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α-Aminoadipate Induces Progenitor Cell Properties of Müller Glia in Adult Mice

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Abstract

PURPOSE—Retinal Müller glia in higher vertebrates have been reported to possess progenitor cell properties and the ability to generate new neurons after injury. This study was conducted to determine the signals that can activate this dormant capacity of Müller glia in adult mice, by studying their behavior during glutamate stimulation.

METHODS—Various concentrations of glutamate and its analogue α-aminoadipate, which specifically binds Müller glia, were injected subretinally in adult mice. Proliferating retinal cells were labeled by subretinal injection of 5′-bromo-2′-deoxyuridine (BrdU) followed by immunohistochemistry. Müller cell fates were analyzed in retinal sections by using double immunolabeling with primary antibodies against Müller and other retinaspecific cell markers. The effects of glutamate and α-aminoadipate were also determined in purified Müller cell cultures.

RESULTS—Although high levels of glutamate induce retinal damage, subtoxic levels of glutamate directly stimulate Müller glia to re-enter the cell cycle and induce neurogenesis in vivo and in purified Müller cell cultures. α-Aminoadipate, which selectively target glial cells, also induced expression of progenitor cell markers by Müller cells in vitro or stimulated Müller cell migration to the outer nuclear layer (ONL) and to differentiate into photoreceptors in vivo.

CONCLUSIONS—Mature Müller glia in adult mice can be induced to dedifferentiate, migrate, and generate new retinal neurons and photoreceptor cells by α-aminoadipate or glutamate signaling. The results of this study suggest a novel potential strategy for treating retinal neurodegeneration, including retinitis pigmentosa and age-related macular degeneration, without transplanting exogenous cells.

The central nervous system (CNS) of adult mammals has long been thought to be incapable of regeneration or self-repair. However, several lines of evidence have revealed that neural stem cells or progenitors exist in the mature CNS and are potentially capable of producing new neurons.[1] Indeed, in certain areas, such as the hippocampus and olfactory bulb, new neurons are continuously generated throughout life.[2,3] Furthermore, various insults or specific cues stimulate the proliferation of endogenous progenitors in regions where neurogenesis does not occur under physiologic conditions.[4,5] These findings suggest that it may be possible to repair

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a damaged CNS by activating dormant endogenous progenitor cells; however, the molecular signal that triggers the neurogenic process by the endogenous progenitor cells remains unknown.

Retinal neurons in higher vertebrates, like those in other parts of the CNS, have a limited ability to regenerate. In gold-fish retina, however, acute neuronal damage stimulates intrinsic photoreceptor progenitors to migrate to the outer nuclear layer (ONL) and produce new photoreceptors and all types of retinal neurons.[6–9] In posthatching chicks (7 days after birth), acute retinal damage caused by a toxic dose of the glutamate analogue NMDA induces Müller glia to re-enter the cell cycle, express neural progenitor cell markers, migrate to the ONL, and at least when examined in culture, differentiate into retina-specific neurons.[10] Müller glia of the rodent retina have a gene expression profile resembling that of progenitor cells[11] and are recently shown to proliferate and produce new neurons in response to acute retinal injury.[12] However, molecular signals that trigger the dedifferentiation and neuroregeneration of Müller glia under these conditions remain largely unknown. In this study, we found evidence that glutamate stimulation itself can induce Müller glia in the adult mouse retina to become progenitor cells and generate new photoreceptors. Based on these observations, we report a highly efficient method of inducing photoreceptor regeneration.

MATERIALS AND METHODS

Subretinal Injection and BrdU Administration

B6/129SF2 mice (age, 2–14 months) were maintained in the animal facility of the Schepens Eye Research Institute. All experimental procedures were approved by the Institute's Animal Care and Use Committee and adhered to the Statement for the Use of Animals in Ophthalmic and Vision Research. Adult mice were anesthetized by intraperitoneal injection of ketamine (62.5 mg/kg) and xylazine (12.5 mg/kg). One microliter of saline or a solution of L-glutamate (0.150 μg) or α-aminoadipate (α-AA; 1 μg) that contained 5′-bromo-2′-deoxyuridine (BrdU; 10 μg) (all from Sigma-Aldrich, St. Louis, MO) was injected slowly through a glass micropipette into the subretinal space. To target neurons in the outer retina, we injected these chemicals subretinally rather than intravitreously. Subretinal injection of either saline or a solution containing glutamate or α-AA caused a transient detachment of the retina from the retinal pigment epithelium layer at the injection site that lasted no longer than 24 hours. Mice were killed 4 to 12 hours and 1 to 21 days after treatment and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde before retinal wholemounts were prepared. In other experiments, after fixation, mouse eyeballs were collected, cryoprotected with 30% sucrose in PBS, embedded in optimal cutting temperature (OCT) compound (Tissue Tek; Sakura Finetek, Torrance, CA), and sectioned at 14 μm.

Histology and Immunohistochemistry

TUNEL was performed as described.[13] For immunostaining, retinal sections were preblocked with PBS containing 3% bovine serum albumin (Sigma-Aldrich) and 0.3% Triton X-100 (Sigma-Aldrich) for 1 hour at room temperature (RT) and incubated with primary antibody overnight at 4°C and with secondary antibody for 2 hours at RT. To identify $BrdU^+$ cells, retinal sections were also treated with HCl (2.0 M) for 1 hour at RT and reacted with anti-BrdU (1:200; Chemicon, Temecula, CA) in PBS containing 1% bovine serum albumin, 1% dimethylsulfoxide, and 0.1% Triton X-100. Other antibodies used were Cy3-conjugated mouse anti-GFAP (1:2000; Sigma-Aldrich); biotin-conjugated mouse anti-isolectin B4 (1:100; Sigma-Aldrich); monoclonal antibodies against glutamine synthetase, NeuN, β-III tubulin, cyclin D3, and nestin and rabbit polyclonal antibodies against recoverin and syntaxin (1:200; all from Chemicon); rabbit anti-pHisH3, PKCα (1:200; Santa Cruz Biotechnology, Santa Cruz, CA); sheep anti-Chx10 (1:200; Exalpha Biologicals, Watertown, MA); mouse anti-rhodopsin

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(Rho1D4;1:4000, courtesy of Robert S. Molday, University of British Columbia, Canada)[14]; rabbit anti-CRALBP (1:5000; courtesy of John C. Saari, University of Washington, Seattle) [15]; rabbit anti-Nr2e3 (1:2000; courtesy of Shiming Chen, Washington University School of Medicine, St. Louis),[16] and secondary antibodies conjugated with fluorescein (Vector Laboratories, Burlingame, CA) or Cy2, Cy3, or Cy5 (Jackson ImmunoResearch Laboratories, West Grove, PA). Rat anti-BrdU (1:80; Novus Biologicals, Littleton, CO) was also used for retinal double staining, followed by reactions with biotinylated anti-rat IgG (1:50, Sigma-Aldrich) and Cy2- or Cy3-conjugated streptavidin (1:100, Jackson ImmunoResearch Laboratories). Retinal sections were also counterstained by a nuclear marker, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 mg/mL in PBS; Sigma-Aldrich) or a nuclear stain (SYTOX green; Invitrogen), to reveal the retinal cellular structure.

Isolation, Culture, and Treatment of Müller Glia

Müller glial cells were isolated as described,[17] cultured in Dulbecco's modified Eagles' medium and F12 (1:1) in the presence of 10% FBS for 7 days, and transferred to serum-free medium (Dulbecco's modified Eagles' medium and F12) containing BrdU (5 μg/mL), L-glutamate (10–500 μM), or α-AA (10–500 μM). After 48 hours, the cultures were fixed with 4% paraformaldehyde and subjected to BrdU labeling, immunohistochemistry, and nuclear stain with DAPI (1 mg/mL in PBS; Sigma-Aldrich).

Cell Culture

Adult mice were killed 1 day after subretinal injection of α-AA (1 μg) and BrdU (32.5 μM). The retinas were dissected 0.5 mm from the ora serrata to avoid contamination with retinal stem cells at the ciliary margin[18] and dissociated with papain as described.[19] Cells (~3×10^5) were plated into four-well culture chambers (Nalge Nunc, Naperville, IL) precoated with poly-L-lysine and laminin (10 μg/mL), and cultured in defined medium,[20,21] containing basal neuronal cell medium (Neurobasal; Invitrogen) supplemented with B27 (Invitrogen), 5% fetal bovine serum (Sigma-Aldrich), insulin (20 μg/mL), brain-derived neurotrophic factor (10 ng/mL), neurotrophin-3 (10 ng/mL), basic fibroblast growth factor (25 ng/mL), epidermal growth factor (50 ng/mL), forskolin (25 μM), cpt-cAMP (0.1 mM), *N*-acetyl-L-cysteine (1 mM), and penicillin-streptomycin. After 7 days, the cells were fixed with 4% paraformaldehyde and processed for immunohistochemistry.

RESULTS

Effect of Glutamate on Retinal Cell Proliferation

To determine whether glutamate stimulation induces retinal damage and neurogenesis from mouse Müller glia, we injected various concentrations of L-glutamate (0.1–50 μg) subretinally in adult mice (age, 2–14 months). Dividing cells in the retina were labeled by subretinal (10 μg) injection of BrdU. A high concentration of glutamate has been shown to induce neuronal apoptosis that can be detected by Tdt-dUTP terminal nick-end labeling (TUNEL) 1 day after treatment.[22,23] $TUNEL^+$ cells were rare in retinal sections of mice treated with saline or <10 μg L-glutamate, but were numerous in those treated with 50 μg L-glutamate (Fig. 1A). Thus, a 10-μg or lower concentration of glutamate was referred to as a subtoxic level of glutamate stimulation. As expected, retinal sections of saline controls contained almost no $BrdU^+$ cells (Fig. 1B).[12,24] In contrast, numerous cells were seen in sections from L-glutamate-treated mice—surprisingly, even after subtoxic doses (1–10 μg) of glutamate stimulation (Fig. 1C), which caused little retinal neuron apoptosis. Most strikingly, $BrdU^+$ cells were present in the ONL (Fig. 1C).

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Proliferating Cells from the Müller Glia

The fact that low doses of L-glutamate induced cell proliferation without causing neuronal apoptosis prompted us to ask whether glutamate, rather than the neural injury signal, directly stimulates Müller glia to re-enter the cell cycle. To test this hypothesis, we injected adult mice (age, 2–9 months) subretinally with α-aminoadipate (α-AA), a glutamate analogue that specifically targets astrocytes and Müller glia.[25–27] After 24 hours, the mice were killed, and the retinas were harvested. $TUNEL^+$ cells were rare after treatment with saline or a low dose (1 μg) of α-AA (Fig. 1A). Similar to observations after subtoxic levels of glutamate stimulation, 1 day after treatment, $BrdU^+$ nuclei were numerous in the ONL of α-AA-treated mice (Fig. 1D), but not in the saline control animals (Fig. 1B). Immunostaining for phospho-histone H3 (pHisH3), which is expressed only by M-phase mitotic cells, was negative in saline-treated retinas (Fig. 1I). In contrast, 1 day after α-AA-treatment, pHisH3-immunoreactive cells colocalized with $BrdU^+$ nuclei were detected (Figs. 1E–H), and 79.7% ± 1.8% of $BrdU^+$ cells were colabeled for $pHisH3^+$, indicating that $BrdU^+$ cells were indeed dividing.

To preclude that $BrdU^+$ cells were undergoing aberrant entry of cell cycle and delayed apoptosis,[28,29] retinal sections were double labeled with TUNEL and anti-BrdU at 1 to 3 days after subretinal injection of BrdU and α-AA. A few $TUNEL^+$ cells were found in retinal sections taken at 24 hours after saline (not shown) or α-AA (Fig. 1J–L) injection. Most $BrdU^+$ cells were not seen to undergo apoptosis, and only 2.4% ± 0.04% of them were colabeled for TUNEL. At 48 hours after injection, no $TUNEL^+$ cells were detected, correlating with the transient retinal detachment induced by subretinal injection. At 3 weeks of α-AA treatment, many $BrdU^+$ cells were still alive and localized to the ONL and INL, suggesting further that these were not apoptotic neurons resuming DNA synthesis before death.

To confirm that the $BrdU^+$ cells originated from Müller glia, we double stained retinal sections for BrdU and Müller glial cell markers glutamine synthetase and cellular retinaldehyde-binding protein (CRALBP; Fig. 2A). Sections were also stained for protein kinase C-α (PKCα; bipolar cells; Fig. 2B), isolectin B4 (microglial cells; Fig. 2C), and recoverin (photoreceptors; not shown). In control retinas, CRALBP staining revealed Müller glial cell bodies in the INL, with long processes spanning the entire retina (Fig. 2A). Twelve hours after α-AA injection, staining for CRALBP (Figs. 2D–F) and glutamine synthetase (not shown) was consistently more intense in the ONL and, at a higher magnification (Figs. 2J–M), they were seen to colocalize with $BrdU^+$ nuclei. One day after α-AA-treatment, CRALBP staining for Müller glia was detected primarily in the ONL, often colocalized with $BrdU^+$ nuclei (Figs. 2G–I). We counted 97.9% ± 0.5% $BrdU^+$ nuclei colocalized with CRALBP labeling. $BrdU^+$ cells did not colocalize with other retinal cell markers (Figs. 2B, 2C). Consistent with that result, subtoxic doses of L-glutamate or α-AA induced little neural damage, activation of microglia in the treated retinas was not dramatic, and few cells stained for BrdU. Induction of reactive gliosis or elevation of GFAP in Müller glia was observed in both saline- and α-AA-injected retinas, and no significant difference in levels of GFAP elevation was observed between saline- and α-AA-injected groups at 1 to 7 days after injection (not shown).

Because retinal pigment epithelial (RPE) cells also express CRALBP and glutamine synthetase, we examined especially the retinal sections taken at the earliest time points, 6 to 8 hours, after α-AA injection. Glutamine synthetase and BrdU double labeling revealed that $BrdU^+$ cells were primarily in the INL (Figs. 3A–C), suggesting that they arose from Müller rather than RPE cells. To demonstrate further that α-AA stimulates Müller glia proliferation and migration, we stained retinal sections with a primary antibody against another Müller glial marker, cyclin D3. It has been reported that Müller glia in the rodent retina upregulate cyclin D3 before re-entering the cell cycle but quickly downregulate it in concert with an exit from mitosis.[30] After saline injection, a moderate level of cyclin D3 was detected in the nuclei of Müller glia, lying as a restricted band in the middle of the INL (Fig. 3D). As early as 6 hours

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after α-AA injection, a dramatic increase of cyclin D3 expression was noted in the nuclei of Müller glia (Fig. 3E). Strikingly, rather than lying in a restricted band, some of the brightly labeled Müller glial cells were seen migrating away from the Müller glial cell layer in the INL and entering the IPL and ONL (Figs. 3F–I). In agreement with the previous report,[30] these cells downregulated cyclin D3, as soon as they exited the cell cycle after entering the ONL. Thus, the data indicate that the $BrdU^+$ cells induced by α-AA injection are Müller cells.

To corroborate this finding, we also used a mouse line expressing a green fluorescence protein (GFP) transgene driven by the GFAP promoter (GFAP-GFP) so that their Müller glia are positive for GFP.[31,32] BrdU and α-AA were injected subretinally into adult wild-type and GFAP-GFP mice. At 24 hours after injection, the retinas were harvested, dissociated, washed to remove unincorporated BrdU, and plated in culture for 6 hours. The cells were stained with primary antibodies specific for retinal neurons and glial cells as well as for GFP. We noted that almost all cells incorporating BrdU were immunopositive for the Müller glia–specific marker CRALBP (Figs. 4A–C), and the glia were also GFP^+ if the cells were isolated from GFAP-GFP mice (Figs. 4D–F). No $BrdU^+$ nuclei were found to colocalize with other retinal neuron markers that have been mentioned herein. The data indicate again that the proliferating cells induced by subtoxic doses of glutamate or α-AA stimulation originated from Müller glia.

Direct Glutamate Stimulation of Müller Glial Proliferation

Next, we studied purified Müller glial cell cultures derived from P14 mouse retinas as described.[17] Most cultured cells displayed fusiform or epithelioid cell morphology (Figs. 5A, 5B) but not for neuronal markers, βIII-tubulin, medium-molecular- weight neurofilament protein, or other retinal cell markers such as recoverin and PKCα. Cell counts revealed that 89.9% ± 3.4% of all cultured cells were $GFAP^+$ or 85.7% ± 2.6% $CRALBP^+$. To assess proliferative capacity, Müller cells were maintained in a serum-free culture medium that contained BrdU. In the absence of mitogens or glutamate stimulation, the cells were quiescent (Figs. 5C–E, 5I). However, within 48 hours after treatment with low doses (0.05–2.5 mM) of L-glutamate or α-AA, the cells became $BrdU^+$ (Figs. 5F–I). In every 100 $BrdU^+$ cells examined, 98.2% ± 3.1% cells were found to be $GFAP^+$. The data further demonstrate that L-glutamate directly stimulated Müller glia to re-enter the cell cycle and exhibit progenitor cell properties.

Differentiation of $BrdU^+$ Cells In Vitro

To test further whether L-glutamate or α-AA-induced $BrdU^+$ cells can generate retina-specific neurons, mice received a subretinal injection of α-AA and BrdU. One day after treatment, retinal cells were obtained from the central retina (to exclude retinal stem cells at the ciliary margin), dissociated, and cultured for 7 to 14 days. Very few cells from saline-treated control cultures survived more than 3 days, but numerous cells from α-AA-treated retina survived more than 7 days, and most were $BrdU^+$. Some of the cells continued to divide for a few days, and a few formed small sphere-like clusters (not shown). After 7 days, the majority of $BrdU^+$ cells displayed differentiated cell morphology and expressed markers of mature retinal neurons or glial cells. Many were GFAP positive (Fig. 6A), but some were positive for βIII-tubulin (Fig. 6B), syntaxin (Fig. 6C), PKCα (Fig. 6D), and recoverin (Fig. 6E). Thus, the $BrdU^+$ cells have the potential to differentiate into various retina-specific neurons.

α-AA Stimulation of Müller Glia Differentiation In Vivo

Next, we studied the fate of proliferating Müller glial cells after α-AA injection in vivo. First, to determine the kinetics of α-AA-induced cell division at various time points after injection, we injected α-AA solution subretinally in the absence of BrdU, and killed the mice at 10 hours and 1, 2, 3, 7, and 21 days after injection. At 4 hours before death, the mice received a single intraperitoneal injection of BrdU, to label proliferating cells. Mouse retinal sections were prepared and immunolabeled for BrdU, and the cells were counted. $BrdU^+$ cells were detected

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10 hours after α-AA treatment, peaked on day 1, decreased rapidly after day 2, and were undetectable after day 7 (Figs. 7A–D), suggesting that a single injection of α-AA causes a transient induction of Müller cell proliferation.

Second, to determine the fate of $BrdU^+$ cells in vivo, retinal sections were collected 1 to 21 days after subretinal injection of α-AA and BrdU and double stained for BrdU and markers of retinal progenitor cells or mature retinal neurons. One day after α-AA injection, $BrdU^+$ cells were spherical and scattered in the IPL and the inner layer of the ONL (Fig. 7B), and many expressed the retinal progenitor cell markers nestin (Figs. 7E–H) and Chx10 (Figs. 7I–L). In control (saline) retinas, only bipolar cells were positive for Chx10, and no $Chx10^+$ cells were detected in the ONL (not shown). Three days after α-AA injection, $BrdU^+$ cells were detected throughout the ONL; some no longer expressed nestin or Chx10. The distribution and nuclear size of $Chx10^+$ bipolar cells were not affected by either saline or α-AA injection (Figs. 7M, 7N). By day 7 after α-AA treatment, most $BrdU^+$ cells in the ONL expressed the nuclear marker for photoreceptor cells Nr2e3[16] (Figs. 8A–D); by day 21, >95% expressed the marker of mature photoreceptor cells rhodopsin (Figs. 8E–H). A few $BrdU^+$ cells were seen in the INL that expressed the Müller glial marker glutamine synthetase (not shown).

To demonstrate further that $BrdU^+$ cells differentiated into photoreceptors or Müller glia, at 14 to 21 days after BrdU and α-AA injection, the retinas were dissociated and plated in culture for 6 hours. The cells were double labeled with primary antibodies against BrdU and recoverin or CRALBP. Consistent with the in vivo observation, many $BrdU^+$ cells were immunopositive for recoverin (Figs. 8I–K), and few were $CRALBP^+$ (Figs. 8L–N). No cells in α-AA-treated retina identified by immunostaining for markers of retinal ganglion cells (Brn-3b) or bipolar cells (PKCα) were $BrdU^+$. Together, these data demonstrate that Müller cells can be induced to dedifferentiate, migrate, and generate new photoreceptors on α-aminoadipate stimulation.

DISCUSSION

In this study, subtoxic doses of glutamate stimulated Müller glia of adult mice to re-enter the cell cycle, become progenitor cells, and generate retina-specific neurons and photoreceptor cells. The notion that Müller glia may serve as a potential source of neuroregeneration[33] and give rise to retinal neurons is not without precedent. In fish and higher vertebrates, neural damage caused by excessive amounts of NMDA activates Müller glia to generate new retinal neurons.[6,10,12] In the present study, glutamate, without causing neural damage, signaled adult murine Müller glia to express progenitor cell markers in vivo. In isolated Müller glial cell cultures, L-glutamate induced Müller glia to lose their glial phenotype, express progenitor cell markers, and divide. Subtoxic doses of L-glutamate in vivo triggered many Müller glia to re-enter the cell cycle. The glutamate analogue α-AA, which targets Müller glia, also induced the cells to dedifferentiate, express the progenitor cell markers nestin and Chx10, migrate to the ONL, and divide. These findings support the emerging view that Müller glia in adult mammals can behave as progenitor cells and serve as a source of neuroregeneration.

A small number of new neurons can be produced in the retina or CNS of adult mammals by acute neural injuries.[4,5] Our data indicated that acute neuronal injury led to the neuronal release of glutamate, which served as a signal to stimulate neurogenesis from progenitor cells in the mature retina and CNS. These data thus suggest a novel function of glutamate in retina or CNS regeneration and self-repair after injury and neurodegenerative diseases.

When examining glutamate or α-AA-induced cell proliferation, we noted that only 2.4% ± 0.04% $BrdU^+$ cells were costained for TUNEL, suggesting that most of the $BrdU^+$ cells were not dying cells resuming DNA synthesis before death. However, because the in vivo TUNEL technique detects apoptotic cells only during a short window (1–2 hours after cell death occurs),

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it is likely that there were more dying $BrdU^+$ cells than we counted. Moreover, some $BrdU^+$ cells might undergo necrosis, which would not be detectable by TUNEL. Nevertheless, a similar number of $TUNEL^+$ cells was also detectable in saline-injected retinal sections within 24 hours after injection, suggesting that this cell apoptosis was not induced by α-AA or glutamate; rather, it was a result of the transient retinal detachment caused by the subretinal injection procedure. The fact that many $BrdU^+$ cells were found alive 3 weeks after α-AA treatment, but not in the saline-treated group, and that these $BrdU^+$ cells expressed mature photoreceptor or other retinal cell markers demonstrates that most of the $BrdU^+$ cells were not apoptotic neurons.

At high levels, α-AA, a dicarboxylic amino acid homologous to L-glutamate, causes selective gliotoxicity by serving as a substrate for glutamate transporters,[34] but acts as a weak antagonist of NMDA receptors at neuronal receptor sites.[26] How glutamate or α-AA stimulates neurogenesis in adult mouse retina is unknown. Nor is it known whether glutamate activates progenitor cell properties by binding to receptors on Müller glia or entering the cells via glutamate transporters. Recent reports suggest several signaling molecules, including TGFβ, Notch, and wnt, that are central to the regulation of postnatal retinogenesis or the progenitor cell properties of Müller glia in vivo.[35,36] It would be most interesting to ask whether glutamate or α-AA stimulation supports progenitor cell proliferation by regulating the signaling events mediated by these molecules to control Müller glia proliferation and dedifferentiation.

In any case, our findings demonstrate that Müller glial cells in the adult mouse retina can be reverted to a progenitor-like state and generate new neurons and photoreceptor cells. New photoreceptor cells in the retina have been generated in lower vertebrates in vivo[8,37] but not in mammals. Our findings suggest that photoreceptor cells may be regenerated in adult mice by manipulation of the glutamate signal. In vivo, glutamateinduced progenitor cells migrated to the ONL and generated primarily photoreceptors. In culture, these cells gave rise to almost all types of retinal neurons and glial cells, including amacrine, bipolar, and photoreceptor cells; astrocytes; and Müller glia. The results suggest that the microenvironment in the ONL of the adult mouse retina supports the generation of photoreceptor cells and that interaction between the progenitor cells and their microenvironment may be important determinants of cell fate. It will be extremely interesting to determine whether newly generated photoreceptors integrate and form functional connections with the existing retinal circuitry.

In conclusion, our findings suggest that it may be possible to manipulate endogenous neural progenitor cells to undergo neuroregeneration and repair in the retina and CNS of adult mammals. Elucidation of the molecular mechanisms underlying glutamate-induced neurogenesis may allow the development of therapies for neurodegenerative disease and CNS injuries without transplanting exogenous cells.

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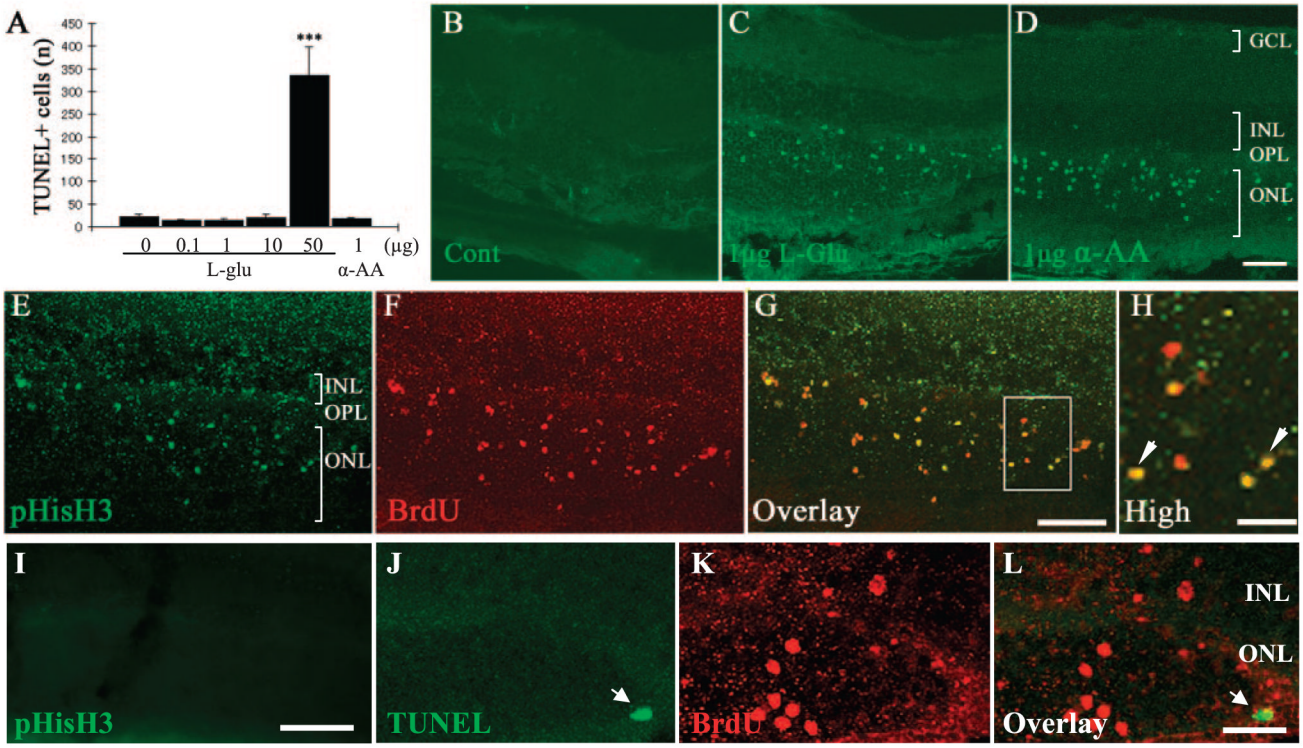

FIGURE 1. Glutamate induced retinal cell apoptosis and cell proliferation in adult mice

(**A**) The number of TUNEL$^+$ cells in vertical sections of the adult mouse retina prepared 1 day after subretinal injection of 1 μL of saline (Cont), L-glutamate (L-glu, 0.1–50 μg), or α-AA (1 μg). Apoptotic cells were significantly increased only after treatment with 50 μg of L-glutamate. ***$P < 0.001$ (two-tailed *t*-test). (**B–D**) BrdU$^+$ cells in retinal sections 1 day after subretinal injection of (**B**) saline (Cont), (**C**) L-glutamate (L-glu; 1 μg), or (D) α-AA (1 μg), followed by retinal injection of BrdU (32.5 μM). Numerous BrdU$^+$ cells were present in the ONL of retinas treated with L-glutamate or α-AA (1 μg) but not in saline-treated control retinas. *Arrows*: BrdU$^+$ cells in the INL or OPL. GCL, ganglion cell layer. (**E–I**) BrdU labeling and pHisH3-immunoreactivity in retinal sections 1 day after subretinal injection of α-AA (1 μg) (**E–H**) or saline (**I**). *Arrowheads*: nuclei double-labeled with BrdU and pHisH3. (**J–L**) Photomicrographs of a retinal section taken at 24 hours after α-AA injection and labeled by TUNEL (*green*) and anti-BrdU (*red*). TUNEL (*arrow*) did not colocalize with anti-BrdU labeling. Scale bar: (**B–G, I–L**) 40 μm; (**H**) 16 μm.

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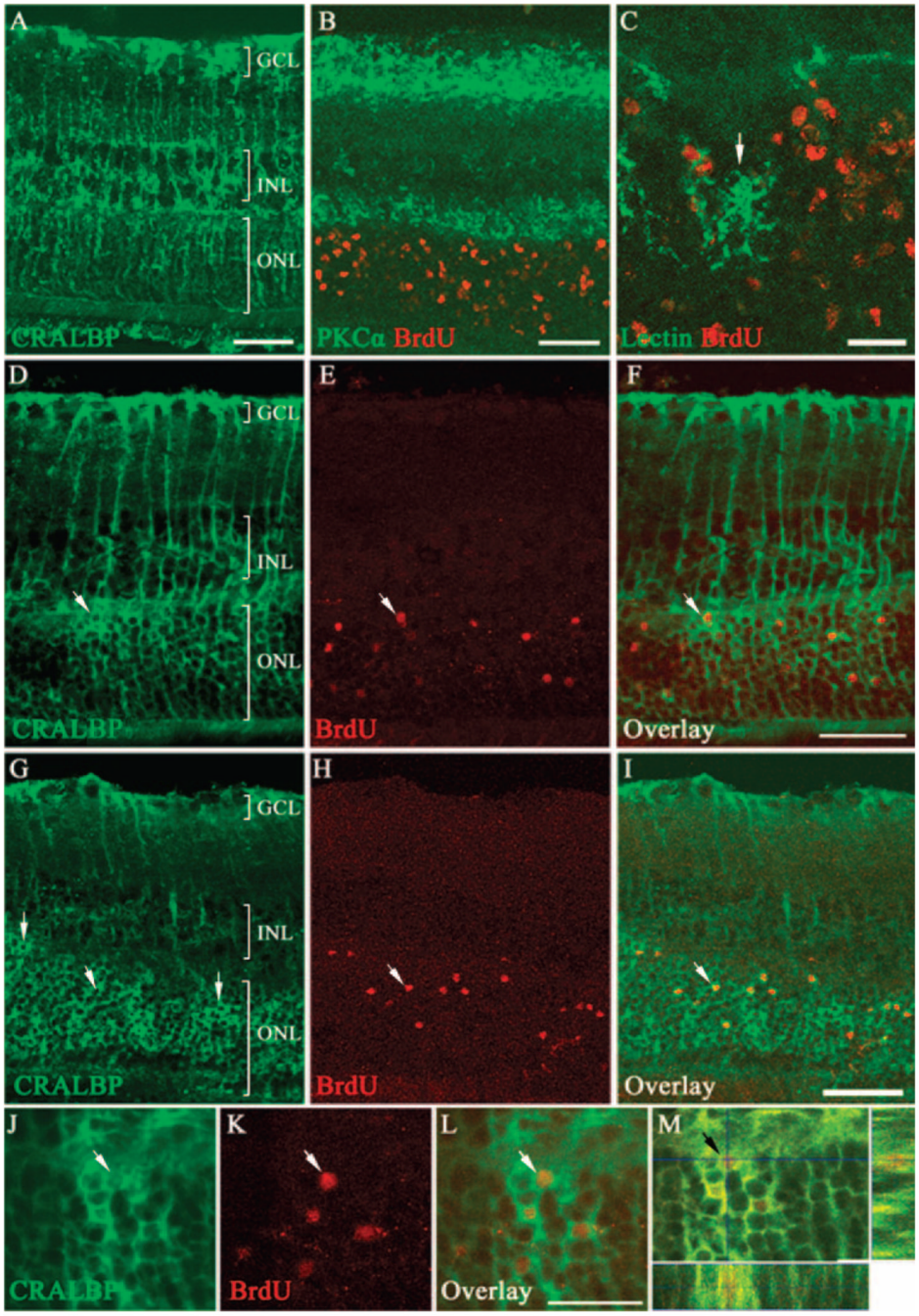

FIGURE 2. Colocalization of BrdU nuclear labeling with Müller glial cell markers

(**A–C**) Retinal sections collected 1 day after saline (**A**) or α-AA treatment (**B, C**) were stained with antibodies against CRALBP (**A**) or double-stained with antibodies against BrdU and either the bipolar cell marker PKCα (**B**) or the microglia marker isolectin (**C, *arrow***). BrdU nuclear labeling did not colocalize with any of these retinal markers in the double-labeling experiment. *Arrows*: isolectin^{+} cells (**C**). (**D–I**) Photomicrographs of retinal sections prepared at 12 hours (**D–F**) and 1 day (**G–I**) after α-AA treatment and double-stained with antibodies against BrdU and CRALBP. *Arrows*: Müller cell nuclei costained by anti-BrdU. One day after α-AA injection, many BrdU^{+}/CRALBP^{+} cells appeared in the ONL. (**J–M**) Orthogonal confocal

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images show colocalization of $BrdU^+$ nuclei in cells labeled with CRALBP in the ONL (***arrows***). ***Arrows***: Müller cell nuclei. Scale bars: 40 μm (**A, B, E–J**); 16 μm (**C, D**).

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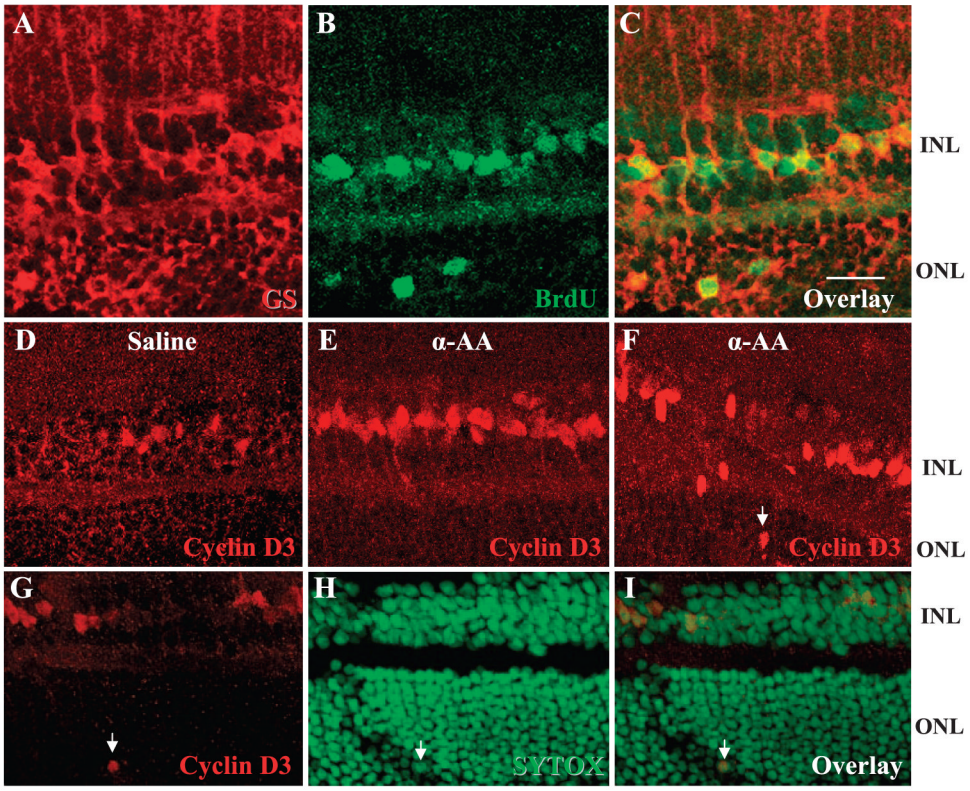

FIGURE 3. Müller glia upregulated cyclin D3 and migrated to the IPL and ONL after α-AA stimulation

(**A–C**) Photomicrographs of retinal sections taken at 8 hours after α-AA treatment and double-labeled by anti-BrdU (***green***) and glutamine synthetase (GS; red). (**D–H**) Retinal sections collected 6 hours after saline (**D**) or α-AA (**E–I**) injection were immunolabeled with a primary antibody against cyclin D3 (**D, E, G**) and counterstained with a ***green*** nuclear marker (**H, I**). ***Arrows***: cell nuclei that migrated into the ONL (**H–I**). Note that the missing cyclin $D3^+$ nuclei in the INL appeared in the IPL and ONL. Scale bars, 40 μm.

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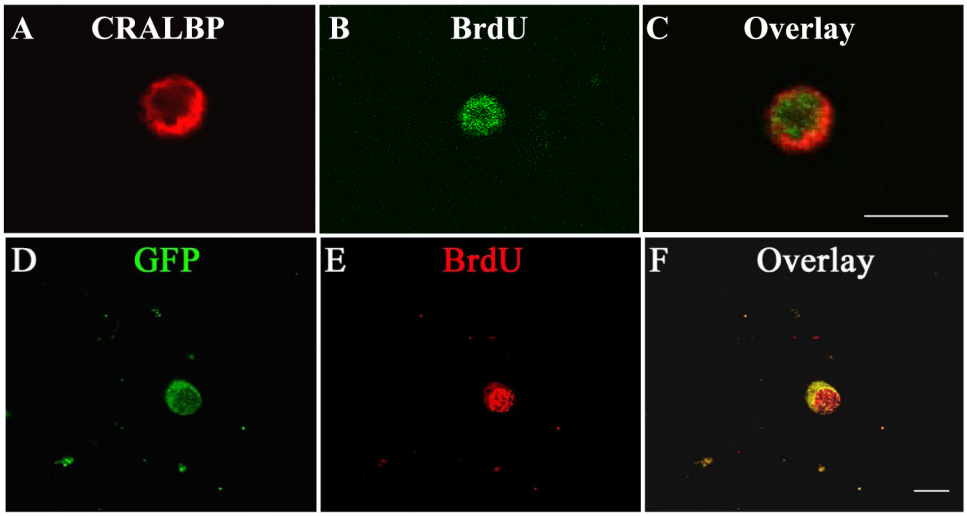

FIGURE 4. BrdU$^+$ cells originating from Müller glia

Retinal cells that were harvested at 24 hours after injection from α-AA-treated wild-type (**A–C**) or GFAP-GFP (**D–F**) mice were dissociated, plated in culture for 6 hours, and double labeled for anti-BrdU and CRALBP (**A–C**) or GFP (**D– F**). Scale bar, 10 μm.

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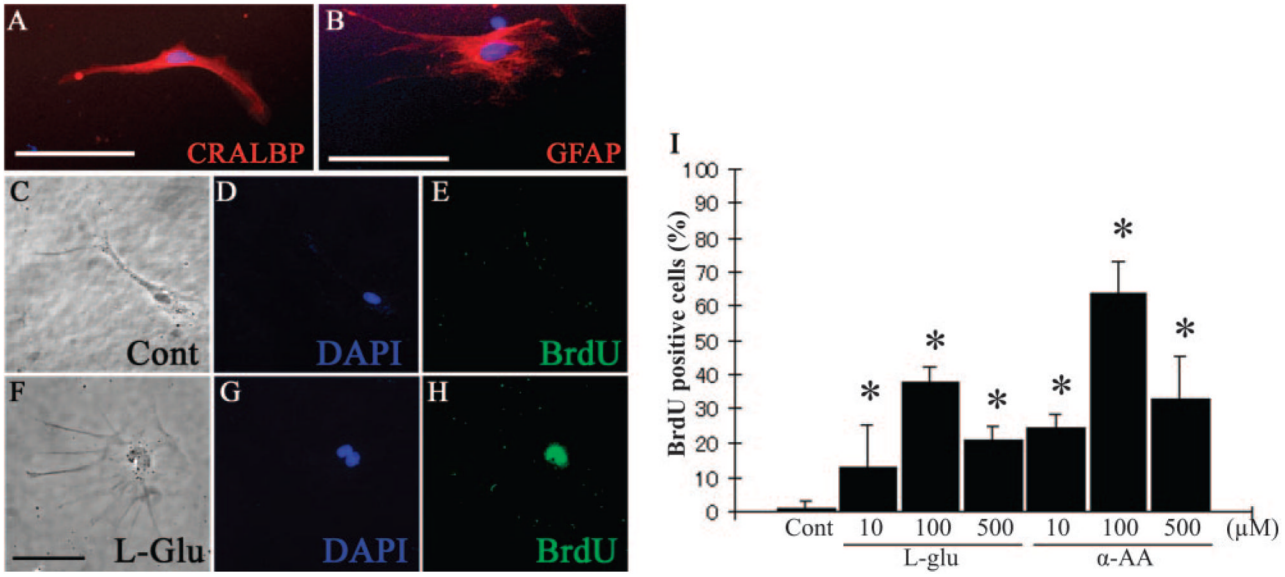

FIGURE 5. Glutamate induced Müller glial proliferation in purified cell cultures

(**A, B**) Photomicrographs of cultured Müller glial cells that were double-stained with DAPI and antibodies against CRALBP (**A**) or GFAP (**B**). (**C–H**) Photomicrographs of Müller glial cultures treated with (**C–E**) control solution or (**F–H**) L-glutamate (0.5 mM) and double-stained with DAPI and antibodies against BrdU. (**I**) Incorporation of BrdU 48 hours after treatment with saline, L-glutamate, or α-AA. Data are the mean ± SD. **P* < 0.001 versus control (two-tailed *t*-test). Scale bar, 10 μm.

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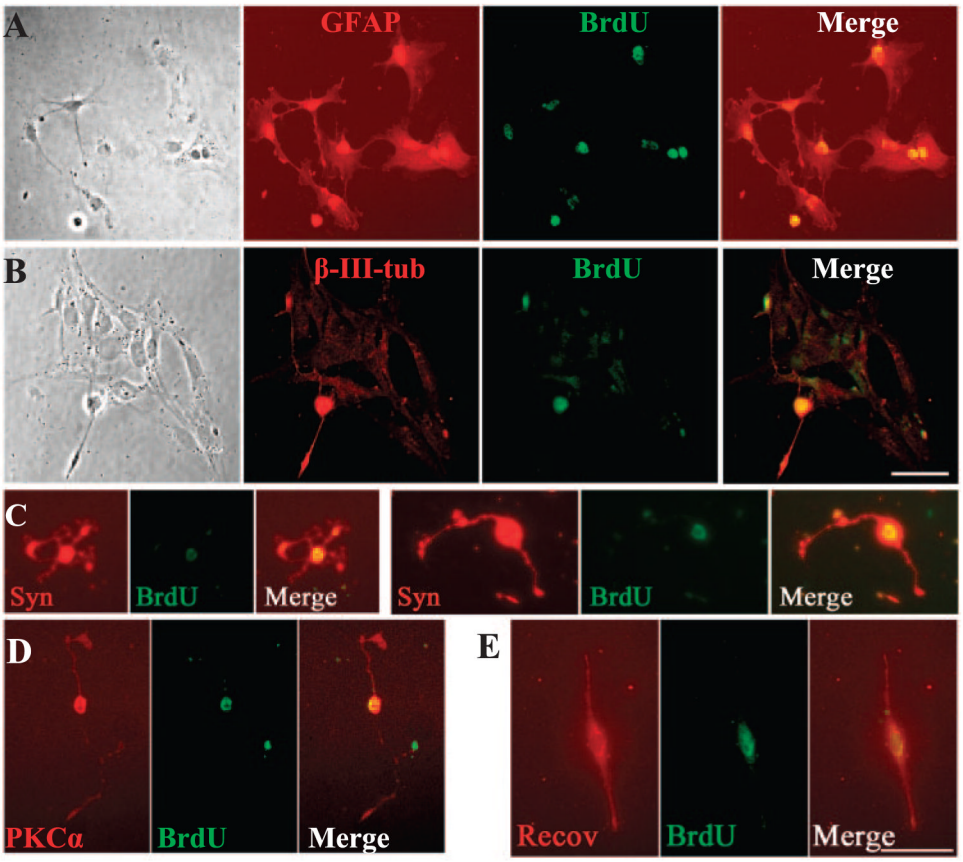

FIGURE 6. Progenitor-like Müller glia produce retina-specific neurons and glial cells in culture

(**A–E**) Photomicrographs of dissociated retinal cells collected 1 day after α-AA and BrdU treatment, cultured for 7 days, and immunostained for (**A**) BrdU and GFAP, (**B**) βIII-tubulin (βIII-tub), (**C**) syntaxin (syn), (**D**) PKCα, and (**E**) recoverin (Recov). Scale bars, 40 μm.

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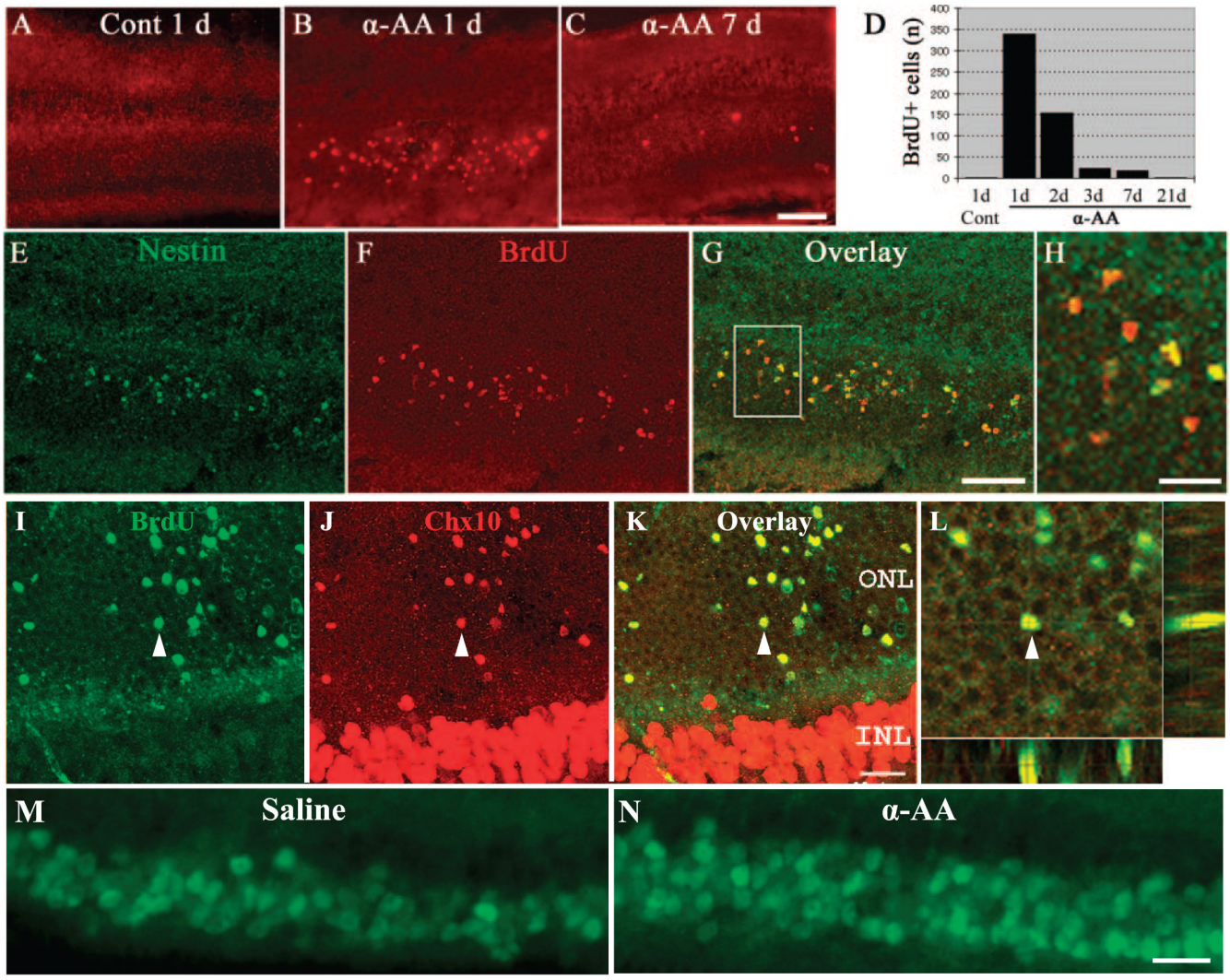

FIGURE 7. BrdU$^+$ cells expressing neural progenitor cell markers at day 1 after α-AA injection in vivo

(**A–C**) Photomicrographs of retinal sections collected from adult mice 0, 1, and 7 days after α-AA treatment and 4 hours after exposure to BrdU. (**D**) BrdU$^+$ cells in the ONL at different times after α-AA treatment. BrdU was injected intraocularly 4 hours before death. (**E–L**) Retinal sections collected 3 days after α-AA treatment were immunostained for BrdU and nestin (**E–H**) or Chx10 (**I–L**). (**M, N**) Bipolar cells in the INL of the adult retina were immunopositive for Chx10. No significant differences in distribution and nuclear size of bipolar cells were noted in (**M**) saline- and (**N**) α-AA-injected group. Scale bars: (**A–G**); 40 μm; (**H, L**) 16 μm; (**I–K, M, N**) 25 μm.

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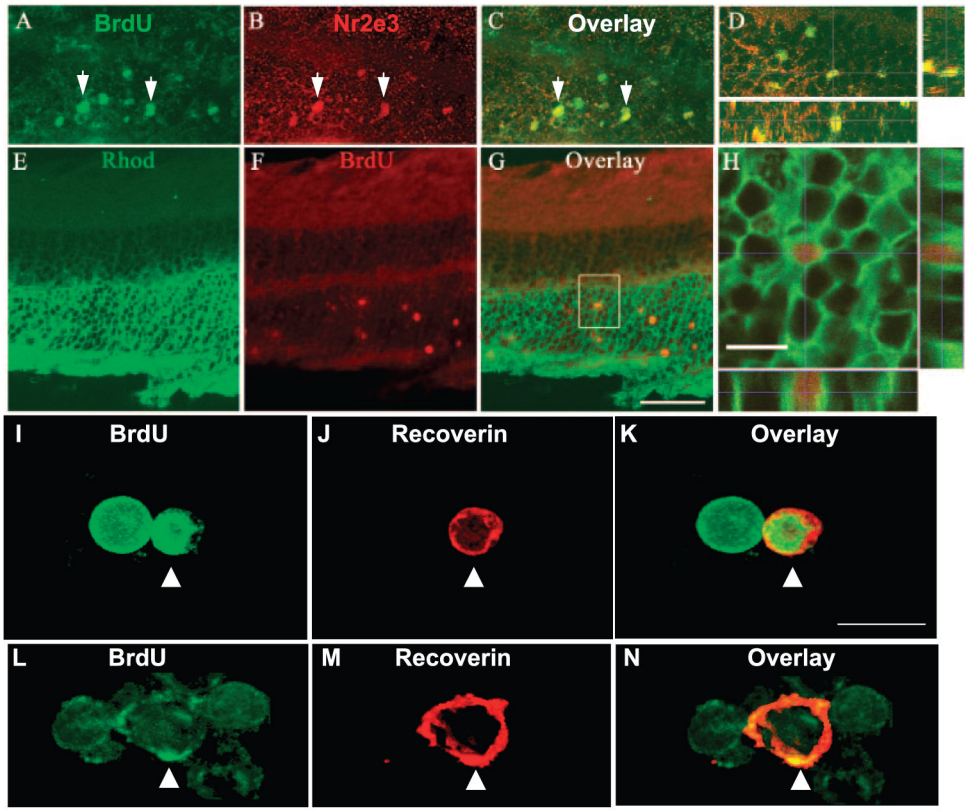

FIGURE 8. BrdU$^+$ cells differentiated and expressed mature photoreceptor cell markers after 7 days of α-AA injection in vivo

(**A–H**) Orthogonal confocal images of retinal sections 7 (**A–D**) and 21 (**E–H**) days after α-AA treatment showing colocalization of BrdU$^+$ nuclei with immunolabeling of the early photoreceptor marker Nr2e3 (**A–D**) and mature photoreceptor marker rhodopsin (Rhod) (**E–H**). (**I–N**) Retinal cells from α-AA-treated mice that were harvested at 21 days after injection were dissociated, plated in culture for 6 hours, and double labeled for anti-BrdU and recoverin. ***Arrows*** and ***arrowheads***: double-labeled cells. Scale bars: (A–G) 40 μm; (H–N) 16 μm.