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Haploinsufficiency for the neurofibromatosis 1 (NF1) tumor suppressor results in increased astrocyte proliferation

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Individuals affected with neurofibromatosis 1 (NF1) harbor increased numbers of GFAP-immunoreactive cerebral astrocytes and develop astrocytomas that can lead to blindness and death. Mice heterozygous for a targeted *Nf1* mutation (*Nf1*+/−) were employed as a model for the human disease to evaluate the hypothesis that reduced *NF1* protein (neurofibromin) expression may confer a growth advantage for astrocytes, such that inactivation of only one *NF1* allele is sufficient for abnormal astrocyte proliferation. Here, we report that *Nf1*+/− mice have increased numbers of cerebral astrocytes and increased astrocyte proliferation compared to wild-type littermates. Intriguingly, primary *Nf1*+/− astrocyte cultures failed to demonstrate a cell-autonomous growth advantage unless they were co-cultured with C17 neuronal cells. This C17 neuronal cell-induced *Nf1*+/− increase in proliferation was blocked by MEK inhibition (PD98059), suggesting a p21-ras-dependent effect. Furthermore, mice heterozygous for a targeted mutation in another GAP molecule, p120-GAP, demonstrated no increases in cerebral astrocyte number. These findings suggest that reduced *NF1* expression results in a cell context-dependent increase in astrocyte proliferation that may be sufficient for the development of astrocytic growth abnormalities in patients with NF1.

Keywords: tumor suppressor gene; glioma; p21-ras; GTPase activating protein; neurofibromin

Introduction

Neurofibromatosis 1 is a common autosomal dominant disorder in which affected individuals develop both benign and malignant tumors at an increased frequency (Bader, 1986; Riccardi, 1992). One of the most common tumors in NF1 is the glioma or astrocytoma (Listernick *et al*., 1997; Gutmann *et al*., 1997). Although these tumors typically occur in the optic pathway and sometimes lead to visual compromise and neurological dysfunction, they are also found in the brainstem and other regions of the brain (Molloy *et al*., 1995; Pollack *et al*., 1996). Advances in our understanding of the molecular pathogenesis of the clinical features of NF1 have resulted from the identification of the *NF1* gene and the elucidation of its protein function. The *NF1* tumor suppressor gene was identified by positional cloning and found to encode a 220–250 kiloDalton cytoplasmic protein termed neurofibromin (Cawthon *et al*., 1990; Viskochil *et al*., 1990; Wallace *et al*., 1990). Neurofibromin is expressed in many cell types and tissues, including neurons, astrocytes, Schwann cells, oligodendrocytes, blood vessels, adrenal medulla, gonadal tissues, and white blood cells (DeClue *et al*., 1991; Gutmann *et al*., 1991, 1995; Daston *et al*., 1992; Golubic *et al*., 1992; Datson and Ratner, 1993; Huynh *et al*., 1992). Sequence analysis demonstrated that a small central domain of neurofibromin shares similarity with the catalytic domain of proteins involved in p21-ras regulation (Ballester *et al*., 1990; Martin *et al*., 1990; Xu *et al*., 1990a,b). These GTPase-activating proteins (GAPs) function to accelerate the inactivation of the p21-ras protein from its active GTP-bound to its inactive GDP-bound form (Bollag and McCormick, 1991). Since p21-ras can transform fibroblasts and increase cell proliferation *in vitro*, neurofibromin has been hypothesized to negatively regulate cell growth by inactivating p21-ras. In support of this mechanism, several studies have shown that neurofibromin suppresses cell growth by inhibiting p21-ras activity in some cell types. These results are consistent with the notion that the tumor suppressor function of neurofibromin is related to its ability to operate as a GAP molecule (Basu *et al*., 1992; DeClue *et al*., 1992; Bollag *et al*., 1996; Largaespada *et al*., 1996). However, little is known about the function of neurofibromin in astrocytes relevant to the development of astrocytomas in individuals affected with NF1.

Previous work from our laboratory and others has demonstrated that increased p21-ras activity is a common feature of sporadic malignant astrocytomas (Gutmann *et al*., 1996; Guha *et al*., 1997). Inhibition of p21-ras activity in astrocytoma tumor cells results in reduced cell proliferation both *in vitro* and *in vivo* (Guha *et al*., 1997). The increase in p21-ras activation in astrocytomas is the direct result of altered signaling through the epidermal growth factor receptor (EGF-R), such that more p21-ras is converted from the inactive GDP to the active GTP bound conformation. In these sporadic astrocytomas, there is no loss of neurofibromin expression (Gutmann *et al*., 1996), arguing that only one alteration in the p21-ras signaling pathway (either EGFR activation or absent neurofibromin) is necessary for astrocytoma development or progression. Since individuals affected with NF1 develop astrocytomas and the *NF1* gene product functions as a p21-ras regulator, we tested the

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hypothesis that reduced *NF1* expression would be sufficient to confer a growth advantage on astrocytes. In this report, we demonstrate that mice heterozygous for a targeted *Nf1* mutation have increased numbers of cerebral astrocytes and increased astrocyte proliferation *in vivo*. We further show that this increase in astrocyte proliferation is dependent on neuronal-like cell contact *in vitro*. Lastly, mice heterozygous for a targeted mutation in another GAP molecule, p120-GAP, fail to demonstrate similar increases in cerebral astrocytes. These results suggest that haploinsufficiency for *Nf1* expression confers a context-dependent growth advantage for astrocytes that may account for the astrocyte growth abnormalities seen in NF1.

Results

Astrocyte NF1 *expression correlates with growth arrest* in vitro

Previous work from our laboratory has demonstrated that *NF1* expression increases in astrocytes in response to cerebral ischemia, pro-inflammatory cytokines, cAMP activation, and tumor formation (Hewett *et al*., 1995; Giordano *et al*., 1996). These results suggest that neurofibromin might function during the astrogliotic response in the brain. In response to cerebral ischemia, the time course of increased neurofibromin expression occurred after 4–7 days, consistent with periods of astrocyte growth arrest and not proliferation (Janeczko, 1988). To determine whether *NF1* expression was associated with growth arrest *in vitro*, primary astrocyte cultures were established from 129sv wild type mice and maintained as a function of days *in vitro*. Parallel cultures were analysed for *NF1* mRNA expression (RT–PCR analysis; Figure 1a), neurofibromin expression (Western blot analysis; Figure 1b), and FACS analysis (Figure 1a). The doubling time of primary astrocyte cultures was approximately 4 days as determined by BrdU pulse-chase FACS analysis (data not shown). We have previously shown that primary astrocytes reach confluence by 12–14 days *in vitro* and undergo growth arrest presumably due to contact inhibition. FACS analysis of parallel cultures demonstrated that 70–80% of the astrocytes were in G_0/G_1 prior to day 14 *in vitro* compared to 94–95% of the astrocytes in G_0/G_1 after that time. *NF1* RNA and protein expression increased dramatically by 10 days *in vitro* when 80% of the astrocytes are in G_0/G_1, just prior to the onset of astrocyte growth arrest. These findings argue that the increase in *NF1* expression is associated with the onset of growth arrest in astrocytes and is consistent with the notion that neurofibromin may function as a growth regulator for astrocytes.

Nf1+/− *mice demonstrate increased numbers of astrocytes* in vivo

In one post-mortem study, increased numbers of astrocytes were detected in the brains of several patients with NF1 (Nordlund *et al*., 1995). It is presumed, although not proven, that these astrocytes have one normal and one mutated *NF1* allele. These results suggest that reduced, but not absent, *NF1* expression might result in abnormal astrocyte proliferation. In keeping with the Knudson two hit hypothesis, loss of both copies of *NF1* by mutation would likely result in the development of an astrocytoma (Knudson, 1971). We reasoned that reduced *NF1* gene expression in astrocytes might lead to mild increases in astrocyte number without overt tumor formation.

Homozygous disruption of *Nf1* in mice leads to embryonic lethality (~E12.5) whereas mice heterozygous for a targeted *Nf1* mutation develop pheochromocytomas and leukemias, but not the typical tumors (neurofibromas, astrocytomas) seen in individuals with NF1 (Brannan *et al*., 1994; Jacks *et al*., 1994). To determine whether a haploinsufficient effect is observed in mice with reduced neurofibromin expression, the number of glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes from *Nf1*+/− mouse brains were counted in several well-defined areas of the brain (corpus callosum, dentate gyrus, cerebellum and hippocampus CA1 region). Increased numbers of

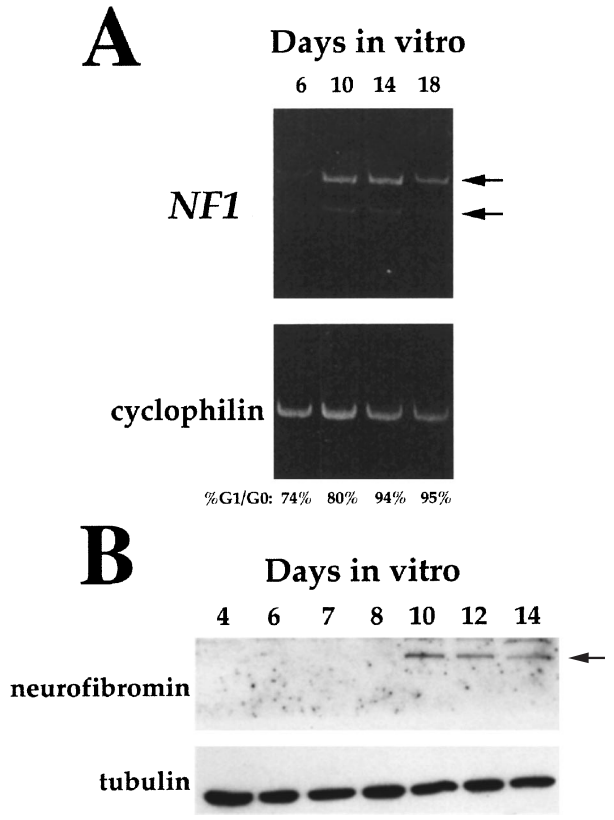

Figure 1 Expression of neurofibromin during astrocyte growth arrest *in vitro*. (**a**) Primary astrocyte cultures were established from sv129 mice and maintained for 6–18 days *in vitro* (DIV) prior to harvest for FACS analysis using the Becton Dickinson FACScan or RNA analysis using semi-quantitative RT–PCR with primers that amplify the GAP-related domain of *NF1* or cyclophilin (internal control for RNA quantity and quality). The arrows denote *NF1* mRNA containing (upper band) or lacking (lower band) exon 23a. The per cent of astrocytes in G_0/G_1 as determined by FACS analysis are shown as a function of days *in vitro*. (**b**) Primary astrocyte cultures maintained for 4–14 days *in vitro* were harvested and equal amounts of total protein were separated by 8% SDS–PAGE for immunoblotting with WA15a (neurofibromin) and tubulin (internal loading control) antibodies. The arrow denotes neurofibromin (220 kDa protein). Increased *NF1* RNA and protein expression was detected by DIV 10 prior to growth arrest as determined by FACS analysis (80–94% of the cells in G_0/G_1)

GFAP-immunoreactive astrocytes were observed in *Nf1+/−* brains compared to wild type littermates (right hippocampus; Figure 2a). Approximately 40% more astrocytes were detected in *Nf1+/−* brains, regardless of strain or sex (Figure 3a) as well as age or brain region (data not shown). This increase in astrocyte GFAP immunoreactivity was also confirmed by Western blotting from homogenized forebrains from pools of at least three mice from each genotype or strain (Figure 2b). In contrast, no increases in mac-1-immunoreactive microglia (Figure 3b) or APC immunoreactive oligodendrocytes (Figure 3c) were observed, arguing that only type 1 astrocytes and not other glial cell lineages were affected by reduced *Nf1* expression.

Nf1+/− *astrocytes lack a cell-autonomous growth advantage* in vitro

To better define the growth advantage of *Nf1+/−* astrocytes, primary astrocyte cultures were established from individual pups in 21 litters. Whereas increased GFAP expression was detected in forebrain homogenates, no differences in GFAP expression were detected in cultured astrocytes from *Nf1+/−* and *Nf1+/+* wild type mice (Figure 4a). Surprisingly, these *Nf1+/−* astrocyte cultures did not demonstrate increased cell proliferation as detected by BrdU incorporation (not shown), direct cell counts (not shown), and thymidine incorporation (Figure 4b). Direct cell counting using Trypan Blue exclusion failed to demonstrate any changes in astrocyte cell viability in *Nf1+/+* compared to *Nf1+/−* cultures (data not shown). Furthermore, no differences in proliferation were detected between *Nf1+/+* and *Nf1+/−* astrocytes under serum starvation conditions, with and without epidermal growth factor stimulation, or in response to serum challenge (not shown). These findings suggested that the increase in astrocyte number observed in the *Nf1+/−* brains did not result from a cell-autonomous defect in astrocyte growth regulation.

Experiments on leukemic cells from *Nf1 −/−* mice have shown that resting, unstimulated cells do not demonstrate increased proliferation and p21-ras activity. Only after granulocyte-macrophage colony stimulating factor (GM–CSF) treatment are the increases in cell proliferation and p21-ras pathway activation manifested (Bollag *et al*., 1996; Largaespada *et al*., 1996). Similar experiments on *Nf1+/−* astrocytes failed to demonstrate differences between wild-type and *Nf1+/−* astrocytes with regard to p21-ras pathway activation after EGF treatment. In these experiments, *Nf1+/+* and *Nf1+/−* astrocyte cultures were serum starved overnight prior to EGF stimulation. Ras pathway activation was measured using phospho-specific MAPK antibodies (Figure 4c) and *in vitro* MAPK assays (not shown). Collectively, these data suggest that no significant cell-autonomous differences in astrocyte proliferation and MAPK pathway activation result from NF1 haploin sufficiency.

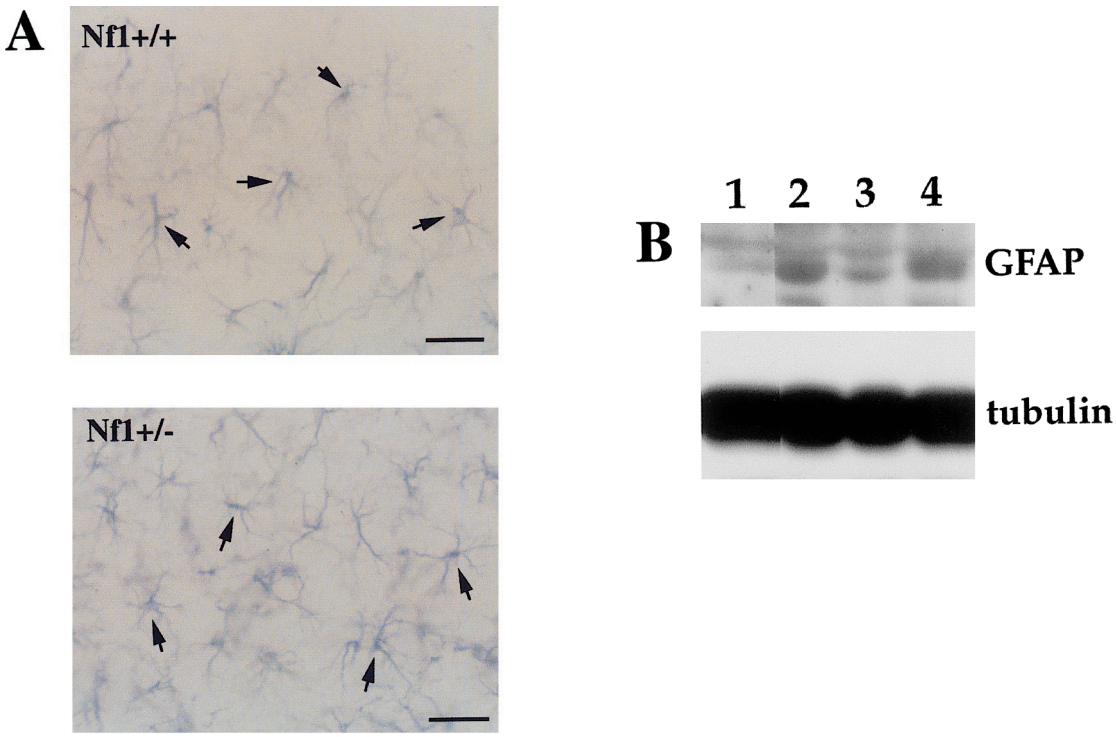

Figure 2 *Nf1+/−* mice demonstrate increased numbers of GFAP-immunoreactive astrocytes. (**a**) Comparable representative 40 μm sections from *Nf1+/−* and wild type littermate sv129 mice were immunostained with GFAP antibodies and developed with Vectastain SG to demonstrate increased numbers of astrocytes in the heterozygous *Nf1* mutant mice. These sections are taken from the hippocampus at 40 × magnification. The bar denotes 50 microns. Arrows point to GFAP-immunoreactive astrocytes. (**b**) Western blot analysis of forebrain homogenates pooled from at least three C57B1/6 (*Nf1+/+*, lane 1 and *Nf1+/−*, lane 2) and sv129 (*Nf1+/+*, lane 3 and *Nf1+/−*, lane 4) mice. Equal amounts of total protein were separated by SDS–PAGE. Increased GFAP is detected in the *Nf1* heterozygote mutant mice compared to the littermate controls. Tubulin is included as a control for equal protein loading

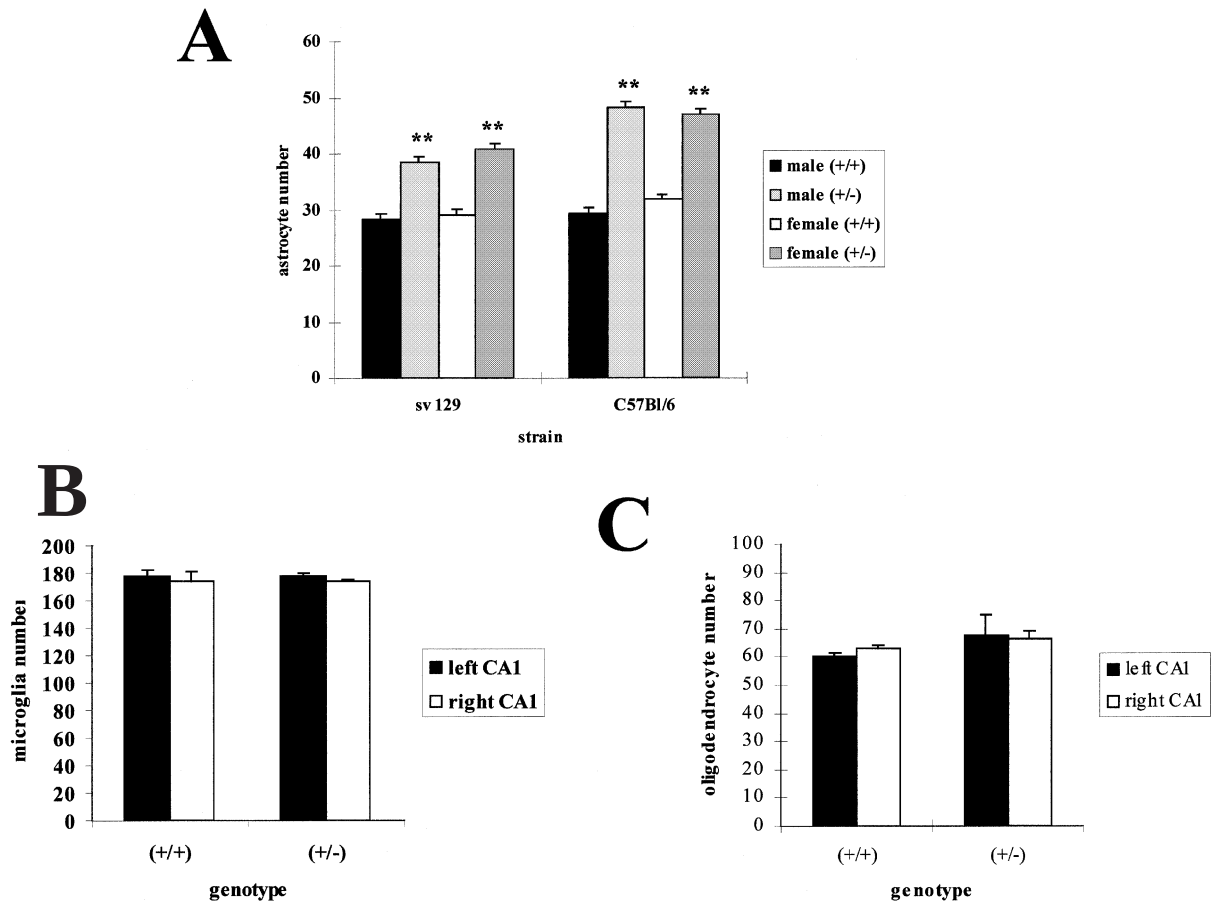

Figure 3 *Nf1*+/− mice demonstrate a specific increase in GFAP-immunoreactive astrocytes. (**a**) Quantitation of the representative results shown in Figure 2a was performed for at least six mice from each genotype, sex, and strain (C57B1/6 and sv129). These counts derive from right hippocampal CA1 sections. Asterisks denote statistical significance ($P<0.001$) by the Student's *t*-test. No changes in the number of mac-1-immunoreactive microglia (**b**) or APC-immunoreactive oligodendrocytes (**c**) were observed in sv129 hippocampal CA1 sections

Increased proliferation of Nf1+/− *astrocytes in response to neuronal contact*

The lack of increased astrocyte proliferation *in vitro* raised the possibility that the observed increases in GFAP-immunoreactive astrocytes in the brain reflected reactive astrogliosis and not increased proliferation (Rizvi *et al*., 1999). Alternatively, it is possible that reduced *Nf1* expression does not confer a cell autonomous growth advantage, but rather an abnormal response to other stimuli. We therefore tested the hypothesis that the increase in astrocyte number in *Nf1*+/− brains reflected an increase in cell proliferation. First, litters of *Nf1* pups (5–8 weeks of age) were injected with 5 μg/g BrdU 48 h prior to perfusion and double-label immunohistochemistry with GFAP and BrdU antibodies. The percentage of astrocytes (GFAP-immunoreactive cells) with nuclear BrdU immunoreactivity in the dentate gyrus and hippocampus CA1 region was determined. We detected an increase in the percentage of proliferating astrocytes in *Nf1*+/− compared to wild type *Nf1*+/+ mice (Figure 5a). On average, approximately twofold increased astrocyte proliferation was observed in the *Nf1*+/− brains.

We reasoned that this increase in astrocyte proliferation might reflect an abnormal response on the part of the *Nf1*+/− astrocytes to neuronal contact or stimuli. To develop an *in vitro* correlate for this neuronal context-dependent effect on astrocyte proliferation, we established primary astrocyte cultures from wild type and *Nf1*+/− littermates grown in the presence or absence of lightly fixed and extensively washed C17 neuronal cells. This cell line was chosen based on previous experiments demonstrating that they display some properties of neurons and can interact with astrocytes (Ryder *et al*., 1990; Weinstein *et al*., 1990). No thymidine incorporation was detected with the fixed C17 cells alone in the absence of astrocytes (not shown). Astrocytes from *Nf1*+/−, but not wild type *Nf1*+/+, mice demonstrated a statistically significant increase in thymidine incorporation in response to C17 cell coculture (Figure 5b). This increase in proliferation ranged between 1.7- and 1.8-fold.

We next determined whether this increase in astrocyte proliferation in response to neuronal-like cell contact resulted in changes in p21-ras pathway activation. Initial experiments failed to demonstrate reproducible changes in MEK activity between *Nf1*+/− astrocyte cultures cultured in the presence or absence of C17 neuronal-like cells (data not shown). Similarly, no significant changes in p38-MAPK or Akt activation were observed (data not shown). Although no changes in p21-ras pathway activity were detected, we reasoned that reduced *Nf1* expression in the context of C17 co-

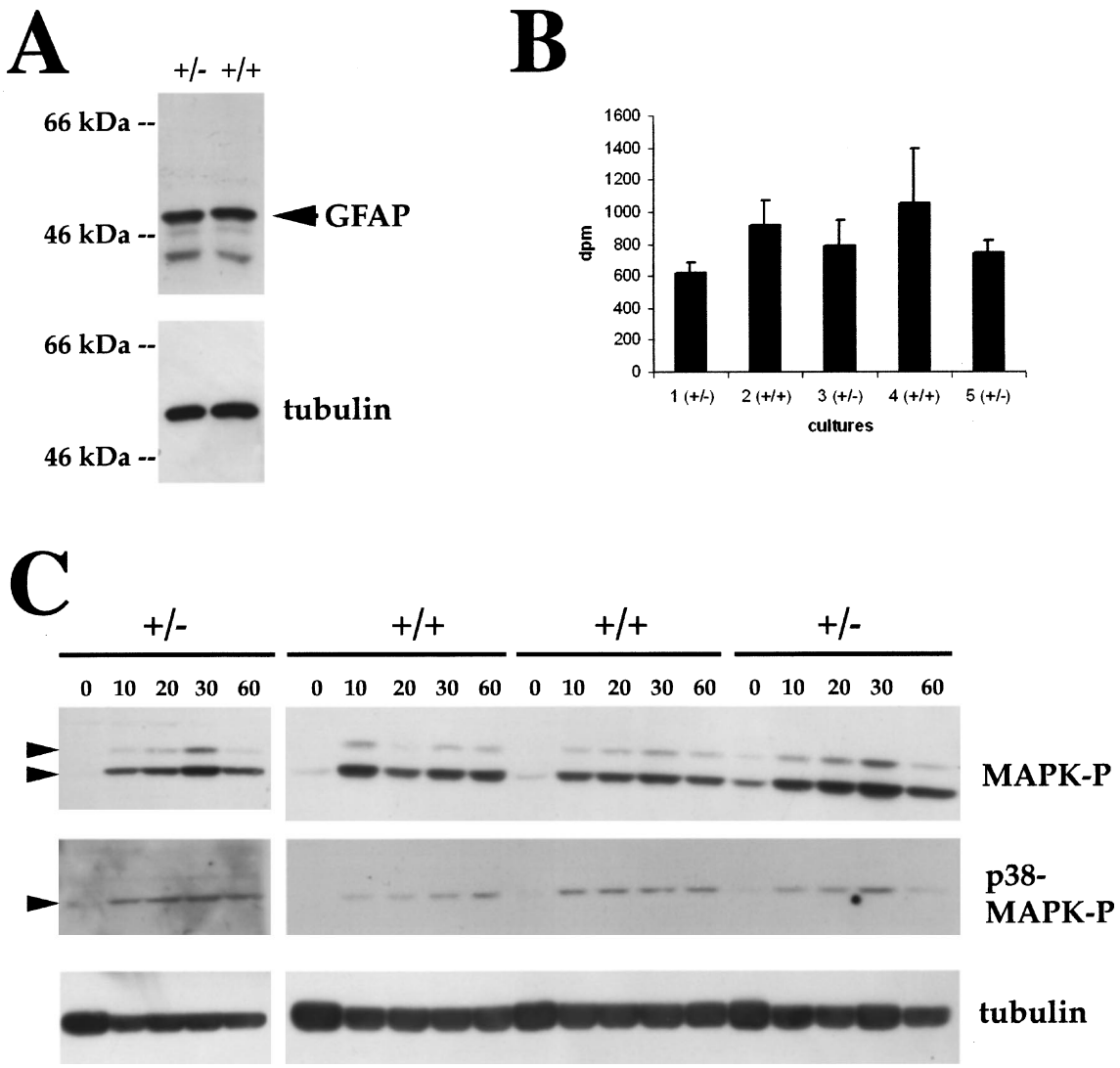

Figure 4 Primary *Nf1* astrocytes fail to demonstrate a cell-autonomous defect in growth regulation. (**a**) Primary astrocyte cultures from sv129 *Nf1+/−* and wild type mice expressed equal amounts of GFAP by Western blotting. Equal amounts of total protein were separated by SDS–PAGE prior to immunoblotting with GFAP and tubulin antibodies. (**b**) Primary astrocyte cultures from sv129 *Nf1+/−* and *Nf1+/+* littermates grown in complete media and analysed by thymidine incorporation failed to demonstrate reproducible differences in proliferation. Error bars represent standard deviations for six duplicate wells. (**c**) Primary astrocytes from two representative sv129 *Nf1+/−* and *Nf1+/+* cultures were serum starved overnight prior to stimulation with EGF (100 ng/ml). Cultures were harvested after 10, 20, 30 and 60 min and analysed using phospho-specific p42/44-MAPK and p38-MAPK antibodies. Tubulin is included as a control for equal protein loading. No differences in the magnitude of the increase in MAPK activation following EGF stimulation were observed between *Nf1+/+* and *Nf1+/−* astrocyte cultures after quantitation by scanning densitometry

culture could result in increased cell proliferation in a p21-ras-dependent fashion, but these changes fell below the threshold of detectability in our assays. To determine whether this context-dependent increase in *Nf1+/−* astrocyte proliferation could be abrogated by p21-ras pathway inhibition, cultures were treated with 50 μM PD98059 (MEK inhibitor). As shown in Figure 5c, PD98059 treatment of *Nf1+/−* astrocytes (cultures #2 and #4) co-cultured with C17 cells reduced the proliferation to levels observed in the astrocytes cultured without C17 cells. In contrast, no effect of PD98059 treatment on proliferation was observed on the wild type astrocytes (cultures #1 and #3). These results suggest that the context-dependent increase in astrocyte proliferation is dependent on p21-ras activation.

It is unlikely that the increase in GFAP expression detected in *Nf1+/−* mouse brains reflected astrogliosis. Although increased GFAP expression was detected in the *Nf1+/−* brains compared with the wild type littermate brains (Figure 2b), no increase in GFAP expression was detected in cultured *Nf1+/−* astrocytes compared to wild type astrocytes *in vitro* (Figure 4a). Furthermore, no significant changes in GFAP expression were noted in *Nf1+/−* astrocytes when co-cultured with C17 cells either (Figure 6).

Increased astrocyte proliferation is a neurofibromin-specific effect

To determine whether any reduction of rasGAP activity is sufficient to result in increased astrocyte

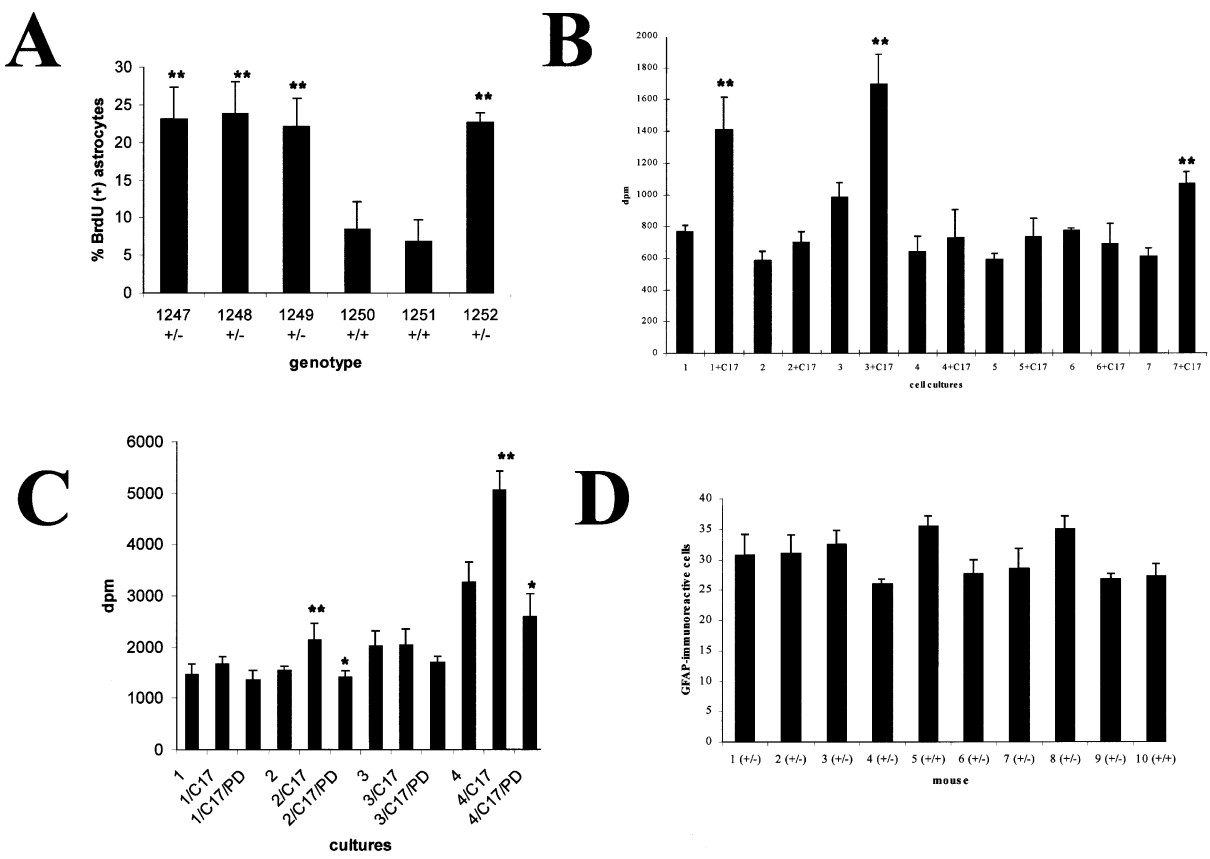

Figure 5 *Nf1*+/− astrocytes when in contact with neurons or neuronal-like cells demonstrate increased cell proliferation *in vitro* and *in vivo*. (**a**) *Nf1*+/− and wild type *Nf1*+/+ sv129 littermates (5–6 weeks of age) were injected with BrdU and the percentage of BrdU-immunoreactive astrocytes determined 48 h later by double-labeled immunohistochemistry. The percentage of proliferating astrocytes was approximately twofold greater in the *Nf1*+/− mice compared to their wild type littermates. Asterisks denote statistical significance ($P<0.001$) using the Student's *t*-test. (**b**) Ten thousand primary astrocytes from sv129 mice were cultured in the presence (+C17) or absence of 10 000 lightly fixed C17 neuronal cells per well for 72 h prior to thymidine incorporation. Increased proliferation (1.7–1.8-fold) was observed in the *Nf1*+/− cultures, but not in the wild type cultures. Asterisks denote statistical significance ($P<0.001$) using the Student's *t*-test. (**c**) Ten thousand primary astrocytes were cultured in the presence (+C17) or absence of 10 000 lightly fixed C17 neuronal cells per well for a total of 72 h prior to the addition of the PD98059 MEK inhibitor for 24 h during the period of tritiated thymidine incorporation. Cultures 1 and 3 represent astrocytes from *Nf1*+/+ wild type mice whereas cultures 2 and 4 are astrocytes from *Nf1*+/− mice. Increased astrocyte proliferation was observed in the *Nf1*+/− cultures in the presence of fixed C17 cells. This increase in proliferation was inhibited by the addition of 50 μM PD98059. The mean and standard deviation are shown for each condition. The astrocytes shown in this experiment represent fresh cultures and are not the same cultures shown in (**b**). (**d**) The number of GFAP-immunoreactive astrocytes in the right hippocampus CA1 region from *Gap*+/− and wild type *Gap*+/+ littermates (5–6 weeks of age) was determined. No differences in the number of astrocytes were detected in *Gap*+/− compared to wild type littermate mice. Error bars denote standard deviations for at least six serial sections from each mouse

proliferation, we examined mice heterozygous for a mutation in the another rasGAP molecule, p120-GAP. Mice homozygous for a targeted mutation in the *Gap* gene are embryonic lethal while heterozygotes fail to develop astrocytic tumors (Henkemeyer *et al*., 1995). We quantitated the number of GFAP-immunoreactive astrocytes in representative regions of the brain and detected no reproducible differences between wild type and *Gap*+/− mice (right CA1 region; Figure 5d). These findings suggest that a general reduction in rasGAP activity is not sufficient to confer a growth advantage for astrocytes and argue that the increase in astrocyte proliferation observed in the *Nf1*+/− mice is a neurofibromin-specific effect.

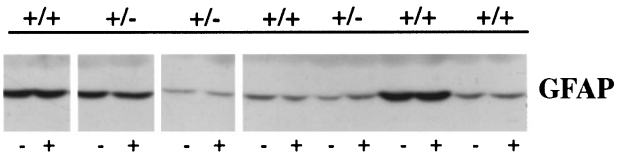

Figure 6 C17 cell co-culture does not result in increased GFAP expression. Ten thousand primary astrocytes were cultured in the presence (+C17) or absence of 10 000 lightly fixed C17 neuronal cells per flask for 96 h prior to harvest in MAPK lysis buffer on ice. Equal amounts of total protein were separated by 10% SDS–PAGE and analysed with GFAP antibodies by Western blot. The *Nf1* genotypes are denoted on the top (+/+ or +/−) while the presence (+) or absence (−) of C17 cells are denoted along the bottom of the figure. No significant changes in the expression of GFAP or tubulin (not shown) were noted in these cultures

Discussion

Since individuals affected with NF1 commonly develop astrocytomas, the *NF1* tumor suppressor gene is thought to function as a negative growth regulator for astrocytes. Neurofibromin is expressed at low levels in resting mature astrocytes both *in vitro* and *in vivo*

(Nordlund *et al*., 1993; Hewett *et al*., 1995). During astrocyte activation *in vitro*, in response to elevations in cAMP levels or in response to pro-inflammatory cytokines, there is a dramatic upregulation of *NF1* mRNA and protein expression (Hewett *et al*., 1995). Similarly *in vivo*, increased *NF1* expression is observed in activated astrocytes in response to cerebral ischemia (Giordano *et al*., 1996) or tumor xenograft implantation (Gutmann, unpublished observations). The time course of increased neurofibromin expression correlates with periods of astrocyte growth arrest and not proliferation, suggesting that upregulation of *NF1* expression is associated with negative growth regulation. In this study, we demonstrate that increased neurofibromin expression precedes astrocyte growth arrest *in vitro*. These results provide indirect evidence that neurofibromin functions as a negative growth regulator for astrocytes.

To provide more direct proof for a growth regulatory function of neurofibromin in astrocytes, we employed an animal model in which mice were heterozygous for a targeted mutation in the *Nf1* gene. These mice are genetically similar to individuals affected with NF1 in that they harbor one wild type and one mutant *NF1* allele in every cell of the body. Previous autopsy studies on NF1 patient brains demonstrated increased GFAP-immunoreactive astrocytes (Nordlund *et al*., 1995). In this report, we demonstrate that *Nf1+/−* mice demonstrate increased numbers of GFAP-immunoreactive astrocytes in the brain, similar to that described for individuals affected with NF1. Recently, Ratner and colleagues have reported similar increases in GFAP-immunoreactivity in brains from mice heterozygous for a targeted *Nf1* mutation, confirming the observations reported herein (Rizvi *et al*., 1999). We further demonstrate that this increase in GFAP-immunoreactive astrocytes reflects increased astrocyte proliferation both *in vitro* and *in vivo*. We show that the increase in *Nf1+/−* astrocyte proliferation is not a cell-autonomous property of these astrocytes but rather appears to be a cell context- and p21-ras-dependent event that requires neuronal-like cell contact. Lastly, we demonstrate that *Gap+/−* mice fail to demonstrate any changes in astrocyte proliferation. These results argue that this astrocyte proliferative effect is neurofibromin-specific and is not reflective of a general rasGAP effect on astrocytes.

Previous studies on the effects of neurons on astrocyte proliferation have demonstrated that neurons and neuronal membranes can exert an inhibitory effect on astrocyte proliferation (Sobue and Pleasure, 1984; Eccleston *et al*., 1989). In these reports, rat central nervous system axolemmal fragments induced phenotypic alterations in cultured astrocytes concomitant with greater than a 50% reduction in tritiated thymidine incorporation. Similarly, co-culture of guinea-pig enteric neurons with rat Schwann cells or astrocytes reduced Schwann cell and astrocyte proliferation *in vitro*. In contrast, guinea-pig dorsal root ganglia neurons or bovine axolemma fractions stimulated astrocyte tritiated thymidine incorporation, similar to what has been reported for Schwann cells (Eccleston *et al*., 1989). The C17 retrovirally immortalized neuronal-like cell line has been shown to inhibit astrocyte and astrocytoma proliferation in a dose-dependent manner (Weinstein *et al*., 1990). However, using primary astrocytes, the ratio of fixed C17 cells to astrocytes was critical: Only 17.4% inhibition was observed at a 1 : 1 ratio whereas 94% inhibition was observed when the C17 : astrocyte cell ratio was 10 : 1. In our experiments, we determined that the maximal C17 cell proliferative effect on the *Nf1+/−* astrocytes was observed at a 1 : 1 ratio with no growth-stimulatory effect observed at a 10 : 1 ratio. Furthermore, the lightly fixed C17 cells were loosely adherent to the astrocytes and, in this fashion, may have behaved more like axonal membranes than living neurons. We are presently exploring the nature of this inhibition using C17 membrane fractions to better characterize the *Nf1+/−* astrocyte growth promoting effect.

The data presented in this report suggest that reduced neurofibromin expression is sufficient to confer a context-dependent growth advantage for astrocytes in the brain. This minor increase in astrocyte proliferation is not sufficient for tumor formation, since no gliomas have been observed in *Nf1+/−* mice (Jacks *et al*., 1994). It may be enough, however, to account for the observation that astrocyte-rich white matter regions appear to be selectively increased in children with NF1 (Said *et al*., 1996; Greenwood *et al*., 1997). Similarly, *NF1* haploinsufficiency may also be sufficient to result in the increase in astrocyte number observed in NF1 patient brains (Nordlund *et al*., 1995).

One of the clinically puzzling features of NF1-associated optic pathway gliomas is the variable behavior of these tumors (Listernick *et al*., 1989; Habiby *et al*., 1995). The majority of these optic pathway gliomas are clinically asymptomatic and do not enlarge to result in visual impairment or blindness. In this fashion, these tumors behave as benign growths. In contrast, some of the NF1-associated astrocytomas grow rapidly and lead to blindness or neurologic compromise as a result of tumor extension. The fact that gliomas do not form as a consequence of *Nf1* haploinsufficiency suggests that additional genetic changes are required.

One of these additional genetic events might involve loss of the one remaining wild type *Nf1* allele, resulting in a complete absence of neurofibromin expression in selected astrocytes in the brain. In agreement with the Knudson two hit hypothesis, loss of both functional *Nf1* alleles might be associated with tumor formation. We would hypothesize that these tumors lacking neurofibromin expression represent the 'benign' clinically non-progressive tumors seen in as many as 10% of patients with NF1. To develop a clinically aggressive optic pathway glioma in NF1, mutations in other tumor suppressor genes (e.g. p53) might be required to facilitate malignant transformation. In support of this notion, cooperation between p53 inactivation and increased ras activity has been reported for Schwann cells, such that both events are required to confer increased Schwann cell proliferation (Ridley *et al*., 1989).

Substantial experimental data exist for a rasGAP growth suppressor function of neurofibromin in some cell types. However, it is not clear whether neurofibromin functions as a negative growth regulator for astrocytes by modulating p21-ras pathway activation.

In this report, we demonstrate that the increase in primary *Nf1+/−* astrocyte proliferation in response to fixed C17 cells is inhibited by blocking MEK activity. These results argue that the increase in proliferation is the result of increased p21-ras pathway activation as a consequence of decreased neurofibromin GAP function. Studies are ongoing in our laboratory to more completely characterize this neurofibromin effect on p21-ras pathway activity in astrocytes.

The development of sporadic astrocytomas involves a multi-step process of genetic and biochemical alterations. One of the most common alterations observed in high grade gliomas is increased activation of p21-ras (Gutmann *et al*., 1996; Guha *et al*., 1997). In the case of sporadic astrocytomas, this increase in p21-ras activity results from abnormal activation of the positive regulators of p21-ras, as a consequence of augmented EGF-R signaling (Guha *et al*., 1997). Activation of the EGF-R results in recruitment of the shc adaptor protein and the hSOS guanosine nucleotide replacing factor (GNRF), culminating in activation of p21-ras by the exchange of GDP for GTP. Inhibition of EGF-R signaling or p21-ras activation in glioma cells results in decreased cell proliferation both *in vitro* and *in vivo*. In a similar fashion, it is possible that loss of neurofibromin expression in NF1 astrocytomas could also result in increased p21-ras activation through absent neurofibromin GAP function. In either case, increased GNRF or decreased neurofibromin GAP activity might provide p21-ras-dependent mitogenic signals for astrocytes relevant to the development of an astrocytoma.

With regard to p21-ras activation and cell proliferation, it is not known whether p21-ras activation is necessary or sufficient for astrocyte proliferation or tumor formation. It is clear that inhibition of p21-ras-induced MAPK activation results in reduced astrocytoma growth, but these experiments have been performed on astrocytoma cell lines with multiple genetic alterations, including mutations at TP53. Experiments are presently underway to determine both *in vitro* and *in vivo* whether p21-ras activation in astrocytes results in increased cell proliferation, neoplastic transformation and astrocytoma tumor development. Further studies on the mechanism of neurofibromin astrocyte growth regulation will likely provide pharmacologic targets for the treatment of these tumors.

Materials and methods

Primary astrocyte cultures

Nf1+/− mice were generously provided by Dr Neal Copeland (National Cancer Institute) (Brannan *et al*., 1994). *Gap+/−* mice have been previously described (Henkemeyer *et al*., 1995). Both are maintained as continuous colonies at Washington University. Heterozygotes and wild type mice were genotyped from tail DNA obtained at the time of weaning using published primer sets. Primary astrocyte cultures (>97% pure astrocytes) were established from postnatal day 1–3 newborn pups as previously described (Hewett *et al*., 1993; Rose *et al*., 1993). Astrocyte purity was assessed using cell type-specific antibodies (GFAP for astrocytes, MAP2 for neurons, and GalC for oligodendrocytes). Briefly, cerebral cortices were dissected and enzymatically digested in 0.09% trypsin for 20–30 min. Cultures were maintained in modified Eagle's medium supplemented with 2 mM glutamine, 20 mM glucose, 10% fetal bovine serum, 10% horse serum, 1% pen-strep and 10 ng/ml epidermal growth factor. Thymidine incorporation was performed by pulsing astrocytes with 1 μCi tritiated thymidine (Amersham) per ml for 24 h in 24 well plates (six wells per culture or condition). Labeled cells were washed in PBS and solubilized in 200 μl of 0.2 M NaOH. Counts were then determined in a scintillation counter. The percentage of astrocytes in G_0/G_1 was determined by FACS analysis on a Becton Dickinson FACScan as previously described (Norton *et al*., 1995).

RNA and protein analysis

Western blotting was performed using WA15a (pan neurofibromin; Gutmann *et al*., 1996), tubulin (clone DM1-A; Sigma), GFAP (Zymed), and activation-specific phospho-MAPK and p38-MAPK (Promega) antibodies with HRP-conjugated secondary antibodies (Sigma) and ECL development (Amersham). Primary antibodies were used according to the manufacturer's recommendations while WA15a was used at a 1 : 300 dilution (Basu *et al*., 1992; Norton *et al*., 1995a,b). For experiments involving MAPK determinations, cultures were serum starved overnight prior to stimulation with serum containing 100 ng/ml EGF for 60 min and collected by direct harvest in MAPK lysis buffer (20 mM Tris, pH 7.5; 10 mM EGTA, 40 mM beta-glycerophosphate; 1% NP40; 2.5 mM $MgCl_2$; 2 mM orthovanadate) on ice. *In vitro* MAPK assays were performed according to published protocols using a rabbit polyclonal ERK2 antibody (Santa Cruz Biotechnology), myelin basic protein (Sigma) and 1 μCi [α-^{32}P]ATP (Amersham) in the presence of DTT.

Forebrain tissue was carefully dissected from freshly euthanized mice of at least 3 months of age and homogenized in RIPA buffer containing protease inhibitors prior to separation by SDS–PAGE. RNA was extracted for semi-quantitative RT–PCR analysis using a previously reported *NF1* primer set that amplifies sequences within the GAP-related domain and a cyclophilin primer set to control for RNA quality and quantity (5′-ATGGTCAACCC-CACCGTGTT-3′ and 5′-CGTTGTAAGTCACCACCCT-3′) (Gutmann *et al*., 1995). RT–PCR products were resolved by non-denaturing PAGE.

C17 neuronal cell co-culture

C17 neuronal cells (generously provided by Dr C Cepko; Harvard Medical School) were maintained in Dulbecco's modified medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Ryder *et al*., 1990). Prior to co-culture with primary astrocytes, C17 cells were lightly fixed in 0.1% paraformaldehyde and washed extensively in media (10 000 cells each per well; 6 wells/condition). In these experiments, 10 000 fixed C17 cells were co-cultured with 10 000 primary astrocytes for 72 h prior to the addition of 1 μCi/well of tritiated thymidine for an additional 24 h. Ten thousand primary astrocytes were cultured in parallel wells in the absence of C17 cells. No thymidine incorporation was observed in the fixed C17 cells when cultured independently in separate wells (data not shown). In the MEK inhibition experiment, PD98059 was added to a final concentration of 50 μM in each well for the 24 h in which the tritiated thymidine was included. For each experiment, the mean and standard deviation of the six wells per condition were determined.

Cell counting

Forty micron sections generated on a microtome were prepared for floating section immunohistochemistry using GFAP (Zymed), APC (Calbiochem) and mac-1 (Chemicon)

antibodies as previously described (Norton *et al*., 1995a,b; Geist *et al*., 1996). Development was accomplished using biotinylated secondary antibodies and ABC Elite (Vector) reagents with DAB or Vector-SG. Landmarks for astrocyte counting were established using a standard mouse brain atlas (Paxinos and Watson, 1986). Only cells containing clearly defined nuclei within the focal plane of the section were counted in at least six consecutive serial sections from at least six mice matched for sex or genotype. BrdU incorporation was performed by a single intraperitoneal injection of 5 μg BrdU (Sigma) per g body weight of mice at least 2 months of age (20–30 gms) 2 days prior to euthanasia and analysed by double labeling immunohistochemistry with BrdU (Sigma) and GFAP antibodies. BrdU was detected using mouse biotinylated secondary antibodies and Vector-SG development (blue reaction product) while GFAP was detected using rat biotinylated secondary antibodies and DAB development (brown reaction product). The percentage of GFAP-immunoreactive cells with BrdU-immunoreactive nuclei was scored.

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