 2.6 ± 0.1-fold increase in M_2 mAChR gene transcription compared with controls (Fig. 6*C*). Thus, conditioned media taken from a Müller glial cell line, but not from two neuroblastoma cell lines, were able to induce retinal M_2 mAChR gene transcription.

DISCUSSION

This work describes the expression of M_2, M_3, and M_4 mAChRs during development of the embryonic chick retina and demonstrates that a protein secreted by the Müller glia of cultured embryonic chick retinal cells may regulate the normal developmental appearance of M_2 mAChRs.

The differential expression of M_2, M_3, and M_4 mAChRs, the dramatic appearance of M_2 mAChRs starting at E10, and the increased number of mAChR-labeled strata in the IPL during development in the chick retina are consistent with data on mAChR expression in chick retina described previously using immunoprecipitation, immunoblot, and solution hybridization analyses (McKinnon and Nathanson, 1995), and with previous studies on receptor localization using autoradiography (Sugiyama et al., 1977) and immunocytochemistry (Fischer et al., 1998).

Millar identified three types of cholinergic amacrine cells in the adult chicken retina (Millar et al., 1987) based on the location of their somata and the depth of stratification in the IPL. Somata labeled with anti-M_2 in the INL appear to represent type III cells, whereas M_2-labeled somata in the GCL appear to represent type II cells. Somata labeled with anti-M_3 antibodies in the INL might also be type III amacrine cells. Somata labeled with anti-M_4 antibodies localized to the IPL and the GCL, with dendrites stratifying into two acetylcholinesterase-positive layers within the IPL. These M_4-labeled cells appear to be type I and type II cells. Thus, the distinct patterns labeled with M_2, M_3, and M_4 antibodies may represent three distinct subsets of amacrine cells.

Although all three subtypes of mAChRs can be found in the inner region of the INL at some stage of development, the M_3 subtype of mAChRs was the only subtype that was found in the OPL (horizontal cells), and in the outer regions of the IPL. These results are consistent with results described in E14 retina (McKinnon et al., 1998) and in the posthatched chicken (Fischer et al., 1998). These neurons appear morphologically to be bipolar cells, although the lack of colocalization of anti-M_3 mAChR immunoreactivity with anti-PKC immunoreactivity indicates that M_3 mAChR immunoreactivity must coincide with a subset of bipolar cells that is PKC α/β-negative. The significance of M_3 mAChR expression in the outer IPL and in the OPL is unclear, because neither acetylcholinesterase (Figs. 1–3) nor choline acetyltransferase (Millar et al., 1987; Prada et al., 1999) immunoreactivity are detected in these areas. Thus, although there does not appear to be a source of ACh in these layers, these receptors may be activated by ACh released from cholinergic neurons in the IPL and the inner INL. For instance, in rabbit retina, mAChRs on glycinergic amacrine cells stimulate the release of glycine, which then inhibits release of ACh from cholinergic amacrine cells (Cunningham et al., 1983; Neal and Cunningham, 1995; Linn, 1998).

The functional significance of each of the mAChR subtypes within the developing chick retina is unclear. In the embryonic chick retina, all three subtypes of mAChRs are expressed at significant levels from E10 onward, and M_3 and M_4 mAChRs are expressed even earlier, at E9. In the chick, this coincides with the period during which amacrine and ganglion cells are establishing and refining their synaptic contacts by spontaneously firing waves of action potentials (for review, see Wong, 1999). Nicotinic cholinergic transmission is involved in the early (E8–E11) but not the late (E12–E19) phases of wave burst activity (Wong et al., 1998). Recently, it has been observed in rabbit retina that cholinergic regulation of spontaneous burst activity undergoes a transition from nicotinic in neonatal retina to muscarinic at postnatal day 3 (Zhao et al., 1999; Zhou and Zhao, 1999). This phenomenon may be similar in the chick retina, because Wong showed that the nicotinic participation in spontaneous wave activity ceases after E11 in chick retina (Wong et al., 1998). During the early phase of wave burst activity in chick retina (E8–E11), there is significant M_4 mAChR expression in the IPL, and both M_2 and M_4 mAChRs can be detected in the INL by E11. During the late phase of spontaneous burst activity (E12–E19), all three mAChR subtypes can be found in the IPL and the INL. This high density of mAChRs suggests that mAChRs might play a significant role in synaptogenesis in the developing chick retina or in subsequent signal processing after hatching.

The antibodies used to obtain the data presented here have been used previously to identify cell type expression mAChRs in cultured chick retina (McKinnon et al., 1998). It was reported that all three subtypes of mAChRs were expressed in ganglion cells, and only M_4 was found in amacrine cells. In the work presented here, both M_2 and M_4 were found in ganglion cells, and all three subtypes were found in amacrine cells. M_4 mAChRs were also found to colocalize with vimentin, a marker for Müller glia, in cultured retina, whereas no mAChRs were found on Müller glia in retinal sections. The cultured retinal cells, having been removed

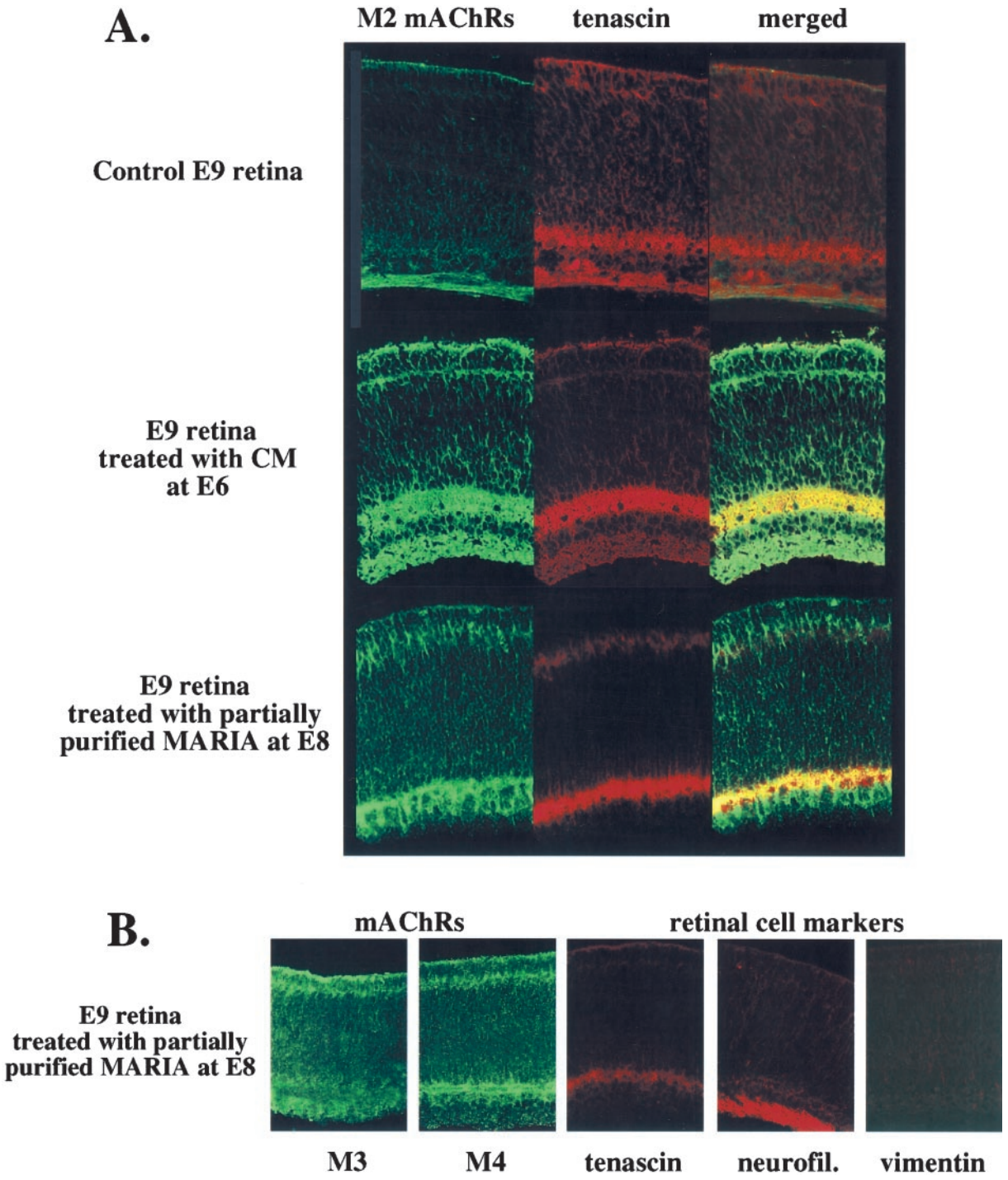

Figure 5. *A*, Application of conditioned medium containing MARIA stimulates precocious expression of M_2 mAChRs. Colocalization of M_2 mAChRs with tenascin, a protein found in amacrine cells. Embryonic retinal sections were prepared from E9 chicks that had been exposed to 1.5 ml of the following: *CM* (injected at E6); *partially purified MARIA* (for purification procedure, see Materials and Methods, injected at E8); *Control*, control serum-free media, injected at E6 or E8. At E9, the retinas were removed and prepared for immunocytochemistry as described in Materials and Methods. mAChRs were immunolabeled with FITC-conjugated secondary antibody (*green*), and tenascin was immunolabeled with Alexa 568-conjugated secondary antibodies (*red*). *B*, Application of partially purified MARIA has no effect on expression of M_3 or M_4 mAChRs, nor does it affect expression of cellular markers. Embryonic retinal sections were prepared from E9 chicks that had been treated at E8 with 1.5 ml of flow through that was collected from a DEAE Sephacel column over which conditioned medium that had been concentrated threefold was passed (see Materials and Methods). mAChRs were immunolabeled with FITC-conjugated secondary antibody (*green*), and cellular markers were immunolabeled with Alexa 568-conjugated secondary antibodies (*red*). See Materials and Methods for specificity of marker antigens. Photographs were taken using confocal microscopy.

from their native neighbors, probably exhibit changes in their receptor expression. This might suggest that there are distinct environmental cues attributable to perhaps secreted proteins or cell–cell interactions, which affect the developmental expression of mAChRs *in vivo*.

The mechanisms that regulate the expression of mAChRs in developing retina are not well defined. Chick retinal cells cultured in the presence of conditioned media containing MARIA collected from mature retinal cultures show an increase in M_2 mAChR mRNA and protein expression, whereas M_3 and M_4 mAChR expression remains unchanged (McKinnon et al., 1998). To see whether MARIA could induce precocious expression of M_2

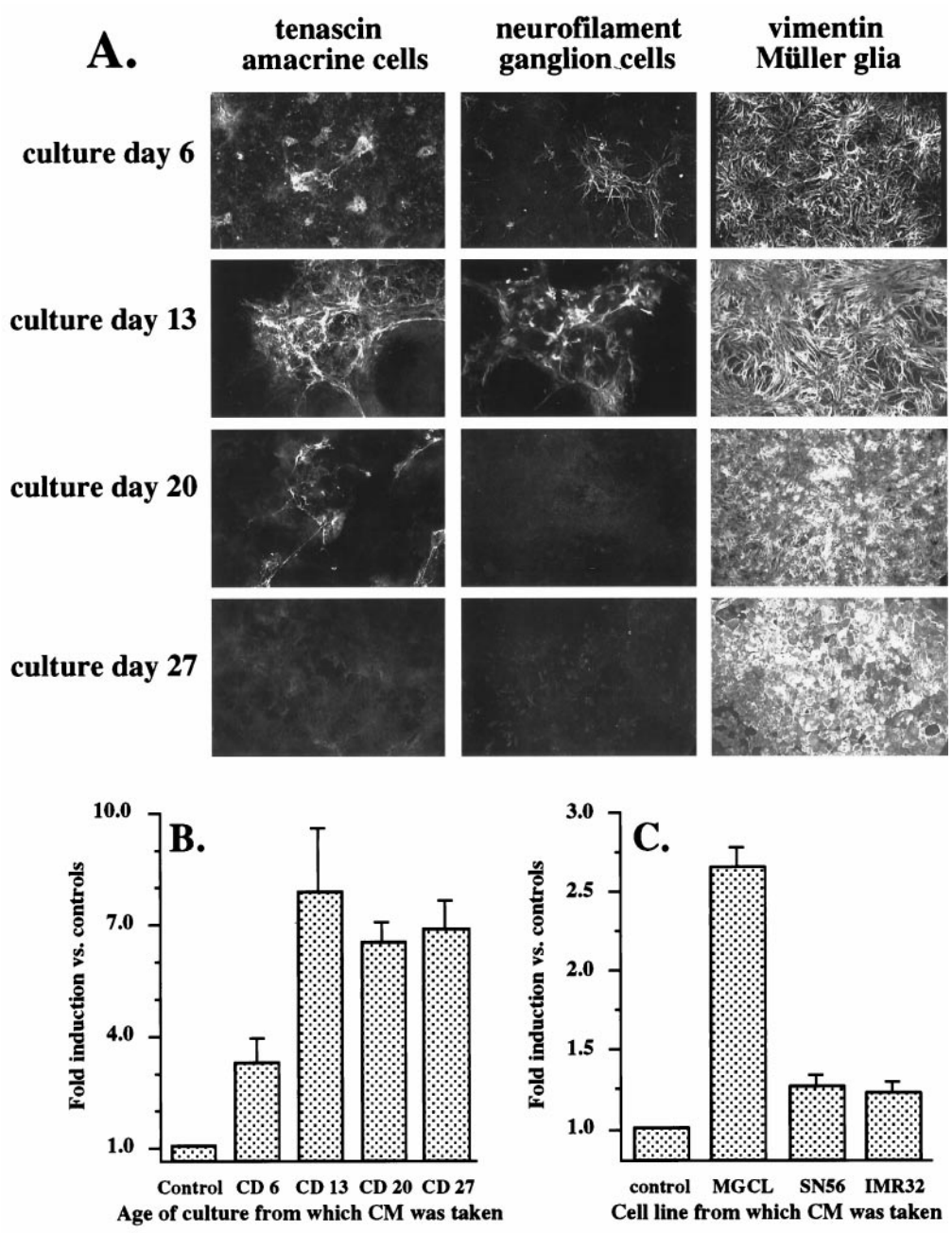

Figure 6. MARIA is found in media conditioned by retinal cultures that are essentially neuron-free. *A*, Immunocytochemistry demonstrating the presence of neuronal cellular markers in retinal cell cultures. Embryonic retinal cultures were prepared from E9 retina and grown on 150 mm plates for 6, 13, 20, or 27 d. Cells were prepared for immunocytochemistry as described in Materials and Methods. Retinal cell markers were immunolabeled with Alexa 568-conjugated secondary antibodies. See Materials and Methods for specificity of marker antigens. All photographs were taken at low power using confocal microscopy. *B*, Embryonic chick retinal cultures were prepared from E8 retina, plated in 24-well plates, and transfected on culture day 1 with the M_2 promoter–luciferase reporter gene construct, as described in Materials and Methods. After transfection, cells were treated for 24 hr with conditioned medium collected on culture day 6 (*CD6*), culture day 13 (*CD13*), culture day 20 (*CD20*), or culture day 27 (*CD27*). *C*, Embryonic chick retinal cultures were prepared from E8 retina, plated in 24-well plates, and transfected on culture day 1 with the M_2 promoter–luciferase reporter gene construct, as described in Materials and Methods. After transfection, cells were treated for 48 hr with concentrated conditioned medium (*CM*) collected from a rat Müller glial cell line (*MGCL*), from SN56 neuroblastoma cells (*SN56*), and from IMR32 neuroblastoma cells (*IMR32*). The data represent luciferase activity/β-galactosidase activity, expressed as fold-increase compared with controls. Values are the mean ± SEM; $n = 3$.

mAChRs *in vivo*, concentrated SFCM and partially purified MARIA were injected into developing eggs at E6 and E8, respectively, and after 24–72 hr, the retinas were evaluated histologically. Because M_2 mAChR expression is not normally detected at E9, changes in M_2 mAChR expression at E9 could be easily detected. In addition, the zone of ceased mitotic activity has spread to the peripheral portion of the retina by E8 (Mey and Thanos, 1992). After 36 hr of treatment with concentrated SFCM and after only 24 hr of treatment with partially purified MARIA, significant M_2 mAChR expression was seen in the IPL of the E9 retina (Fig. 5), whereas no expression was seen in control retina. The expression pattern of M_2 mAChRs in the MARIA-treated retinas was similar to the pattern of expression seen at E15 and later. These data indicate that exogenously applied MARIA can specifically induce M_2 but not M_3 or M_4 receptor expression *in vivo*, resulting in precocious expression of M_2 mAChRs in tenascin-positive amacrine cells. Thus, it appears that MARIA has similar effects on M_2 mAChR expression *in vivo* as it does on M_2 mAChR expression *in vitro*, suggesting that it may be responsible for its normal developmental appearance *in vivo*.

MARIA can be found in media conditioned by mature cultured retinal cells, suggesting that it is a secreted protein (McKinnon et al., 1998). However, because retinal cultures contain a mixture of retinal neurons and Müller glia, the cellular source of MARIA had not been identified previously. We demonstrate here that the Müller glia appear to be the cellular source of MARIA. The appearance of MARIA has been shown to be culture age-dependent (McKinnon et al., 1998). We have demonstrated that the first appearance of M_2 mAChRs *in vivo* occurs at E10, and the level of expression increases dramatically over time. The Müller glia also initially appear in significant numbers at E10, and these numbers increase over time. The concomitant appearance of M_2 mAChRs *in vivo* with the developmental appearance of significant numbers of Müller glia is consistent with the hypothesis that the Müller glia is the cellular source of MARIA. To demonstrate more conclusively that the Müller glia secrete MARIA, we demonstrated that retinal cells in culture were essentially neuron-free after 20–27 d but still contained Müller glia. We found that the activity contained by the 13, 20, and 27 d cultures was greater than that contained by the 6 d cultures (Fig. 6*A*,*B*). Finally, MARIA can

be found in media conditioned by a rat Müller glial cell line but not in media conditioned by two different neuroblastoma cell lines (Fig. 6*C*). It is not known whether a signal from the neurons is required for the initiation of MARIA secretion, nor can it be ruled out that the neurons might also secrete MARIA. However, the Müller glia appear to be a strong cellular source of MARIA in the chick retina.

The data presented here describe the normal developmental expression of M_2, M_3, and M_4 mAChRs in the embryonic chick retina. These data also describe a potentially novel regulatory factor secreted by the Müller glia that appears to be responsible for the normal developmental appearance of M_2 mAChRs in the chick retina. Together, these data are important for understanding the role that mAChRs play in regulation of embryonic retinal function and development.

REFERENCES

Bartsch S, Husmann K, Schachner M, Bartsch U (1995) The extracellular matrix molecule tenascin: expression in the developing chick retinotectal system and substrate properties for retinal ganglion cell neurites *in vitro.* Eur J Neurosci 7:907–916.

Blusztajn JK, Venturini A, Jackson DA, Lee HJ, Wainer BH (1992) Acetylcholine synthesis and release is enhanced by dibutyryl cyclic AMP in a neuronal cell line derived from mouse septum. J Neurosci 12:793–799.

Bonaventure N, Jardon B, Sahel J, Wioland N (1989) Neurotransmission in the frog retina: possible physiological and histological correlations. Doc Ophthalmol 72:71–82.

Bradshaw AD, McNagny KM, Gervin DB, Can GM, Graf T, Clegg DO (1995) Integrin a2b1 mediates interactions between developing embryonic retinal cells and collagen. Development 121:3593–3602.

Catsicas M, Bonness V, Becker D, Mobbs P (1997) Spontaneous Ca^{+2} transients and their transmission in the developing chick retina. Curr Biol 8:283–286.

Cunningham JR, Dawson C, Neal MJ (1983) Evidence for a cholinergic inhibitory feed-back mechanism in the rabbit retina. J Physiol (Lond) 340:455–468.

Feller MB, Wellis DP, Stellwagen D, Werblin FS, Shatz CJ (1996) Requirement for cholinergic synaptic transmission in the propagation of spontaneous retinal waves. Science 272:1182–1187.

Fischer AJ, McKinnon JA, Nathanson NMN, Stell WK (1998) Identification and localization of muscarinic acetylcholine receptors in the ocular tissues of the chick. J Compar Neurol 392:273–284.

Halvorsen SW, Nathanson NM (1981) *In vivo* regulation of muscarinic acetylcholine receptor number and function in embryonic chick heart. J Biol Chem 256:7941–7948.

Hoffmann H-D (1988) Development of cholinergic retinal neurons from embryonic chicken in monolayer cultures: stimulation by glial derived factors. J Neurosci 8:1361–1369.

Jardon B, Bonaventure N, Scherrer E (1992) Possible involvement of cholinergic and glycinergic amacrine cells in the inhibition exerted by the ON retinal channel and the OFF retinal channel. Eur J Pharmacol 210:201–207.

Johnson JA, Nathanson NM (1994) Differential requirements for p21ras and protein kinase C in the regulation of neuronal gene expression by nerve growth factor and neurokines. J Biol Chem 269:18856–18863.

Linn DM (1998) A comparison of the inhibitory actions of glycine and GABA on acetylcholine release from the rabbit retina. Vis Neurosci 15:1057–1065.

McKinnon LA, Nathanson NM (1995) Tissue-specific regulation of muscarinic acetylcholine receptor expression during embryonic development. J Biol Chem 270:20636–20642.

McKinnon LA, Gunther EC, Nathanson NM (1998) Developmental regulation of the cm2 muscarinic acetylcholine receptor gene: selective induction by a secreted factor produced by embryonic chick retinal cells. J Neurosci 18:59–69.

Mey J, Thanos S (1992) Development of the visual system of the chick: a review. J Hirnforsch 33:673–702.

Millar TJ, Ishimoto I, Chubb IW, Epstein ML, Johnson CD, Morgan IG (1987) Cholinergic amacrine cells of the chicken retina: a light and electron microscope immunocytochemical study. Neuroscience 21:725–743.

Nathanson NM (1987) Molecular properties of the muscarinic acetylcholine receptor. Annu Rev Neurosci 10:195–236.

Neal MJ, Cunningham JR (1995) Baclofen enhancement of acetylcholine release from amacrine cells in the rabbit retina by reduction of glycinergic inhibition. J Physiol (Lond) 482:363–372.

Penn AA, Riquelme PA, Feller MB, Shatz CJ (1998) Competition in retinogeniculate patterning driven by spontaneous activity. Science 279:2108–2112.

Prada F, Medina JI, Lopez-Gallardo M, Lopez R, Quesada A, Spira A, Prada C (1999) Spatiotemporal gradients of differentiation of chick retina types I and II cholinergic cells: Identification of a common postmitotic cell population. J Comp Neurol 410:457–466.

Reh TA (1992) Cellular interactions determine neuronal phenotype in rodent retinal cultures. J Neurobiol 23:1067–1083.

Rosoff ML, Wei J, Nathanson NM (1996) Isolation and characterization of the chicken m2 acetylcholine receptor promoter region: induction of gene transcription by leukemic inhibitory factor and ciliary neurotrophic factor. Proc Natl Acad Sci USA 93:14889–14894.

Rotundo RL (1984) Purification and properties of the membrane bound form of acetylcholinesterase from chicken brain. J Biol Chem 259:13186–13194.

Sambrook J, Fritsch EF, Maniatis T (1989) Standard protocol for calcium phosphate-mediated transfection of adherent cells. In: Molecular cloning: a laboratory manual (Ford N, Ferguson M, eds), pp 16:33–16:36. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sarthy VP, Brojianm SJ, Dutt K, Kennedy BN, French RP, Crabb JW (1998) Establishment and characterization of a retinal Müller cell line. Invest Ophthalmol Vis Sci 39:212–216.

Schmidt M, Humphrey MF, Wässle H (1987) Action and localization of acetylcholine in the cat retina. J Neurophysiol 58:997–1015.

Sernagor E, Grzywacz NM (1996) Influence of spontaneous activity and visual experience on developing retinal receptive fields. Curr Biol 6:1503–1508.

Skorupa AF, Klein WL (1993) Developmentally regulated secreted factors control expression of muscarinic receptor subtypes in embryonic chick retina. J Neurochem 60:2087–2097.

Sugiyama H, Daniels MP, Nirenberg M (1977) Muscarinic acetylcholine receptors of the developing retina. Proc Natl Acad Sci USA 74:5524–5528.

Wess J (1996) Molecular biology of muscarinic acetylcholine receptors. Crit Rev Neurobiol 10:69–99.

Willbold E, Reincke M, Lance-Jones C, Lagenaur C, Lemmon V, Layer PG (1995) Müller glia stabilizes cell columns during retinal development: lateral cell migration but not neuropil growth is inhibited in mixed chick-quail retinospheroids. Eur J Neurosci 7:2277–2284.

Wong RD (1999) Retinal waves and visual system development. Annu Rev Neurosci 22:29–47.

Wong WT, Sanes JR, Wong ROL (1998) Developmentally regulated spontaneous activity in the embryonic chick retina. J Neurosci 18:8839–8852.

Zhao D, Cornett L, Zhou ZJ (1999) Consistency and modulation of spontaneous waves of excitation in the developing rabbit retina. Soc Neurosci Abstr 25:721.3.

Zhou ZJ (1998) Direct participation of starburst amacrine cells in spontaneous rhythmic activities in the developing mammalian retina. J Neurosci 18:4155–4165.

Zhou ZJ, Zhao D (1999) Age-dependent and receptor type-specific role of acetylcholine in spontaneous waves of retinal excitation. Soc Neurosci Abstr 25:1042.