Published in final edited form as:

Neuron Glia Biol. 2008 February ; 4(1): 43–55. doi:10.1017/S1740925X09000076.

Activity-dependent neuron–glial signaling by ATP and leukemia-inhibitory factor promotes hippocampal glial cell development*

Jonathan E. Cohen and R. Douglas Fields

AUTHOR'S ADDRESS Nervous Systems Development and Plasticity Section, National Institutes of Health, NICHD, USA

Abstract

Activity-dependent signaling between neurons and astrocytes contributes to experience-dependent plasticity and development of the nervous system. However, mechanisms responsible for neuron–glial interactions and the releasable factors that underlie these processes are not well understood. The pro-inflammatory cytokine, leukemia-inhibitory factor (LIF), is transiently expressed postnatally by glial cells in the hippocampus and rapidly up-regulated by enhanced neural activity following seizures. To test the hypothesis that spontaneous neural activity regulates glial development in hippocampus via LIF signaling, we blocked spontaneous activity with the sodium channel blocker tetrodotoxin (TTX) in mixed hippocampal cell cultures in combination with blockers of LIF and purinergic signaling. TTX decreased the number of GFAP-expressing astrocytes in hippocampal cell culture. Furthermore, blocking purinergic signaling by P2Y receptors contributed to reduced numbers of astrocytes. Blocking activity or purinergic signaling in the presence of function-blocking antibodies to LIF did not further decrease the number of astrocytes. Moreover, hippocampal cell cultures prepared from LIF $-/-$ mice had reduced numbers of astrocytes and activity-dependent neuron–glial signaling promoting differentiation of astrocytes was absent. The results show that endogenous LIF is required for normal development of hippocampal astrocytes, and this process is regulated by spontaneous neural impulse activity through the release of ATP.

Keywords

Activity dependent; cytokine; glial differentiation; neuron–glia signaling; synaptic plasticity

INTRODUCTION

Many studies have established that glial morphology, proliferation and differentiation are regulated by environmental enrichment (Sirevaag and Greenough, 1991; Jones *et al.*, 1996; Kronenberg *et al.*, 2007), visual cortical activity (Friedman and Shatz, 1990; Muller, 1990; Gargini *et al.*, 1998), sensory deprivation through monocular occlusion (Hawrylak and Greenough, 1995) or activity blockade using TTX (Barres and Raff, 1993), but the signaling mechanisms communicating functional activity to glia and in turn controlling glial development are largely unknown. Many aspects of neuronal development are influenced by glia, including neuron survival, differentiation, migration, dendritic morphogenesis, synapse formation, elimination and plasticity. All of these neuronal processes can be regulated by neural

*Marc R Freeman served as Editor-in-Chief for this manuscript.

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Correspondence should be addressed to: R. Douglas Fields, Nervous System Development and Plasticity Section, National Institutes of Health, NICHD Bldg. 35, Room 2A211, MSC 3713, 35 Lincoln Drive, Bethesda, MD 20892, USA, phone: +1 301 480-3209, fax: +1 496-9630, email: fieldsd@mail.nih.gov.

impulse activity in fetal and early postnatal development (Spitzer, 2006), suggesting that identifying molecular mechanisms regulating activity-dependent glial development could contribute to a better understanding of experience-dependent development and plasticity in neural circuits.

Here we test a specific molecular mechanism that could regulate the development of hippocampal astrocytes in response to changes in neural impulse activity. This involves inter-cellular communication between neurons and glia through ATP released from electrically active neurons, stimulating the subsequent release of the cytokine leukemia-inhibitory factor (LIF) to promote astrocyte differentiation.

Cytokines, including LIF, are known to regulate cell proliferation and differentiation during development and in response to injury (Bauer *et al.*, 2007), but these signaling molecules have not been implicated in experience-dependent development and plasticity. Interestingly, postnatal LIF expression in the rodent hippocampus is transient and absent under normal conditions in the adult. We tested whether the transient increase in LIF during development was critical for normal hippocampal development by using LIF $-/-$ mice and determining whether GFAP-expressing astrocytes are involved in this process in an activity-dependent manner *in vitro*.

The results support a role of LIF during normal development, but this activity-dependent process of regulating glial development may have relevance to developmental disorders that result from abnormal functional activity or disease causing inflammation and the release of cytokines, including drugs of abuse during critical periods of early postnatal development.

OBJECTIVES

The primary goals of this study were to test:

- Whether spontaneous neural activity regulates the differentiation of hippocampal astrocytes in cell culture
- Whether ATP released from electrically active neurons promotes astrocyte differentiation via a mechanism requiring LIF signaling
- Whether the loss of the LIF gene results in the development of fewer GFAP⁺ astrocytes

MATERIALS AND METHODS

Animals

Mice with a targeted deletion of the LIF gene were generously provided by Dr. Colin L. Stewart (NCI Frederick, Maryland) and maintained on a CD1 background (Stewart *et al.*, 1992). CD1 wild-type (wt) mice were used as controls. For the experiments, LIF $-/+$ females were bred with LIF $-/-$ males and genotyped by tail biopsies using DirectPCR (Viagen, Biotech Inc., Los Angeles, CA) and PCR supermix (Invitrogen, Carlsbad, CA). Timed-pregnant Sprague-Dawley rats were also used in this study. All procedures conformed to NIH animal welfare guidelines and approved animal study protocols.

Cell culture

Dissociated hippocampal cell cultures were prepared from timed-pregnant Sprague-Dawley rats (~E18.5) or CD1 wt and LIF $-/-$ mice (E17). In the case of LIF $-/-$ mice, individual hippocampi were processed prior to genotyping results. Hippocampi were dissected out in ice-cold Pucks' D1 buffer, dissociated by incubation in 0.25% trypsin for 15 min at 37°C and

trituted with fire-polished Pasteur pipettes. Hippocampal cells were plated at 130 cells/mm² on poly-L-lysine (Sigma)-coated coverslips in Neurobasal (Invitrogen, Carlsbad, CA) containing 2% B27, 25 μ M glutamate, 2 mM Glutamax-1, 100 U/ml Pen–100 μ g/ml Strep and 10% FBS. Medium was replaced 2 h later with medium lacking glutamate and FBS; half-changes were made every 3–4 days until treatments with drugs or cytokines at 7 days *in vitro* (DIV). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Semi-quantitative real-time RT-PCR

RNA was extracted from hippocampus and hippocampal cell cultures using TRIzol® reagent (Invitrogen). Hippocampi were rapidly dissected from P14 wt and LIF $-/-$ brains in ice-cold Pucks' D1 and homogenized in glass–Teflon homogenizers containing TRIzol® reagent. Total RNA (2 μ g) was reverse transcribed with Superscript II using oligo-dT (Invitrogen, Carlsbad, CA). For PCR, reactions were diluted 10-fold in RNase/DNase-free water. Semi-quantitative, real-time PCR was performed on a Roche LightCycler using the FastStart DNA Master SYBR Green 1 PCR reaction mix (Roche Diagnostics, Indianapolis, IN). Data analysis was performed using LightCycler Software (Roche Diagnostics) with quantification and melting-curve options. Fluorescence signals were quantified by the second derivative maximum method using LightCycler data analysis software to obtain crossing point values (*C_p*) and slope; PCR efficiency was determined by serial dilutions of cDNA template. Fold changes in amplified transcripts calculated as differences in threshold cycle (ΔC_p) between samples and the relative expression ratio (*R*) of target genes were calculated based on *E* and ΔC_p of the treated and untreated cDNA. For all transcripts, *R* was expressed as a ratio to the housekeeping gene glyceraldehyde phosphate dehydrogenase. All RT-PCR measurements were performed in duplicate. Primer sequences are listed in Table S1 in supplementary materials.

Immunofluorescence microscopy

Hippocampal cell cultures were fixed in 4% paraformaldehyde containing 4% sucrose and 10 mM EGTA for 30 min at 4°C. For immunofluorescence of hippocampal sections, brains from wt and LIF $-/-$ mice were fixed in 4% paraformaldehyde containing 4% sucrose and 10 mM EGTA overnight on a rocking platform at 4°C. The following day, brains were incubated in PBS containing 4% sucrose prior to preparing 100 μ m sections on a Vibratome (Vibratome St. Louis, MO). Coverslips or sections were rinsed three times for 5 min with PBS–4% sucrose, permeabilized with 0.1% Triton X-100 for 5 min, and quenched with 50 mM ammonium chloride and 50 mM glycine for 10 min. Samples were blocked with 3% normal goat serum (NGS) (Jackson ImmunoResearch, West Grove, PA) in PBS at room temperature (RT) for 1 h, followed by incubation with primary antibodies (diluted in 3% NGS) overnight at 4°C. Primary antibody dilutions and sources are listed in supplemental Table S2. Following 4 $\times$ 5 min rinses with PBS, highly cross-absorbed secondary antibodies coupled to Alexa-488, Alexa-568 or Alexa-633 (Molecular Probes, Eugene, OR) diluted 1:1000 in 3% NGS were incubated for 2 h at RT. Samples were then counterstained with Hoechst 33342 at a 1:5000 dilution for 5 min. All images (512 $\times$ 512 or 1024 $\times$ 1024 pixel resolution) were acquired on a Zeiss 510 NLO confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) equipped with a 20 $\times$ (0.8 NA) lens, and both 40 $\times$ (1.3 NA) and 63 $\times$ (1.4 NA) oil-immersion lens using appropriate laser lines and excitation/emission filters. Glial and neuronal cell densities were quantified by triple-labeling for astrocytes (GFAP⁺), oligodendrocyte precursors (NG2⁺), and neurons (MAP2⁺) and nuclei (Hoechst 33342).

Proliferation assays

Hippocampal cell cultures were incubated with 10 μ M BrdU at 10 DIV for 48 h following treatment with either 1 μ M TTX or 1 ng/ml LIF. At 12 DIV, coverslips were processed for

immunocytochemistry. Prior to blocking non-specific binding, coverslips were treated with 2 N HCl for 30 min at 37°C and neutralized with 0.1 M borate buffer (pH 8.5). Cells were triple labeled for glial cells (GFAP⁺ or Vimentin⁺), proliferating cells (BrdU⁺) and neurons (MAP2⁺), and counterstained for nuclei (Hoechst 33342).

Integrated morphometric analysis

Randomly chosen fields (450 × 450 μm) for wt (66 fields from 4 samples) and LIF ^{-/-} (63 fields from 4 samples) hippocampal cell cultures that had at least a single GFAP⁺ astrocyte were acquired on a Zeiss LSM NLO with a 40× oil-immersion lens. Fourteen morphological parameters were measured on each cell by an integrated morphometry package (MetaMorph, Molecular Dynamics, Sunnyvale, CA). To directly compare astrocyte morphology between wt and LIF ^{-/-} mice hippocampal cell cultures, several parameters were directly compared: cell area, area of holes within cell boundaries, cell perimeter and cell shape factor ($4\pi A/P^2$, where A is the area and P is the perimeter). Measurements were compared by unpaired t -test.

Pharmacological treatments

All pharmacological treatments were made by replacing culture medium with fresh medium at 7 DIV containing drugs at final concentration: apyrase grade VI, 30 U/ml, TTX Na⁺-citrate, 1 μM (Sigma, St. Louis, MO); PPADS tetrasodium salt, 50 μM, suramin hexasodium salt, 50 μM (Tocris Biosciences, Ellisville, MO); rat LIF 1 ng/ml (Chemicon, Billerica, MA), LIF function-blocking antibody, 200 ng/ml (R&D Systems, Minneapolis, MN). For LIF release assays, half-medium changes were made on 14 DIV cultures of mouse astrocytes and mixed neuronal cultures containing purinergic agonists: 2-MS, αβ-methylene-ATP, ATPγS or UTP (100 μM final concentration).

LIF ELISA

Mouse hippocampal astrocyte and mixed neuronal cultures were treated at 14 DIV with purinergic agonists for 24 h. Conditioned medium was collected and pooled from three replicate wells for each condition and concentrated on 3 kDa MWCO glycerin-coated Microsep concentrators to ~100 μl (Pall Life Sciences, East Hills, NY). Each sample was assayed in duplicate for LIF content using a Quantikine LIF ELISA assay (R&D Systems, Minneapolis, MN) on a Victor Wallac microtiter plate reader as previously described (Ishibashi *et al.*, 2006).

Extracellular measurement of ATP

Hippocampal cell cultures at 7 and 12 DIV were superfused with sterile-filtered recording saline containing (in mM) NaCl, 145; KCl, 4.5; MgSO₄, 0.8; CaCl₂, 1.8; HEPES, 10; glucose, 10 at pH 7.3 for 15 min using a peristaltic pump (1.4 ml/min[43]). After 15 min, the pump was switched off in order to condition the medium for an additional 5 min. The pump was switched on again and superfusate collected in 30 s fractions. Cultures were then superfused in recording saline containing 1 μM TTX. The fractions were either assayed immediately or flash frozen. ATP measurements including known standards were made using a luciferin–luciferase ATP determination kit (Sigma, St. Louis, MO). For each sample, two individual measurements (5 s integration time) were made on a Lumat LB 9507 Berthold luminometer (Berthold Technologies USA, Oak Ridge, TN) using 100 μl of conditioned medium and 100 μl of enzyme mixture.

Data and statistical analysis

All values are reported as mean ± standard error of mean. For each condition analyzed, 20 randomly chosen fields were acquired per coverslip (n) and % change in cell number was

calculated by manually counting either GFAP⁺, NG2⁺ or MAP2⁺ cells and total cells (Hoechst 33342) per microscopy field (40× objective, 225 × 225 μm) and normalizing to mean cell numbers per cell type. For experiments assessing proliferation, coverslips were immunostained with cell-specific markers, MAP2 and BrdU[44]. Gain and offset settings were optimized for each fluorescence channel and cells were scored positive if immunoreactivity was associated with a cell nucleus. For multiple comparisons between samples, one-way ANOVA and Dunnet's post-hoc test were used. For drug effects on NG2⁺ cells, a non-parametric Kruskal-Wallis test was used because the oligodendrocyte precursor cells (OPC) cell frequency was not normally distributed, but rather followed a Poisson distribution as expected for rare events. Extracellular levels of ATP were calculated by normalizing luminescence counts to a calibrated ATP standard curve. Descriptive statistics, including Student's *t*-test, one-way ANOVA and post-hoc tests were used to assess statistical significance using Sigma Plot 10.0 software (SPSS, Chicago, IL) or Minitab (Minitab Inc., State College, PA). Morphometric analysis was performed using MetaMorph as described above (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Previously we determined that the synthesis and secretion of LIF from astrocytes can be regulated by electrical impulses in dorsal root ganglion axons to stimulate myelination by oligodendrocytes (Ishibashi *et al.*, 2006). The hypothesis that a similar molecular mechanism could regulate differentiation of hippocampal GFAP-positive astrocytes in an activity-dependent manner was investigated.

Normal cell cultures prepared from hippocampus contain a mixture of neurons and glial cells. The cellular composition of our hippocampal cell cultures was determined by using several immunocytochemical markers for glial precursor cells (GPC), astrocytes, oligodendrocyte precursors and neurons. We found that from 7 to 12 DIV, there was an increase in immunoreactivity for GFAP and S100β, as well as morphological changes in GPC expressing vimentin and nestin, becoming more complex (Fig. 1A). By 12 DIV, neurons accounted for 27 ± 1% of total cells in mixed cultures (Fig. 1B). In all, 22 ± 1% of total cells were GFAP⁺ and 13 ± 1% expressed NG2. Cells negative for the markers GFAP, MAP2 and NG2 (GPCs) made up 39 ± 2% of total cell numbers (*n* = 34).

LIF is required for development of hippocampal glia

Immunocytochemistry showed strong LIF receptor (LIF-R) expression on NG2⁺-OPC and GFAP⁺-astrocytes in hippocampal cell cultures at 7 and 12 DIV (Fig. 2A). Neurons also stained strongly for LIF-R at these time points with diffuse staining in the dendrites and strong nuclear staining as has been reported in cells previously (Gardiner *et al.*, 2002). LIF immunoreactivity was present in both neurons and GFAP⁺ cells (Fig. 2B) with punctate staining in the processes (see arrowheads). (We also observed weak staining for LIF in both vimentin⁺ and NG2⁺ cells.) LIF, absent in the adult hippocampus, is induced in an activity-dependent manner in neurons and astrocytes following seizures (Yamakuni *et al.*, 1996; Jankowsky and Patterson, 1999; Rosell *et al.*, 2003). These studies support the observation of LIF immunoreactivity in hippocampal cultures and its potential role as an activity-dependent signaling molecule[45].

Hippocampal cell cultures prepared from LIF^{-/-} mice had significantly fewer GFAP⁺ astrocytes (12 ± 1%, *n* = 10) compared with wt mice (17 ± 2%, *n* = 8) (*P* < 0.05) consistent with the *in vivo* findings (Bugge *et al.*, 1998; Koblar *et al.*, 1998). There were no significant effects on either neuronal cell numbers or OPC cell numbers in LIF^{-/-} mice. (OPCs were present at very low density in both wt and LIF^{-/-} mouse cultures compared to rat cultures, <5% of total cells vs. 13 ± 1%, *n* = 34.)

Astrocytes from LIF $-/-$ mice were morphologically different from astrocytes derived from wt mice. GFAP immunoreactivity was decreased in astrocytes in hippocampal cell cultures from LIF $-/-$ mice and they had fewer, less stellate processes (Fig. 3A). The morphological differences were confirmed by automated computer morphometric analysis of astrocytes from wt and LIF $-/-$ hippocampal cell cultures using several parameters for comparison, including cell area, hole area, perimeter and shape factor. This analysis confirmed that astrocytes derived from LIF $-/-$ mice were smaller and were morphologically less complex than astrocytes derived from wt mice (Table 1). Dendritic processes (Fig. 3B) and spines (Fig. 3B, inset) immunostained for MAP2 appeared morphologically normal in LIF $-/-$ cultures. This suggests that endogenous LIF is necessary for normal development of hippocampal astrocytes.

Conversely, low concentrations of LIF (1 ng/ml) added to hippocampal cell cultures significantly increased the numbers of GFAP-positive astrocytes by $29 \pm 6\%$ ($P < 0.001$) and OPCs ($P < 0.05$) by $39 \pm 16\%$ ($P < 0.001$) (Fig. 2C). Both the length and thickness of GFAP⁺ processes were increased in cultures treated with LIF, and the intensity of GFAP immunoreactivity was elevated (Fig. 2D, arrowhead in LIF-treated culture). In contrast to effects on astrocytes, LIF had no significant effect on the number of neurons or total cell numbers. Moreover, LIF treatment did not alter the proliferation rate in culture as measured by BrdU incorporation into cells undergoing mitosis. Proliferation rate in LIF-treated cultures was $33 \pm 3\%$ of total cells compared to $35 \pm 2\%$ in untreated cultures (supplemental Fig. 1).

Consistent with the effects of exogenous LIF in increasing GFAP⁺ cells in hippocampal cultures, blocking the effects of endogenous LIF with a LIF-function blocking antibody (LIF-FBA) significantly decreased the numbers of GFAP⁺ cells by $14 \pm 4\%$ ($n = 20$, $P < 0.01$) and decreased overall expression of GFAP (Fig. 2C). This finding suggested that endogenous levels of LIF in normal hippocampal cell cultures are sufficient to activate the LIF-R and promote differentiation of GFAP⁺ cells. As a control, normal goat IgG at 200 ng/ml did not affect astrocyte, OPC or neuronal cell numbers in hippocampal cell cultures. (Addition of LIF or blockade with LIF-FBA did not change total cell numbers in these experiments.) Exogenous LIF also increased mRNA transcript levels for GFAP by 1.7-fold at 12 DIV and 2.2-fold at 14 DIV. Conversely, levels of GFAP mRNA were reduced by 1.5-fold and 1.4-fold in hippocampal cell cultures treated with LIF-FBA at 12 and 14 DIV, respectively.

Immunofluorescence staining for GFAP in hippocampus was less intense in sections prepared from LIF $-/-$ mice compared to CD1 wt mice (Fig. 4A). Moreover, GFAP transcript was reduced by 2.1 ± 0.3 -fold in LIF $-/-$ mice ($P < 0.05$) (Fig. 4B). There were no significant changes in transcript levels for other astrocyte genes that were tested with the exception of the inwardly rectifying potassium channel, Kir4.1, which was modestly increased in LIF $-/-$ hippocampus (1.20 ± 0.04 -fold, $P < 0.05$). Conversely, treatment of mixed hippocampal cell cultures with 1 ng/ml LIF decreased Kir4.1 expression by both RT-PCR and immunocytochemistry.

These results show that the number of GFAP⁺ cells in hippocampal cell cultures is increased by endogenous levels of LIF and can be further increased by supplementing the basal levels of LIF. These results in cell culture are consistent with the reduced number of GFAP⁺ cells observed in the hippocampus of LIF $-/-$ mice (Fig. 4A), suggesting that LIF secretion is required for normal hippocampal development during the neonatal period.

Spontaneous neuronal impulse activity regulates glial differentiation

The hypothesis that activity-dependent signaling between neurons and glial cells could regulate glial development was then tested by blocking spontaneous impulse activity in hippocampal cell cultures. Although activity-dependent regulation of glial development has been demonstrated by many *in vivo* studies, the molecular signaling mechanisms are difficult to

isolate *in vivo*. Therefore, spontaneous neural impulse activity was blocked in hippocampal cell cultures with TTX (1 μ M) and after 5 days of treatment, the cellular composition of the cultures was characterized using immunocytochemical markers of astrocytes, oligodendrocytes, neurons and undifferentiated progenitor cells.

The results showed that at 7 DIV, when hippocampal cell cultures are gradually becoming spontaneously active (Basarsky *et al.*, 1994), the majority of non-neuronal cells expressed vimentin, 3CB2 and nestin, with a lower number of cells expressing the astrocytic markers, GFAP and S100 β . These features are characteristic of GPC predominating in these early cultures (Fig. 1A). By 12 DIV, GFAP-expressing cells greatly increased, and accounted for $22 \pm 1\%$ of total cells ($n = 34$ cultures). This increase parallels astrocyte development *in vivo* (Catalani *et al.*, 2002; Wei *et al.*, 2002; Raponi *et al.*, 2007).

Blocking spontaneous neural impulse activity with TTX significantly decreased the number of GFAP⁺ cells at 12 DIV by $19 \pm 3\%$ ($P < 0.001$) (Fig. 5A). Transcript levels for GFAP were significantly increased by 1.6 ± 0.2 -fold ($n = 4$, $P < 0.05$) and 1.9 ± 0.2 -fold ($n = 4$, $P < 0.01$) at 12 and 14 DIV, respectively (when normalized to 7 DIV). In contrast, cultures in which spontaneous impulse activity was blocked from 7 DIV onwards, GFAP transcript levels did not significantly increase by either 12 or 14 DIV (blocking spontaneous activity with TTX-reduced GFAP transcript levels by $14 \pm 5\%$ and $22 \pm 5\%$ at both 12 and 14 DIV, respectively). GLT-1 transcript increased by 2.8 ± 0.3 -fold ($n = 4$, $P < 0.005$) and 3.8 ± 0.6 -fold ($n = 4$, $P < 0.005$) at 12 and 14 DIV, respectively (when normalized to 7 DIV); in the presence of TTX, transcript levels were reduced $21 \pm 7\%$ and $21 \pm 10\%$ at both 12 and 14 DIV, respectively.

This is consistent with fewer numbers of GFAP⁺ cells in cultures lacking spontaneous activity. In addition, the expression of GFAP in individual cells was also reduced. In particular, the GFAP⁺ cell processes on astrocytes grown in cultures where electrical activity had been blocked appeared less robust than in hippocampal cell cultures with spontaneous activity (Fig. 5B).

In contrast, blocking spontaneous activity did not significantly decrease the number of MAP2⁺ neurons (n.s.), NG2⁺ OPC (Fig. 5A), and there was no effect on total cell numbers (n.s.[47]). Blocking neural impulse activity did not significantly affect either overall cell proliferation rate ($35 \pm 2\%$ of total cells vs. $32 \pm 3\%$ in the presence of 1 μ M TTX, $n = 6$, $P > 0.1$) or proliferation of GFAP⁺ cells (25 ± 4 vs. $24 \pm 2\%$, $n = 6$, $P > 0.1$). As expected, the majority of proliferating cells were vimentin⁺ progenitor cells ($60 \pm 5\%$ of BrdU⁺ cells, supplemental fig. 1), accounting for $42 \pm 2\%$ of total cells in culture.

These results support a role for spontaneous activity in promoting hippocampal glial cell differentiation by increasing the number of GFAP⁺ cells. We therefore sought to identify the cellular signaling mechanism responsible for activity-dependent regulation of glial development in hippocampus. Although many growth factors could be released in an activity-dependent manner from neurons to affect glial development, purinergic signaling has recently become appreciated as an important component of activity-dependent neuron–glial signaling (Fields and Stevens, 2000; Fields and Burnstock, 2006; North and Verkhratsky, 2006). In other contexts, previous research has shown that purinergic signaling through P₂Y receptors on astrocytes increases the expression (Yamakuni *et al.*, 2002) and release of LIF (Ishibashi *et al.*, 2006). By hypothesis, activity-dependent release of ATP from developing hippocampal neurons could regulate astrocyte development by inducing LIF release from hippocampal astrocytes.

Spontaneous activity and ATP release promote astrocyte differentiation through P₂Y receptors

ATP can be released by neuronal impulse activity, and ATP analogs acting through P₂Y receptors can regulate astrocyte morphology (Neary *et al.*, 1994; Abbracchio *et al.*, 1995; Bolego *et al.*, 1997) as well as developmental changes in neural progenitor cells and GPC differentiation into astrocytes (Lin *et al.*, 2007). If spontaneous impulse activity in hippocampal cell cultures also releases ATP, activation of purinergic receptors on hippocampal cells in the astrocyte lineage could induce transcription of the LIF gene, resulting in an activity-dependent increase in LIF. This would couple the potent actions of LIF in regulating differentiation of glial cells to the state of functional activity in developing hippocampus. Accordingly, the concentration of ATP and LIF in hippocampal cell cultures should be reduced by blocking spontaneous activity with TTX, and LIF concentration should be increased by agonists of ATP receptors. Moreover, interfering with either activity-dependent regulation of ATP or LIF signaling in hippocampal cell cultures should mimic the effect of blocking spontaneous impulse activity with TTX, in regulating the number of GFAP positive astrocytes in hippocampal cell cultures.

Measurements showed that the ATP concentration in culture medium increased from 1.25 ± 0.15 to 1.54 ± 0.18 pM between 7 and 12 DIV, paralleling the increasing amount of spontaneous impulse activity developing over this developmental period. Blocking spontaneous impulse activity with 1 μ M TTX for 15 min significantly decreased the levels of ATP in the culture medium by $35 \pm 7\%$ at 7 DIV ($n = 10$, $P < 0.005$, two-tailed t -test) and $30 \pm 7\%$ at 12 DIV ($n = 11$, $P < 0.005$, two-tailed t -test), indicating that ATP release is regulated by spontaneous neural impulse activity in hippocampal cell cultures.

Immunocytochemical staining revealed that glial progenitor cells in hippocampal cell cultures at 7 DIV have receptors for extracellular ATP (P₂Y₁ and P₂Y₂ receptors) (Fig. 6A, see arrowhead and compare to Fig. 1A). By 12 DIV, GFAP⁺ cells developed strong punctate staining for P₂Y receptors in processes adjacent to neuronal MAP2⁺ dendrites (Zhu and Kimelberg, 2004) consistent with a possible role for P₂Y receptor in neuron–glial signaling by ATP released in an activity-dependent manner from hippocampal neurons.

To test the effects of activity-dependent purinergic signaling on differentiation of hippocampal glia, purinergic signaling was blocked with specific ATP receptor antagonists and disrupted by an enzyme (apyrase) that rapidly degrades extracellular ATP. The results showed that interfering with purinergic signaling by either 30 U/ml apyrase ($n = 22$) or the P₂Y antagonists 50 μ M PPADS ($n = 10$) or 50 μ M suramin ($n = 10$) significantly decreased the numbers of GFAP⁺ cells by $21 \pm 3\%$ (apyrase), $13 \pm 4\%$ (PPADS) and $20 \pm 6\%$ (suramin), respectively (Fig. 6B) ($F_{81,4} = 6.34$, $P = 0.0001$, one-way ANOVA). The degree of inhibition by apyrase was comparable to that seen in the following treatment with 1 μ M TTX ($n = 22$) (compare to Fig. 5A), consistent with the hypothesis that impulse activity affects hippocampal development through ATP signaling. Blockade of purinergic signaling with apyrase, PPADS or suramin did not affect total cell numbers, or neuronal numbers, similar to the results of TTX treatment. To our knowledge, this is the first study to demonstrate that neuronal activity, acting via ATP release, contributes to astrocyte development.

LIF regulates glial differentiation by coupling with activity-dependent purinergic signaling

To test the hypothesis that LIF is released in an activity-dependent manner following activation of extracellular ATP receptors, primary cultures of mouse hippocampal astrocytes were treated with purinergic agonists, $\alpha\beta$ -methylene-ATP, ATP γ S, UTP and 2-MS at 100 μ M for 24 h and conditioned media was assayed for LIF concentration by ELISA. Basal LIF concentration in astrocyte cultures was 19.0 ± 0.9 pg/ml. Treatment with ATP γ S ($P < 0.1$, $n = 3$) and UTP (P

< 0.05 , $n = 3$) induced LIF release from astrocytes (Fig. 7A), whereas 2-MS and $\alpha\beta$ -methylene-ATP were inactive, thus implicating P_2Y_2 receptors in mediating LIF secretion from astrocytes. In mixed hippocampal cell cultures, ATP γ S also significantly increased LIF release above basal (0.11 ± 0.01 pg/ml, $P < 0.01$, $n = 3$).

To test the hypothesis that the LIF-mediated effects on glial cell differentiation are regulated by spontaneous activity, hippocampal cell cultures were incubated in LIF-FBA, together with TTX to block spontaneous impulse activity or apyrase to block purinergic signaling. The results showed that hippocampal cell cultures treated with TTX ($-19 \pm 5\%$, $P < 0.05$), apyrase ($-25 \pm 5\%$, $P < 0.005$) or LIF-FBA ($-26 \pm 9\%$, $P < 0.05$) had significantly less astrocytes compared with untreated cultures ($n = 8$) (Fig. 7B). Treatment with either LIF-FBA + TTX ($-24 \pm 2\%$, $P < 0.005$) or LIF-FBA + apyrase ($-27 \pm 4\%$, $P < 0.005$) did not further decrease astrocyte numbers, consistent with the hypothesis that spontaneous activity and purinergic signaling regulate glial development through LIF signaling. For confirmation of this conclusion, cultures were supplemented with low concentrations of LIF to determine whether exogenous LIF could rescue the effect of activity blockade on GPC differentiation. Indeed, addition of 1 ng/ml LIF to TTX-treated cultures restored GFAP $^{+}$ cell numbers to levels of spontaneously active cultures ($P < 0.05$, $n = 6$).

Many other activity-dependent factors are likely to contribute to the development of hippocampal astrocytes. To determine the extent to which activity-dependent regulation of hippocampal development acts through LIF synthesis and secretion via purinergic receptor signaling, experiments were conducted on LIF $-/-$ hippocampal cell cultures. Although both 1 μ M TTX and 30 U/ml apyrase significantly decreased the number of GFAP $^{+}$ cells by $39 \pm 6\%$ ($P < 0.05$) and $42 \pm 2\%$ ($P < 0.01$) in wt cultures, neither TTX ($9 \pm 14\%$ reduction, n.s.) nor apyrase ($1 \pm 16\%$ reduction, n.s.) significantly decreased the number of astrocytes in LIF $-/-$ cultures (Fig. 8A). There were no significant effects on either neuronal cell numbers or OPC cell numbers with TTX or apyrase in wt or LIF $-/-$ mice. Thus, LIF signaling is required for the increases in GFAP $^{+}$ cells produced by ATP receptor activation or spontaneous neural impulse activity.

An alternative hypothesis to explain the lack of effects of apyrase and TTX on GFAP $^{+}$ cells is that the glial population is different in LIF $-/-$ hippocampal cell cultures. To test this hypothesis, hippocampal cell cultures from LIF $-/-$ cultures were grown from 2 to 7 DIV in the presence of 0.1 ng/ml murine LIF. LIF $-/-$ cultures grown in the presence of LIF had significantly more GFAP $^{+}$ cells compared to LIF $-/-$ cultures alone ($P < 0.05$, $n = 4$); however, neither TTX nor apyrase significantly decreased the number of GFAP $^{+}$ cells (Fig. 8B). The lack of activity-dependent effects may also be due to impairment in either electrical activity or transmitter; however, the majority of neurons within the hippocampus differentiate embryonically prior to the expression of LIF. Moreover, although LIF promotes neuronal cell survival, neuronal cell numbers were not significantly different in LIF $-/-$ hippocampal cultures. These results do not exclude the possibility of other activity-dependent signals contributing to hippocampal development, but the data indicate that activity-dependent regulation of glial differentiation induced by blocking sodium-dependent action potentials or extracellular ATP in these experiments requires LIF.

CONCLUSIONS

- LIF is required for normal astrocyte development in hippocampus. Mixed hippocampal cell cultures derived from LIF $-/-$ mice have reduced numbers of GFAP $^{+}$ cells.
- Blocking electrical activity with TTX decreases the number of GFAP $^{+}$ cells and this is rescued by exogenous LIF.

- Hippocampal astrocytes from LIF $-/-$ mice lack activity-dependent regulation through either neuronal activity or ATP release.
- LIF function-blocking antibodies prevent the activity-dependent increase in GFAP⁺ cells by ATP.

DISCUSSION

These studies have identified an activity-dependent mechanism whereby spontaneous activity of hippocampal neurons regulates astrocyte development. We have presented several lines of evidence to support the contribution of neural impulse activity to astrocyte differentiation, in part through neuron–glial signaling via ATP and LIF. First, we have identified ATP as a neuron-derived signal that acts on glia in an activity-dependent manner to promote GPC differentiation into astrocytes in mixed hippocampal culture. Similar mechanisms may regulate glial development in other brain regions and this in turn could regulate neuronal development. For example, radial glial cells serve several functions during development, including migration of neurons and astrocytes and functioning as a progenitor cell pool for astrocytes (Goldman, 2003). Kriegstein and colleagues (Weissman *et al.*, 2004) have demonstrated that radial glial cell migration and differentiation are regulated through purinergic signaling mediated by P₂Y receptors. Progenitor cell proliferation from neurospheres can be regulated in an autocrine manner by pulsatile release of ATP from the progenitor cells themselves that decreases over several days *in vitro* (Lin *et al.*, 2007).

Activated microglia are a potential source of extracellular ATP and LIF and may contribute to regulating astrocyte differentiation and reactivity. We did not find any microglia in these hippocampal cell cultures using the microglial markers CD11b, CD68 and F 4/80.

Blocking spontaneous activity with TTX did not completely block ATP release from hippocampal cell cultures. Non-synaptic release mechanisms likely contribute a significant amount of extracellular ATP. However, blocking P₂Y receptors with either PPADS or suramin decreased numbers of GFAP⁺ cells to similar levels seen for activity blockade (Fig 6 and Fig 7). Furthermore, apyrase, which depletes all extracellular ATP in hippocampal cell cultures, did not completely inhibit astrocyte differentiation. ATP signaling through P₂Y receptors regulates both astrocyte morphology and differentiation (Neary *et al.*, 1994; Abbracchio *et al.*, 1995; Bolego *et al.*, 1997). However, these studies examined direct effects of adding exogenous ATP and agonists on astrocytes. It is clear that astrocyte differentiation is regulated by many factors in addition to purinergic signaling as astrocyte differentiation persisted in the absence of extracellular ATP (Bonaguidi *et al.*, 2005). More importantly, this is the first study to demonstrate that spontaneous impulse activity in neurons promotes astrocyte differentiation in an ATP-dependent manner.

One of the major findings is that LIF, a member of the IL-6 family of cytokines, is required for hippocampal development *in vitro* in an activity-dependent manner. LIF, originally identified as cholinergic differentiation factor in the peripheral nervous system (Yamamori *et al.*, 1989; Bamber *et al.*, 1994), regulates stem cell proliferation, inflammation and injury (Bauer *et al.*, 2007). LIF expression is absent in the adult rat and is rapidly up-regulated by seizures in the hippocampus in an activity-dependent manner (Minami *et al.*, 2002; Holmberg and Patterson, 2006). Seizure-induced LIF promotes formation of reactive astrocytes in the dentate and CA1 and may act in a neuroprotective manner. In contrast to the role of LIF as a pro-inflammatory cytokine during injury, evidence for a role of LIF in hippocampal development has not been previously demonstrated (Lemke *et al.*, 1996). Cardiotrophin-1 (another member of the IL-6 family of cytokines) acts as a neuron-derived signal promoting astroglial development during late embryonic development of the cortex (Barnabe-Heider *et al.*, 2005); this study suggested that LIF may play a role in glial development postnatally. We

have found that LIF expression in the hippocampus is transiently increased during early postnatal development.

Glial cells undergo a rapid and transient expansion from progenitor cells within the first several weeks of life, which coincides with increasing neuronal activity in the hippocampus (Catalani *et al.*, 2002). Our results support a role for emerging neuronal activity in promoting astrocyte differentiation *in vivo*. We found that blocking endogenous LIF signaling decreased the number of GFAP-expressing astrocytes; blocking both LIF signaling in combination with either TTX or apyrase was not additive, supporting the concept that spontaneous activity promotes glial differentiation in part through LIF (Fig. 7B). Furthermore, hippocampal cell cultures prepared from LIF $-/-$ mice had reduced numbers of astrocytes, and blocking activity with either TTX or apyrase did not further reduce astrocyte cell numbers (Fig. 8A). We rescued the phenotype of reduced astrocyte numbers in LIF $-/-$ cultures by adding back LIF (from 2 to 7 DIV) but found that regulating spontaneous activity and ATP in the absence of exogenous LIF (from 7 to 12 DIV) did not affect numbers of GFAP⁺ cells (Fig. 8B). These findings demonstrate that LIF is required as an activity-dependent factor in astrocyte development.

We found that LIF did not alter the proliferation rate of vimentin⁺ cells in hippocampal cell cultures (supplemental Fig. 1). Proliferation of radial glial neural stem cells and glial progenitors is not affected in the adult dentate of LIF $-/-$ mice (Muller *et al.*, 2008). In contrast, over-expression of LIF promotes neural stem cell self-renewal (Bauer *et al.*, 2007); the proliferative effects of LIF are likely restricted to the subventricular zone.

Poland and colleagues (Pechnick *et al.*, 2004) found that LIF $-/-$ mice had reduced immobility in the forced swim test, suggesting that these mice had elevated levels of anxiety; additionally, rodents injected with LIF during early postnatal development display increased GFAP immunoreactivity and decreased pre-pulse inhibition in an acoustic startle test by adolescence (Watanabe *et al.*, 2004). These behavioral studies argue for the involvement of LIF in postnatal brain development at least in part through alterations in glia. The importance of astrocytes and morphological contacts formed between astrocytic processes and synapses is apparent as astrocytes and astrocyte-conditioned media promote synapse formation (Pfrieger and Barres, 1997; Ullian *et al.*, 2001) and both LTP and LTD are affected by astrocytes acting through several mechanisms. Astrocytes directly regulate NMDA-receptor-dependent LTP and plasticity through glial-derived D-serine (Yang *et al.*, 2003; Panatier *et al.*, 2006), ATP and adenosine (Pascual *et al.*, 2005; Fields and Burnstock, 2006), glutamate reuptake and release (Haydon and Carmignoto, 2006) and extracellular K⁺ handling (Wallraff *et al.*, 2006; Djukic *et al.*, 2007; Ge and Duan, 2007). Our findings on activity-dependent astrocyte differentiation by ATP and LIF *in vitro* and the observation of behavioral impairments in LIF $-/-$ mice (Pechnick *et al.*, 2004) and LIF over-expressing mice (Pechnick *et al.*, 2004) suggest that LIF may be important in hippocampal development and plasticity *in vivo* through astrocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank Peter Wadeson and Daniel Abebe for assistance with experimental animals, Tatiana Cohen and Alexander Dityatev for critical comments on a previous version of the manuscript, and Olena Bukalo and Philip R. Lee for insightful discussion on this study. We also thank Colin Stewart for generously providing LIF $-/-$ mice for this study. This work was supported by the intramural research program at National Institutes of Health, National Institute of Child Health and Human Development.

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Fig. 1. Differentiation of GPC in hippocampal culture

(A) Mixed hippocampal cell cultures express markers for GPC and neurons. Cultures immunostained at either 7 or 12 DIV for glial cell markers: nestin, vimentin, 3CB2 or S100 (Alexa 568) and triple-labeled with GFAP (Alexa 488) and MAP2 (Alexa 633). At 7 DIV, the majority of glial progenitor cells do not express GFAP and stain strongly for nestin and vimentin. GPC have a less complex morphology than GFAP-expressing cells with few, short processes. By 12 DIV, vimentin expression is down-regulated and astrocytes have a more stellate appearance with increased expression of GFAP and the radial glial marker 3CB2. Expression for the mature astrocyte marker, S100 β , was also increased by 12 DIV. Scale bar = 25 μ m. (B) MAP2⁺ neurons made up $27 \pm 1\%$ of total cell numbers in these cultures, whereas

GFAP-expressing astrocytes made up $22 \pm 1\%$. Rat hippocampal cell cultures at 12 DIV were predominately comprised of cells negative for the markers GFAP, MAP2 and NG2. The majority of these non-neuronal cells express low levels of vimentin and nestin.

Fig. 2. LIF alters both astrocyte cell number and type in hippocampal cell cultures

(A) LIF-R was ubiquitously expressed on all cell types at 7 and 12 DIV. LIF-R (Alexa 488), GFAP and NG2 (Alexa 568), and MAP2 (Alexa 633). Expression was moderately strong in neuronal processes as well as in GFAP⁺ cells and OPC. By 12 DIV, LIF-R was still present in astrocyte processes, OPCs and neurons. Note that strong expression of LIF-R is present in the nucleus with more diffuse staining in processes (see Gardiner *et al.*, 2002). Scale bar = 25 μ m. (B) LIF is expressed in neurons and astrocytes from both 7 and 12 DIV hippocampal cell cultures. Arrowheads indicate punctate staining in MAP2⁺ and GFAP⁺ processes. Scale bar = 20 μ m. (C) LIF significantly increased the numbers of astrocytes and OPCs in culture and depleted the GPC pool ($n = 22$) compared to untreated controls ($n = 34$). Blocking function of

LIF with a LIF-FBA (LIF FBA, 200 ng/ml) significantly decreased the number of astrocytes ($n = 20$, one-way ANOVA, $F_{73,2} = 22.93$ and $P = 0.0001$). GFAP⁺ astrocytes and NG2⁺ OPCs comprised 22 ± 1 and $13 \pm 1\%$ of total cells, respectively. *** $P < 0.005$ and ** $P < 0.01$. (D) LIF at 1 ng/ml increased both the number of GFAP⁺ cells and level of GFAP immunoreactivity. LIF also increased the number of OPCs in culture as well as the intensity of NG2 immunoreactivity. Arrowhead denotes GFAP⁺ processes that have become thickened and are longer with increased stellate appearance compared to untreated cultures. Scale bar = 25 μ m.

Fig. 3. Reduced numbers and morphological differences of hippocampal astrocytes from LIF $-/-$ mice

(A) Astrocytes derived from LIF $-/-$ mice exhibited a simpler morphology with reduced GFAP immunoreactivity compared to wt cultures. In hippocampal cell cultures derived from LIF $-/-$ mice, there were fewer astrocytes per field and astrocytes had less ramifications. Astrocytes from wt hippocampal cell cultures had a more stellate appearance. Scale bar in both = 25 μ m. (B) Representative MAP2 staining of neurons derived from wt and LIF $-/-$ hippocampal cell cultures. Neurons from LIF $-/-$ cultures were healthy with dendritic branching and the appearance of spines (arrowheads correspond to inset). Scale bar = 25 μ m.

Fig. 4. Reduced expression of GFAP in hippocampus of LIF $-/-$ mice

(A) Hippocampal sections (100 μ m) from P14 wt and LIF $-/-$ mice were immunostained for GFAP (Alexa 488) and Hoescht 33342. GFAP staining was decreased in sections prepared from LIF $-/-$ hippocampus compared to sections prepared from wt hippocampus (scale bar = 200 μ m). GFAP $^{+}$ -astrocytes appeared less stellate in LIF $-/-$ hippocampus as well (insets a–c, scale bar = 50 μ m). (B) Transcript levels for astrocyte genes in hippocampus. LIF $-/-$ mice ($n = 3$ hippocampi) had significantly reduced levels of GFAP compared to wt mice ($n = 3$ hippocampi), $P < 0.05$). Conversely, Kir4.1 was modestly increased in LIF $-/-$ mice.

Fig. 5. Spontaneous neuronal activity regulates glial differentiation

(A) Blocking spontaneous activity with 1 μ M TTX for 5 days decreased the percentage of GFAP-expressing astrocytes by $19 \pm 3\%$ ($n = 32$, *** $P < 0.001$). There were no significant effects on either OPCs or neurons. GPCs were not significantly decreased with TTX. Mixed hippocampal cell cultures were primarily comprised of neurons ($27 \pm 1\%$), astrocytes ($22 \pm 1\%$) and OPCs ($13 \pm 1\%$). Cells negative for the markers GFAP, MAP2 and NG2 (GPCs) comprised $39 \pm 2\%$ of total cell numbers ($n = 34$). (B) Immunocytochemistry for GFAP, NG2 and MAP2 in cultures treated with 1 μ M TTX for 5 days. Untreated astrocytes have a stellate appearance with multiple thick processes. TTX-treated astrocytes have both reduced numbers

of astrocytes and reduced GFAP expression with correspondingly thinner processes. Arrowheads denote differences in astrocyte morphology (scale bar = 25 μm).

Fig. 6. P2Y receptor signaling regulates astrocyte differentiation

(A) P₂Y receptors were predominately expressed on GPC at 7 DIV. Arrowhead indicates strong receptor expression on simple GPCs. These cells were 20–50 μ m in diameter with short processes. By 12 DIV, expression of both GFAP and P₂YRs increased. Note co-localization of P₂Y₁ and P₂Y₂ receptors with GFAP⁺ processes. Neuronal processes were also positive for P₂YRs. Arrowheads indicate astrocyte processes with co-localized P₂Y₁ (upper figure) and P₂Y₂ (lower figure) receptor expression. Scale bar = 25 μ m. (B) Hippocampal cell cultures were treated for 5 days with either 1 μ M TTX (n = 22) to block spontaneous activity or either 30 U/ml apyrase (n = 22), 50 μ M PPADS (n = 10) or 50 μ M suramin (n = 10) to interfere with purinergic signaling by degrading extracellular ATP or blocking P₂Y receptors. Blocking

activity or purinergic signaling significantly decreased the percentage of GFAP-expressing astrocytes with no effects on neurons (GFAP⁺ astrocytes comprised $22 \pm 1\%$ of total cells). One-way ANOVA, $F_{81,4} = 6.34$ and $P = 0.0001$. Direct comparison of apyrase-treated cultures to untreated, spontaneously active cultures demonstrated that the percentage of NG2⁺-OPCs were significantly reduced from $13 \pm 1\%$ in untreated cultures to $9 \pm 2\%$ with apyrase ($P = 0.041$, Kruskal–Wallis non-parametric test). (Note: A non-parametric test was used here as the NG2⁺-OPC population did not follow a normal distribution but rather a Poisson distribution in the mixed cultures.) * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$.

Fig. 7. Effects of LIF on glial precursors is downstream of activity and purinergic signaling
 (A) P $_2$ Y purinergic agonists increased LIF secretion from hippocampal astrocytes. Hippocampal astrocytes were treated for 24 h with 100 μ M 2-MS, $\alpha\beta$ -M-ATP, ATP γ S or UTP, and media was concentrated and assayed by ELISA. UTP and ATP γ S increased LIF secretion (basal levels of LIF in conditioned media was 19.0 ± 0.9 pg/ml, $n = 3$). (B) Blocking LIF and either spontaneous activity or purinergic signaling decreased GFAP $^{+}$ -astrocyte numbers to the same extent as blocking activity or LIF function by itself. Apyrase significantly decreased numbers of OPCs, independent of effects of LIF. One-way ANOVA, $F_{4,15} = 4.23$, $P = 0.003$. GFAP $^{+}$ -astrocytes and NG2 $^{+}$ -OPCs comprised 23 ± 2 and $10 \pm 1\%$ of total cells, respectively. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$.

Fig. 8. Activity-dependent astrocyte differentiation is suppressed in LIF ^{-/-} hippocampal cell cultures

(A) Blocking activity or purinergic signaling significantly decreased numbers of astrocytes in wt mice. In contrast, inhibition of spontaneous activity with TTX or ATP by apyrase did not significantly alter astrocyte numbers in LIF ^{-/-} hippocampal cell cultures. Astrocyte numbers were reduced by $32 \pm 6\%$ in LIF ^{-/-} cultures compared to wt cultures. One-way ANOVA, $F_{4,5} = 2.5$ and $P = 0.044$. * $P < 0.05$ and ** $P < 0.01$. (B) Addition of 100 ng/ml LIF to LIF ^{-/-} hippocampal cell cultures significantly increased numbers of GFAP⁺ astrocytes ($P < 0.05$, $n = 4$). Blocking either spontaneous activity with 1 μ M TTX or ATP signaling with apyrase did not significantly decrease numbers of astrocytes in LIF-treated LIF ^{-/-} cultures.

Table 1Integrated morphometry analysis of wt and LIF $-/-$ astrocytes.

IMA parameter	Wild type (<i>n</i> = 258)	LIF $-/-$ (<i>n</i> = 215)
Area (μm^2)	2400.2 ± 102.0 ***	1788.3 ± 98.2
Hole area (μm^2)	911.7 ± 230.8 **	188.5 ± 57.2
Perimeter (μm)	1223.7 ± 55.9 **	1079.3 ± 56.6
Shape factor	0.062 ± 0.007 *	0.039 ± 0.003

Integrated morphometry analysis was performed on astrocytes immunostained with GFAP. For wt and LIF $-/-$ mouse hippocampal cell cultures, $450 \times 450 \mu\text{m}$ random fields were imaged for GFAP immunoreactivity. Images were taken at $0.44 \mu\text{m}/\text{pixel}$ resolution with a $40\times$ objective (1.3 NA); *n* corresponds to number of astrocytes measured from 66 (wt) and 63 (LIF $-/-$) fields from 4 coverslips each.

 $P < 0.001$

**
 $P < 0.01$

*
 $P < 0.05$.

Shape factor was calculated according to the equation: $4\pi A/P^2$, where *A* is the area and *P* the perimeter. This measure ranges from 0 to 1 between extremes of a line and a perfect circle. The quantitative morphometry supports the conclusion that in the absence of LIF, hippocampal astrocytes are less complex.