Primary Culture of Rat Cerebral Astrocytes

Biology Honors Thesis

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ABSTRACT

Neurogenesis has been shown to occur in certain areas of the adult rat brain: the subventricular area and the hippocampus (Palmer et al., 2000). Numerous studies, including Gage et al. (1995) and Young et al. (2000), have shown that neurons can be grown *in vitro* and grafted back into the adult rat hippocampus and retina. The present research was conducted to better understand the glial environment of the developing neurons by growing and purifying a primary culture of astrocytes. Primary tissue was removed from the cerebral cortex of two-day old neonatal rats. The tissue was grown both as explants and from a cell suspension in 25 cm^2 culture flasks. The resulting cultures showed great morphological diversity and survived repeated passages. After 12 days the flasks were shaken to dislodge the oligodendrocyte layer from the other cell layers. The culture was grown and then shaken again, preferentially leaving only astrocyte cells. The remaining cells were grown in chamber slides to confluency, and then fixed in methanol. The slides were stained using an anti-GFAP antibody and visualized using fluorescence- or peroxide-conjugated immunohistochemistry. The results of immunofluorescence showed staining in a large percentage of the cells. Peroxidase staining weakly labeled the same percentage of cells. The successful astrocyte culture provides clues to understanding the role of astrocytes in neural development.

INTRODUCTION

The fully-grown, adult mammalian brain has long been assumed to be physiologically immutable. This inability to restore lost neurons and nerve function has been identified as a complicating factor in neurological diseases such as Parkinson's disease, Alzheimer's disease, and Huntington's disease (Eriksson et al., 1998). Studies in the past decade have shown that sections of the brain, namely the hippocampus region, the subgranular zone, and the ventricular zone, retain plasticity throughout life (see Palmer et al., 2000). Cells, therefore, continue to be produced in certain areas throughout adult life. Neurogenesis in the hippocampus is intriguing because this region is intimately associated with spatial learning and memory (Nilsson et al., 1999).

Studies (Gage et al., 1995; Young et al., 2000) have shown that neurons can be grown *in vitro* and grafted back into the adult rat hippocampus and retina. These findings suggest two possible methods for therapy: *ex vivo* manipulation of the cells and grafting, or *in vivo* activation of stem cells already present by neurotrophins or growth factor injections to induce repair (Shihabuddin et al., 1999). Neural progenitor cells can be grown *in vitro* without losing their ability for differentiation. However, neurons must be supported physically and metabolically by glial cells such as astrocytes and oligodendrocytes. Astrocytes make up half of the cell population in adult mammalian brains, and provide structural, metabolic, and tropic support for neurons (Song et al., 2002). Understanding the growth of glial cells, especially astrocytes, is a key to repairing or growing new neurons.

Multiple types of astrocytes have been identified in the brain; glial fibrillary acidic protein (GFAP) serves as the main marker for all mature cultured astrocytes (Spina

Purrello et al., 2002). The functional and regional differences in astrocytes may represent unequal influences on brain development. Growth factors produced by astrocytes exert their influence via G-protein signaling cascades (Spina Purrello et al., 2002).

In preparation for this experiment, tissue culture techniques using neuroblastoma cells were learned. Neuroblastoma cells are tumor cells that exhibit abnormal growth and differentiation. Therefore, it was important to make the change in methods to mortal cells taken directly from animal tissue once sterile tissue culture techniques were learned. The neuronal cells in neonatal rats are growing and proliferating rapidly and are well-suited for establishing *in vitro* cultures. Cells from the cerebrum and hippocampus of rats were removed and cultured *in vitro*. The cells were characterized morphologically, and attempts were made to isolate the astrocyte population. The resulting population was characterized by testing for expression of GFAP (glialfibrillary acidic protein), a mature astrocyte marker.

MATERIALS AND METHODS

Brain Dissection

Two 2-day old neonatal rat pups from the Geneseo Vivarium were sacrificed in a CO_2 chamber according to current college IACUC (Institutional Animal Care and Use Committee) guidelines. The procedure was adapted from *A Dissection and Tissue Culture Manual of the Nervous System* (Shahar, de Vellis, Vernadakis & Haber, 1989). Using microdissecting scissors, the skin was opened at the midline of the head, cutting from the base of the skull to the mid-eye area. After folding back the skin flaps with the

scissors, the skull was cut at the midline fissure, without cutting into the brain tissue. The raised skull cap was removed with the curved forceps, applying slight pressure. The brain was then released from the skull cavity by running a microspatula underneath and along the length of the brain from the olfactory lobes to the beginning of the spinal cord. After gently transferring the brain to a 60 mm Petri dish, it was rinsed with a squirt of modified DMEM/F12 culture medium, containing 10% FBS, 1% glutamine and gentamicin antibiotic, from a syringe to remove the adhering blood and moisten the tissue. An identical procedure was performed for the second brain. The brains were then moved to a second Petri dish and placed in a tissue culture hood. Using micro-dissecting forceps, each brain was transferred to the inverted lid of a 35 mm Petri dish that was resting on a pad of sterile gauze. While steadying the brain with the forceps, the cerebrum was separated from the cerebellum and brain stem, and the cerebral hemispheres were separated from each other by gently teasing along the midline fissure with the sharp edge of a second forceps. The cortex (grey matter containing the cell bodies) was deflected and peeled away, leaving behind the white matter. The meninges were gently peeled from the individual cortical lobes that were immediately placed into a fresh 60 mm Petri dish containing modified DMEM/F12 culture media.

Tissue Digestion

Cortices were dissociated into a cell suspension using mechanical digestion. After digestion, any remaining undigested tissue was plated as an explant in modified DMEM/F12 culture medium. Cells were plated in 75 cm^2 tissue culture flasks at a concentration of $15x10^6$ cells in 11 ml of medium. Incubating the flasks at 37^oC in a

moist 5% CO_2, 95% air atmosphere for 48-72 hours before moving, allowed the cells sufficient time to adhere and begin multiplying. The medium was changed at this time and every 48-72 hours until the cells were ready to be used for culture on slides.

Culture purification

After incubating the primary cultures for 7-9 days, the medium was changed completely (11 ml), and the caps were tightened. Flasks were wrapped in plastic, placed on a shaker platform in a horizontal position with the medium covering the cells, and were shaken at 350 rpm for 6 hours at 35^{o} C to separate the oligodendrocytes from the astrocytes. The flasks were changed with 10 ml of medium and replaced on the shaker for 18 hours. After removing the flasks, the contents were aseptically poured into a new 75 cm^2 flask, and placed in the incubator. The shaken flasks were again changed with 10ml of fresh medium and the shaking process was repeated for an additional 24 hours. Contents were then aseptically poured into a new 75 cm^2 flask, and placed in the incubator. When confluent, cells were passed from the flasks into sterile Petri dishes containing glass slides. The secondary cultures were grown to confluency, rinsed in Dulbecco's Phosphate Buffered Saline, and the slides were fixed for 10 minutes in methanol. Concurrently, secondary cultures were grown in plastic chamber slides and 16-well slides and then fixed in methanol. Lastly, a sample of the cells was frozen in liquid nitrogen and thawed and re-grown one month later.

Fluorescence Immunostaining

Slides were incubated for 30 minutes at room temperature in a blocking solution containing 10% normal goat serum (NGS), 1% bovine serum albumin (BSA) and 0.3% Triton X-100 in wash buffer. This incubation blocks non-specific antibody binding sites. Primary antibody T5 Rabbit Anti-GFAP (donated by Eileen Gardner, William Paterson University) was diluted 1:1000 with an antibody diluent (1% BSA and 0.3% Triton X-100 in wash buffer.) Slides were incubated overnight at 4^{o}C. Between antibody incubations, the slides were washed in PBS. The secondary antibody, fluorescein-conjugated Goat-anti-Rabbit IgG (Oncogene) was diluted 1:40 *in* antibody diluent and incubated for 90 minutes at room temperature. Cells were examined with an Olympus fluorescence microscope, and pictures were taken using a live video digital camera.

Immunoperoxidase Staining

Slides were stained using a Mouse and Rabbit UniTect ABC kit (Oncogene). They were first incubated in the kit's blocking solution diluted in 1 ml of PBS wash buffer. The primary antibody (T5 polyclonal rabbit anti-GFAP) was diluted 1:1000 and slides were incubated overnight at 4^{o} C. After rinsing in PBS 3 times for 5 minutes each, the slides were incubated in the biotinylated secondary antibody for 30 minutes at room temperature. After rinsing, the slides were incubated in the ABC reagent for 30 minutes at room temperature. Cells were visualized using DAB substrate (Zymed), incubating for 5 minutes at room temperature. A methylene blue counterstain was used to stain cell

bodies and processes. A wet mount was prepared and pictures were taken using a live video digital camera and a light microscope.

RESULTS

Primary cultures were successfully grown from both tissue explants and plated cell suspensions of rat neuronal cells. Photographs were taken of cells under a light microscope. Figure 1 shows the clusters of cells seen in the cultures. The cells began to extend processes from the cell body within a day of plating. In general, cells in isolation grew a greater number of processes than those surrounded by a high density of other cells. Some cells appeared to be extending processes towards each other.

Figure 2 shows representative morphologies of cells from the primary and secondary cultures. Cells were most commonly found growing in clusters or small groups, as seen in Figures 2A and 2B. Solitary cells (Figures 2C and 2E) often had large numbers of processes extending from the cell body. A unique morphology was observed in cells cultured from both rats; two long processes extended from opposite poles of the cells in a straight line (Figure 2D).

Anti-GFAP (glialfibrillary acidic protein) antibodies tag the microfibers in the cytoplasm of astrocytes. Using a fluorescein-conjugated secondary antibody specific for the GFAP primary antibody caused the entire cell to glow bright green with a dark spot where the nucleus was located (Figure 3). This immunohistochemical staining was performed to positively identify the astrocytes growing in culture. Cells labeled with green fluorescence were identified as astrocytes. The comparison of cells under light

microscope and fluorescence allowed identification of astrocytic and non-astrocytic cells (Figure 4 A & B and C & D). All cells present in the culture could be seen under the light microscope, but only astrocytes were visible under fluorescent light. The cultures were found to be 90% pure, containing mostly astrocytes, based on the results of the immunofluorescent staining. The peroxidase-conjugated immunohistochemistry (Figure 5) provided only a weak brown stain; however, the stain was present in a large number of cell bodies. An interesting morphology was found in the peroxidase-conjugated cells; mounds of cells were present in several cultures (Figure 5F).

The cultures were maintained and passed over a four-week period, then frozen in liquid nitrogen for one month. The cells were thawed and grown again for a period of 12 weeks. Cells that were frozen in liquid nitrogen were successfully re-established as cultures in 25 cm^2 flasks. However, the cultures did not grow to confluency and could not be successfully passed to chamber slides or Petri dishes.

DISCUSSION

This project demonstrates a successful primary culture of rat astrocytes *in vitro*. Difficulty in keeping the harvested tissue sterile was anticipated, but a primary culture was successfully established after only one surgery period. The initial primary culture most likely contained neurons, astrocytes, oligodendrocytes, other glial cells, endothelial cells, and undifferentiated cells. The shaking method of separating cells allowed for the selection of astrocytes, which are more readily adherent (Shahar, de Vellis, Vernadakis & Haber, 1989). The resulting culture contained cells with the typical star-shaped morphology of astrocytes; however, morphology could not be an accurate indication of

cell type because the cells were grown in aphysiologic conditions. For this reason, cells were stained with a marker against GFAP, a cytoplasmic protein found in astrocytes.

Although many cells appeared to be growing towards one another and perhaps making connections, this study could not confirm this without a more specific assay for neuronal connections. The cells that grew in long straight lines (Figure 2D) appeared to grow with a purpose, perhaps responding to cues from other cells. Cells can be induced to grow along scores on the growth surface (Spina Purrello et al., 2002), thus it is possible that the cells grew along scratches in the culture dishes. However, this explanation is unlikely because the capped culture flasks generally have a very smooth surface. The clusters of cells in culture were most likely clonal populations from a single plated cell or a result of explant adherence. The presence of mounds of cells (Figure 5F) could not be explained. These are possibly aggregations of cells left over from the explant procedure, but also warrant further investigation.

Although the cultures were determined to be highly pure (90%), the astrocytes presented showed a great variation in morphology. One reason for this variety could be the aphysiologic conditions under which the cells were grown. Normally, cells in the brain grow at an oxygen concentration of about 2%. Atmospheric oxygen concentrations are usually 20%; at high oxygen concentrations, many cells in the body have been shown to exhibit unusual morphologies (Morrison et al., 2000).

Cultured neurons may have therapeutic value in treating neurodegenerative diseases. Understanding the supportive role of astrocytes and other glial cells is among the first essential steps in growing neurons. Besides providing physical and metabolic support to neurons, astrocytes have been reported to play an active regulatory role in

neuronal development (Song et al., 2002). Eriksen and Druse (2001) examined the role of astrocytes on the developing serotonergic (5-HT) system, and its vulnerability to damage by ethanol in the early stages of development. Their research suggested that the protective effects of 5-HT agonists are mediated by astrocyte-produced factors. Astrocytes, therefore, are an integral part of the developmental process. Hippocampal astrocytes can instruct stem cells to differentiate into neurons (Song et al., 2002). Additionally, astrocytes regulate formation and transmission in synapses. Song and colleagues found that primary neurons were not sufficient to cause multipotent progenitor cells to differentiate, but astrocytes taken from the hippocampus could cause the cells to accept a neuronal fate. In this study, adult stem cells dependent on FGF-2 (Fibroblast Growth Factor) gave rise to neurons when cultured on a feeder layer of neonate hippocampal astrocytes. This finding supports the view that neural stem cells are widely distributed throughout the central nervous system and depend on environmental cues to regulate differentiation. Additionally, Sond and colleagues (2002) found that both diffusible and membrane-bound factors influence proliferation and differentiation of neurons. Thus, one possibility for neurogenesis might be due to signals produced by regionally specific astrocytes (Song et al., 2002). Nerve growth factor promotes the survival and differentiation of cholinergic neurons in the basal forebrain (Jurič and Čarman-Kržan, 2001). Although normally produced by neurons, nerve growth factor production is up regulated in astrocytes during times of rapid glial proliferation, such as early developmental periods or after injury.

Based on current research, the importance of a primary culture of astrocytes is elevated from a learning experiment to a tool for medical and therapeutic discovery. The

astrocytes cultured in this experiment were taken from a number of different areas of the cortex, so the effect of different regions on cell growth cannot be determined. Future experiments could examine the neurotropic abilities and the growth factors produced by astrocytes from different areas of the brain.

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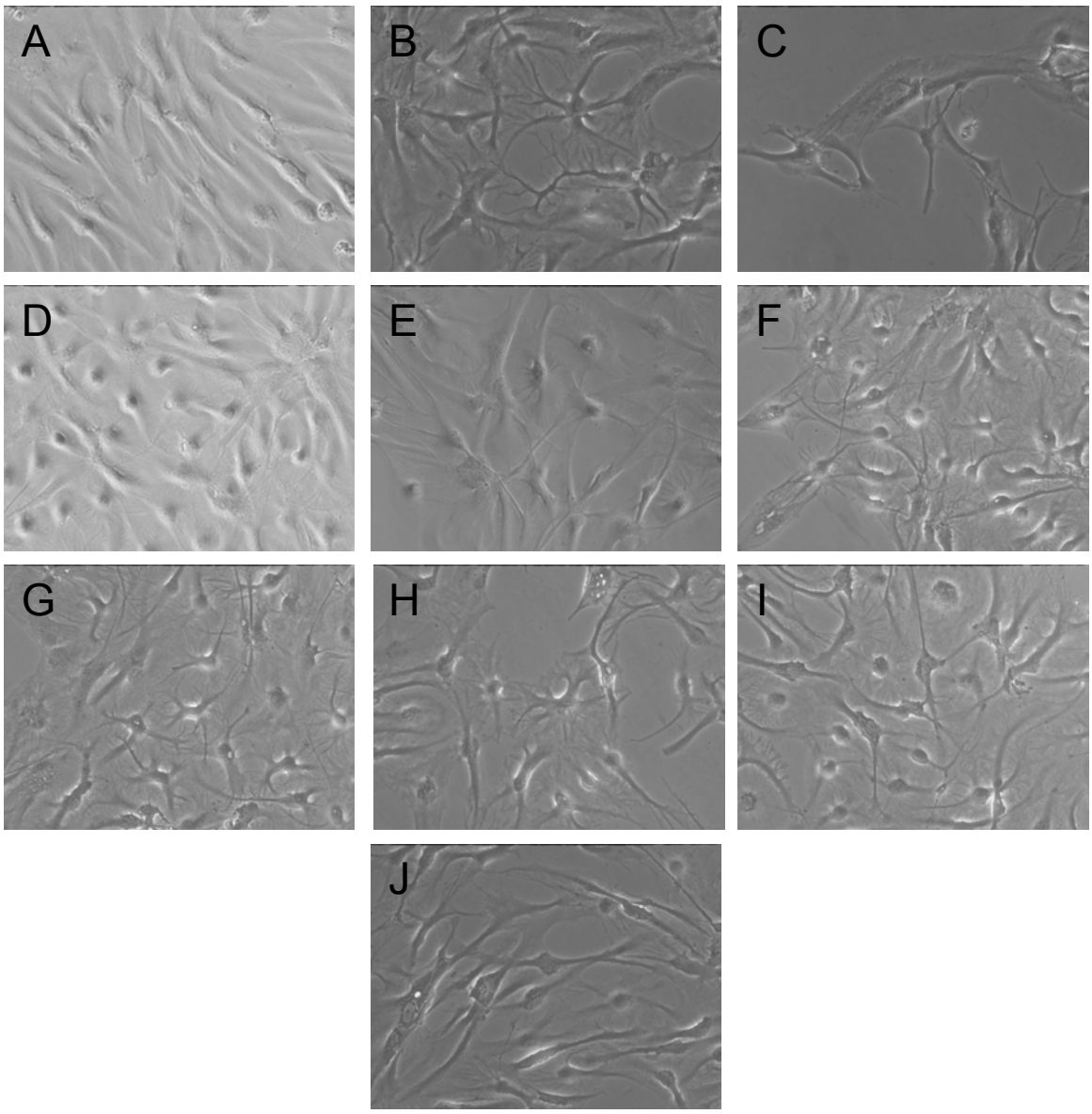

Figure 1: Light microscope pictures of primary astrocyte culture. Letters A –J demonstrate the variety of morphologies observed using phase contrast microscopy (magnification 42.5X).

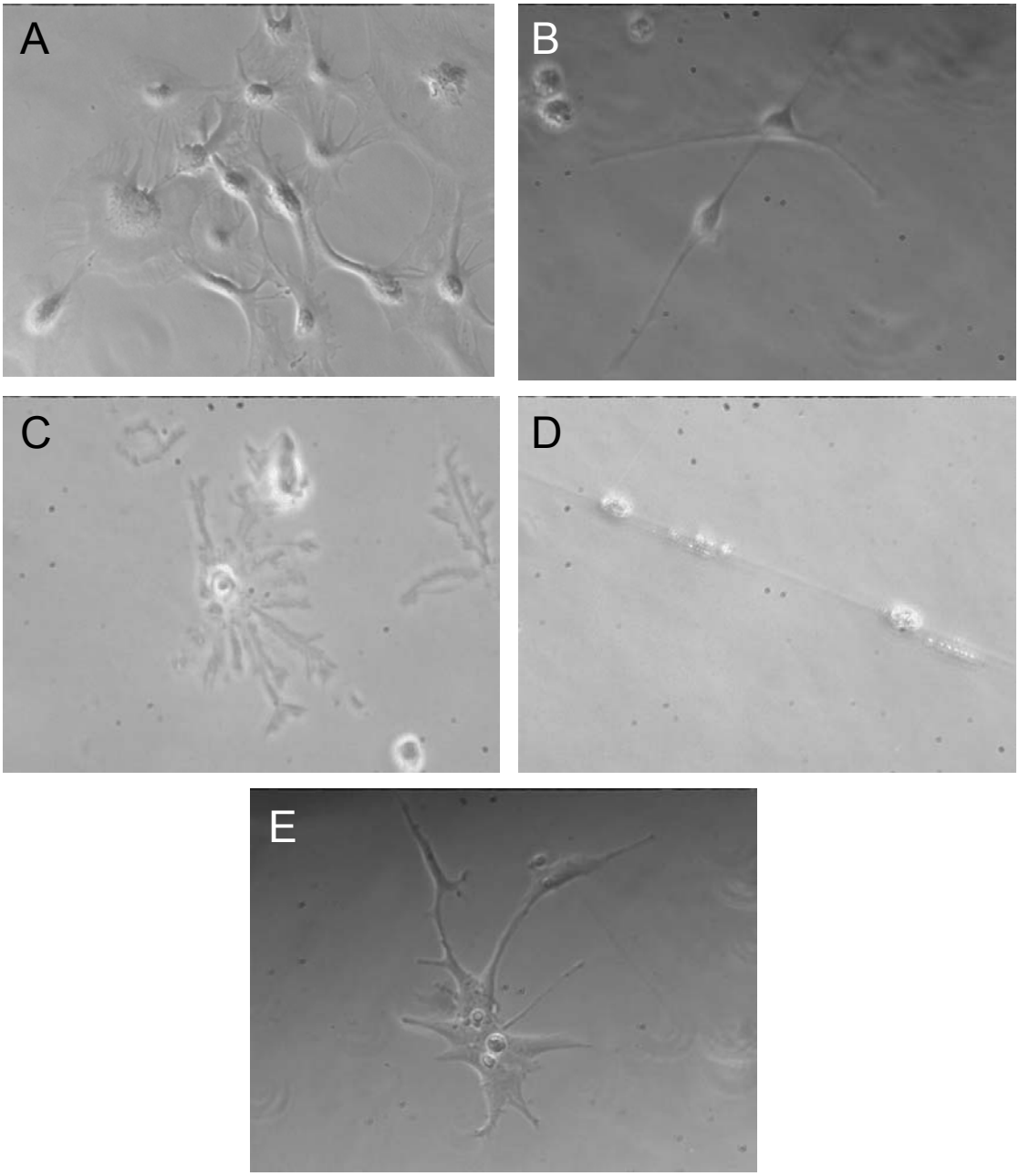

Figure 2: Representative morphologies (A-E) of cells in culture at higher magnification (85x). Figures 2a and 2b show cells growing in clusters while figures 2c and 2e show solitary cells. Figure 2d shows cellular processes extending form opposite poles in a straight line.

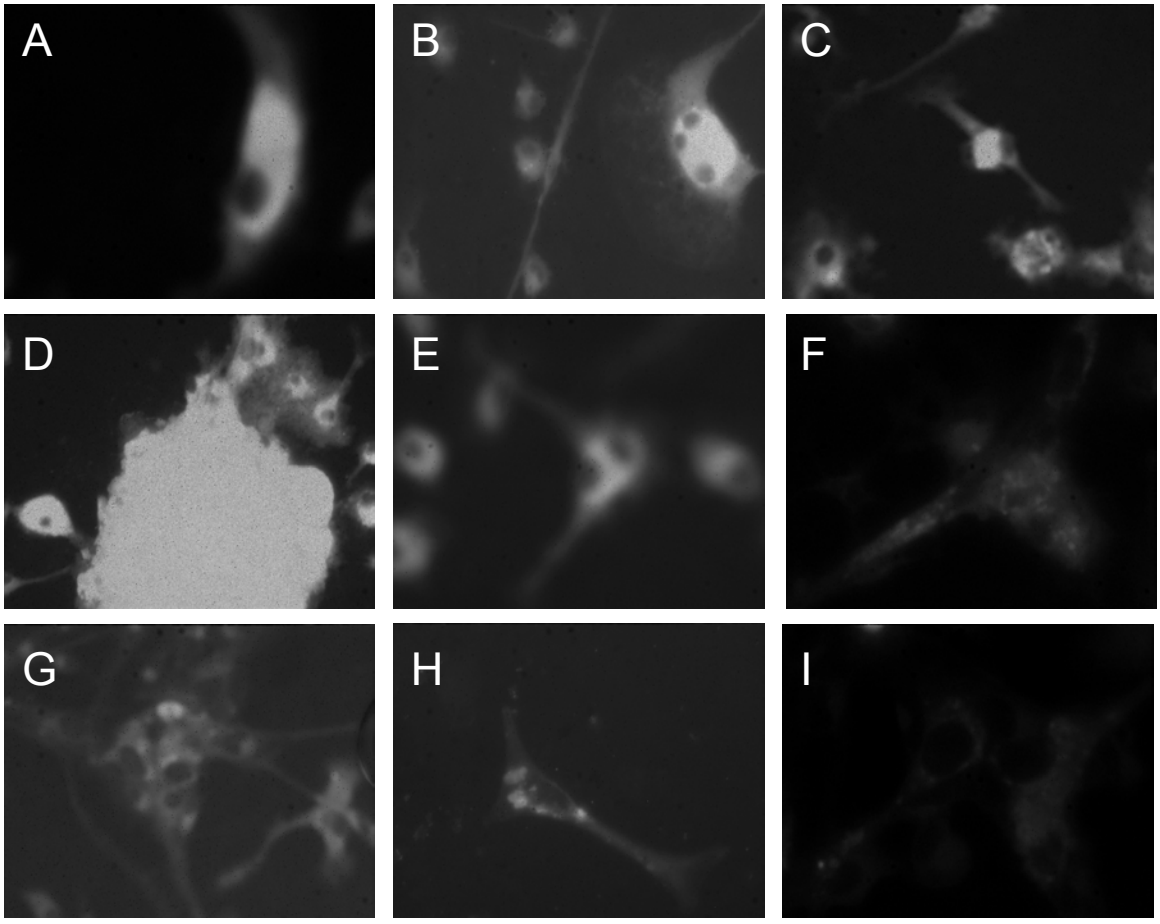

Figure 3: Fluorescence images of nine (A-I) GFAP stained astrocytes (magnification 170x). Positive cells were tagged with anti-GFAP antibodies. Figure 3D represents a large cluster of positive cells.

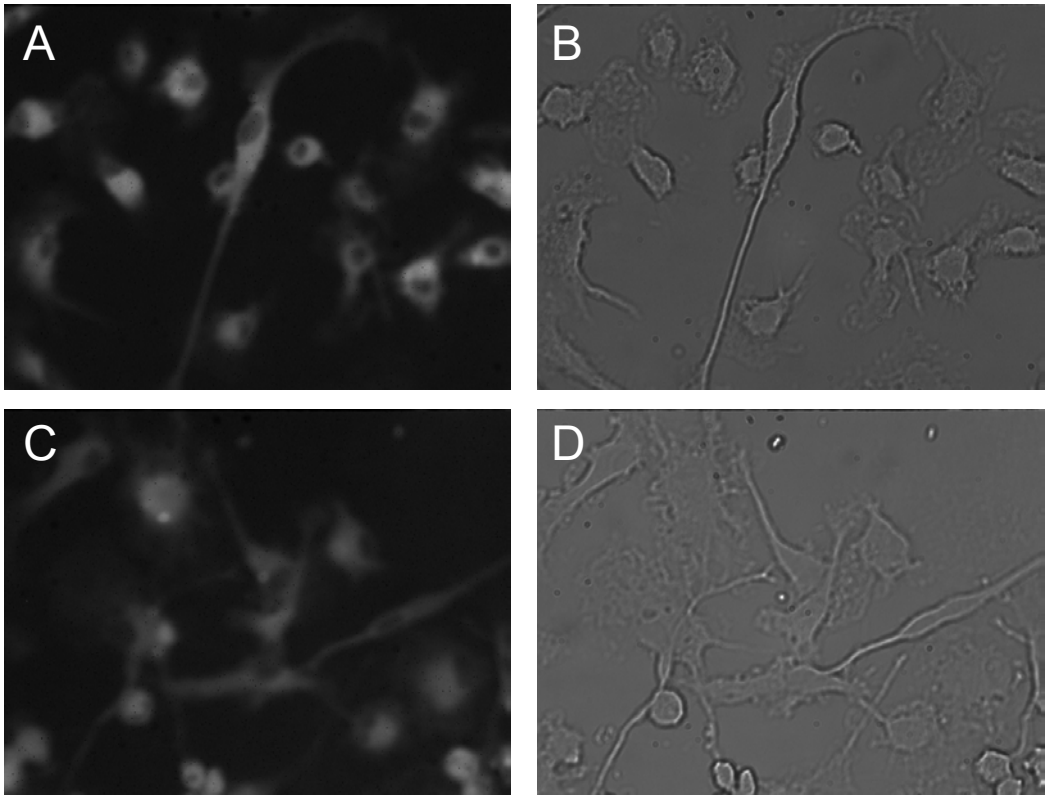

Figure 4: Astrocytes under fluorescent and normal light. Figures 4A & 4B and 4C & 4D are identical pictures taken from two sites in the culture. Cells that appear in both views are positively identified as astrocytes (magnification 170x).

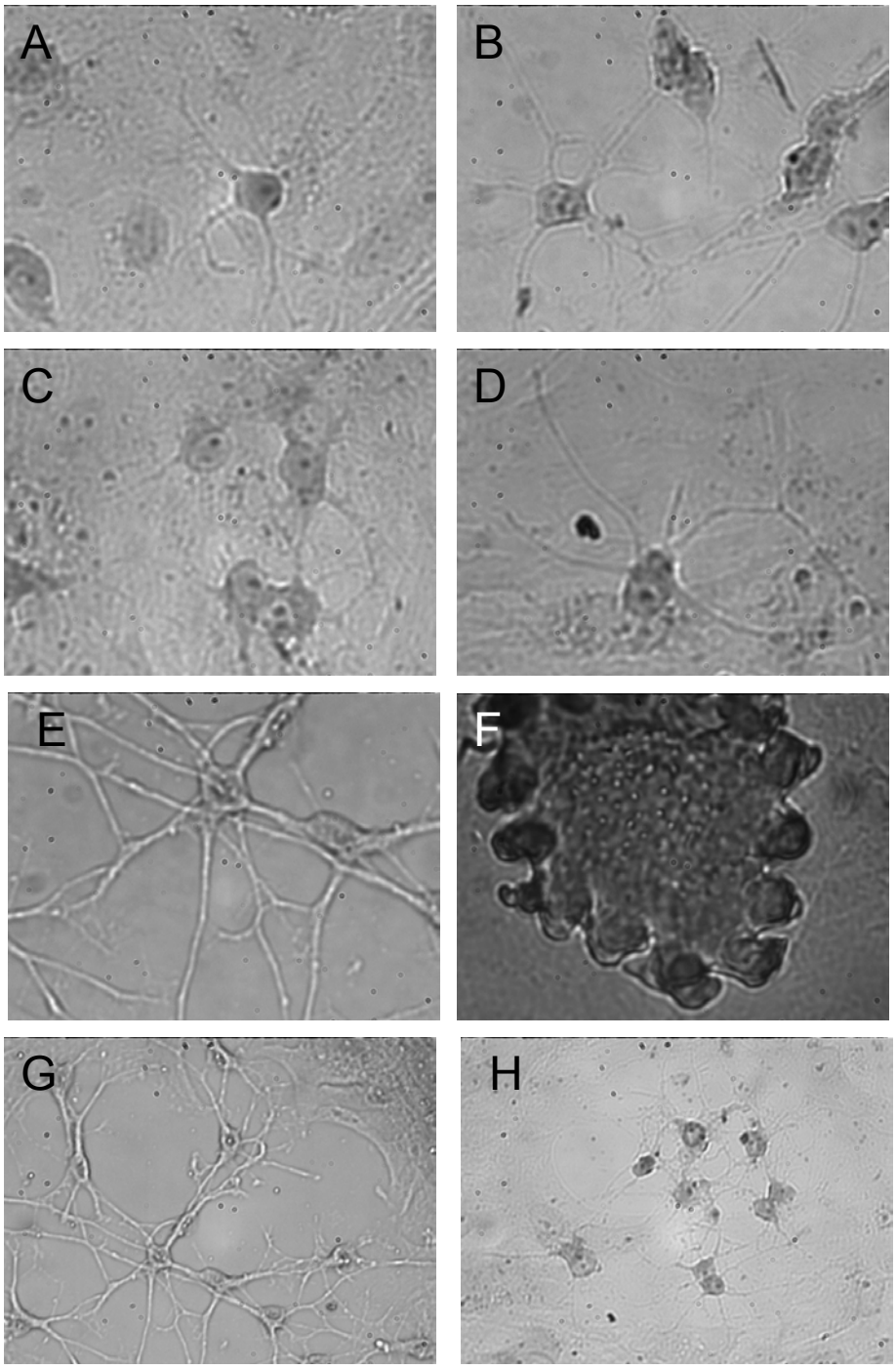

Figure 5: Representative images of astrocytes (A-H) stained with anti-GFAP antibodies followed by a biotin conjugated second antibody, ABC reagent and DAB substrate (magnification A-F, 170x G-H, 85x). Positive cells showed a reddish-brown precipitate around the nucleus.