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Toll-Like Receptor Ligands and CD154 Stimulate Microglia to Produce a Factor(s) That Promotes Excess Cholinergic Differentiation in the Developing Rat Basal Forebrain: Implications for Neurodevelopmental Disorders

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ABSTRACT: Maternal inflammation plays a role in the etiology of certain neurodevelopmental disorders including autism and schizophrenia. Because maternal inflammation can lead to activation of fetal microglia, we have examined effects of inflamed microglia on cultured neural progenitors from rat embryonic septal region and basal forebrain. These cells give rise to cholinergic neurons projecting to cortex and hippocampus. Microglia stimulated with lipopolysaccharide (LPS), peptidoglycan, Poly I:C and CD154 produce conditioned media (CM) that promotes excessive numbers of cholinergic neurons and levels of choline acetyltransferase (ChAT) activity 6–8 times that of untreated cultures. Expression of the neural-specific transcription factor MATH1 increases substantially within 1 h of plating in LPS-CM. Untreated cultures do not attain equivalent levels until 6 h. By contrast, expression of glial-related transcription factors in LPS-CM-treated cultures never attains the elevated levels of untreated cultures. LPS-CM-treated clones derived from individual progenitors labeled with a LacZ-expressing retrovirus showed >2.5-fold increase in the percentage of cholinergic cells compared with untreated clones. Thus, CM from activated microglia prompts excess cholinergic differentiation from undifferentiated progenitors suggesting that microglial inflammation during critical stages can lead to aberrant brain development. **(*Pediatr Res* 61: 15–20, 2007)**

Increasing evidence suggests that prenatal exposure to infectious agents or toxins with associated maternal inflammation may play a role in the etiology of neurodevelopmental disorders including autism and schizophrenia (1–3). In the case of autistic subjects, serum and cerebrospinal fluid (CSF) levels of inflammatory cytokines and chemokines are elevated (4–6), persistent microglial activation attends the disorder (5), and there is a high correlation with the incidence of gastrointestinal inflammatory symptoms (7). Together these studies have led to the hypothesis that inflammatory immune responses in the pre- and/or perinatal period may contribute to the etiology of certain neurodevelopmental abnormalities.

Maternal inflammation can manifest itself in the embryonic or fetal brain as activated or inflamed microglia (8,9), cells capable of producing cytokines, chemokines, and damaging reactive oxygen species. While many studies reveal the deadly role of inflamed microglia on mature neurons and glia, fewer have examined the effects of microglial activation on neuronal development. These, however, have suggested that microglial activation during embryogenesis has deleterious effects on white matter (10) and compromises cerebellar Purkinje cell development (11).

Microglia migrate from blood islands in the yolk sac to populate the embryonic brain as early embryonic day 10 (E10) (12), but their role in normal development is not clear. The junction of the telencephalon and rostral diencephalon as well as the medial walls of the telencephalic vesicles attract concentrations ("hot spots") of microglia from E13 onward, at least until E17 (13). The ameboid (*i.e.* activated) morphology of fetal microglia has suggested that their main function is the pruning of excess neurite outgrowth and the phagocytosis of dying neurons and neuritic debris. However, they are not always found in association with dying cells (14), and their ability to elaborate nerve growth factor (15), brain-derived neurotrophic factor (16), glial cell line-derived neurotrophic factor (16), IGF (17) as well as growth factors of the IL-6, heparin-binding, and transforming growth factor-beta (TGF-β) families (18) suggest alternative functions.

We have investigated the effect(s) of microglia on developing neural progenitors of the septal region and adjacent basal forebrain (BF). These will develop into an almost continuous cluster of cholinergic neurons stretching from the medial septum rostrally to the globus pallidus caudally, and projecting to cortex, hippocampus, amygdala, and thalamus. Disruption of cortical cholinergic innervation produces alterations in cortical cytoarchitecture and disturbed cognitive function (19). It is noteworthy that autistic children have a pathologic excess of cholinergic neurons in this region (20,21).

The clustering of microglia in the area of cholinergic differentiation suggests a role in normal development, but also leaves microglia poised to produce aberrant effects should they become inflamed. In previous studies, we and others have shown that a factor or cocktail of factors from interferon-γ-

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Abbreviations: BF, basal forebrain; **ChAT,** choline acetyltransferase; **LPS,** lipopolysaccharide; **PGN,** peptidoglycan; **PolyI:C,** Polyinosinic acid:polycytidylic acid

treated microglia can profoundly affect cholinergic differentiation in this brain region (22–24). However, since microglia can become activated through multiple stimuli with varying outcomes (25), we sought to determine which sorts of microglial stimuli might affect cholinergic differentiation and to determine further what neural- or glial-specific transcription factors might be involved in the conversion to the cholinergic phenotype. We have found that Toll-like receptor agonists as well as CD154 stimulate microglia to produce a factor or cocktail of factors that promotes cholinergic differentiation from undifferentiated progenitors in this brain region.

MATERIALS AND METHODS

Materials. Lipopolysaccharide (LPS; from *E. coli* 026:B6) and peptidoglycan (PGN; Sigma Chemical Co.-Aldrich, St. Louis, MO); Polyinosinic acid/polycytidylic acid (poly I:C; Calbiochem, San Diego, CA); goat polyclonal antibody against ChAT (Chemicon, Temecula, CA). The BAG retrovirus was a gift from Dr. Marla B. Luskin, Emory University. CD154-expressing baculovirus-infected Sf9 cells were provided by Dr. Michael Berton at the University of Texas Health Science Center in San Antonio, TX. Using procedures outlined by Dr. Berton (26) these particular cells were prepared in the laboratory of Dr. Virginia Sanders at Ohio State University. HAPI cells were a gift from Dr. James R. Connor, Penn State University, M.S. Hershey Medical Center.

Microglial cultures. Enriched microglial cultures were prepared from neonatal Sprague-Dawley rat cortices as described by us previously (22,23) Final treatment of the microglia occurs in serum-free Opti-MEM™ with an N2 supplement (N2 medium; Invitrogen, Carlsbad, CA). Conditioned medium (CM) was collected after 24 h and filtered before use on BF cultures.

CD154-expressing baculovirus-infected Sf9 cells were used to ligate CD40. They were used routinely at a ratio of 1:10 (Sf9 cells:microglia). Untransfected Sf9 cells were used as a control.

HAPI cells were treated identically.

Basal forebrain cultures. The septal region with tissue immediately ventral to it is dissected from rat embryos at embryonic day 15 (E15). Dissociated cells are plated onto poly-lysine coated 35 mm tissue culture dishes at a density of $1.2–1.6 \times 10^5$ cells per cm^2. Cultures are grown in low serum-containing N2 medium with penicillin (25 U/mL) and streptomycin (25 μg/mL). Microglial CM is added at plating and remained in the cultures for the full 5–6 d unless otherwise indicated. N2 medium alone, CM from unstimulated microglia, and N2 medium incubated for 24 h without microglia but containing additives were included as controls. All procedures using animals were approved by the Rutgers University Animal Care and Facilities Committee.

Choline acetyltransferase (ChAT) assay. ChAT activity was assayed using the method of Fonnum (27) as modified by Martinez *et al.* (28) and used by us previously (22,23). In this assay, cultured cells are homogenized in 10 mM EDTA containing 0.5% Triton X-100. Two microliters of the supernatant is transferred to a fresh microtube to which 5 μL of incubation medium is added. The incubation medium contains EDTA (17 mM), sodium phosphate (50 mM), sodium chloride (0.3M), choline bromide (8 mM), eserine (0.06 mM), and acetyl CoA + ^{14}C acetyl-CoA (0.2 mM; PerkinElmer, Boston, MA). After a 60-min incubation at 37°C ^{14}C-acetylcholine is extracted into a solution containing 5 mg/mL tetraphenylboron in acetonitrile and counted in Ecoscint O® (National Diagnostics, Atlanta, GA) scintillation fluid.

Protein determination was assessed using the Bio-Rad protein asssay.

BrdU incorporation. BrdU incorporation was assessed colorimetrically using the Cell Proliferation ELISA kit (Roche Applied Science, Indianapolis, IN) according to manufacturer's instructions. Final determinations were made by measuring absorbance at 450 nm.

Immunohistochemistry and cell counting. Cells were cultured with and without CM in poly-lysine-coated Lab-Tek® (Nunc, Rochester, NY) slides at a density of 3.5×10^5 cells/well. Cells were fixed with 4% paraformaldehyde, taken through a graduated series of alcohols, washed and incubated with antibody to ChAT (Chemicon, 1:200) for 48 h at 4°C. The secondary was biotinylated rabbit anti-goat (1:100). The antigen-antibody complex was visualized using the Elite™ ABC kit (Vector Labs, Burlingame, CA). ChAT+ cells were assessed as a proportion of the total numbers of neurons within a 50 mm^2 field in three separate wells. Only neurons with more than 2 dendrites were counted.

Retroviral labeling. E14 cells were cultured with and without CM in poly-lysine-coated Lab-Tek® slides as above. The lacZ-containing retrovirus (BAG; (29)) was introduced 18 h after plating. The retrovirus, provided at a concentration of 1.5×10^5 particles/mL, was diluted 1:25 for a final concentration of 6,000 particles/mL. Six hours after the addition of retrovirus, the medium was removed and fresh medium added.

Combined immunocytochemistry and histochemistry. At the end of 6 d *in vitro*, cells were prepared for the simultaneous histologic and immunocytochemical detection of lacZ and ChAT using methods used by us previously (22).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA is extracted using Ultraspec™ RNA Isolation Reagent (Biotecx Laboratories, Inc., Houston, TX). cDNA is produced from 1 μg of RNA by using random hexamer and MMLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instruction. For real-time PCR, cDNA was amplified using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. After amplification, two additional cycles were used for a dissociation curve to verify that the signal was generated from a single target amplicon and not from primer dimers or contaminating DNA. Serially diluted cDNA of each sample was amplified to measure the efficiencies of PCR and to draw the standard curve for each sample to calculate relative concentration of target message. The PCR products and their dissociation curves were detected using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). GAPDH mRNA was used as an internal control. Rat-specific primers are listed in Table 1.

RESULTS

Conditioned medium from LPS-, PGN-, Poly I:C-, and CD154-treated microglia enhance ChAT activity in E15 BF cultures. In previous studies we found that treatment of microglia with IFN-γ led to the production of a factor or cocktail of factors that enhanced cholinergic differentiation in

Table 1. *Primer pairs used for RT-PCR analysis*

Gene of interest	Primers pairs
Mash1	F:GTCCTGTCGCCCACCATCT (1293-1311)
	R:GGGTCGTAGGATCCCTCGTC (1393-1374)
Math1	F:TGAAAGTGCGGGAACAACTG(1433-1452)
	R:CCCATTCACCTGTTTGCTGG (1533-1514)
NeuroD1	F: CATTCGCATCATGAGCGAGT (1105-1124)
	R:GCACAGTGGATTCGTTTCCC (1205-1186)
NeuroD3	F:GACTTCACCTATGGCCCGG (889-907)
	R:AAGCCCTAGTGGTACGGGATG (989-969)
Hes5	F: CAACAGCAGCATTGAGCAGC (148-167)
	R: CGGCCATCTCCAGGATGTC (248-230)
Id1	F:GACTCCAGCCCTTCAGGAGG (532-551)
	R:GATCCGTCAAGAGTCCCGTG(632-613)
Id2	F:GCCTTCAGTCCGGTGAGGT (65-83)
	R:AGCAGACTCATCGGGTCGTC (165-146)
Id3	F:GGATGAGCTTCGATCTTGCC (500-519)
	R:GGCCACCCAAGTTCAGTCCT (600-581)

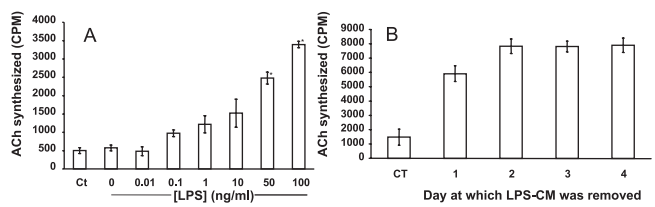

Figure 1. (*A*) Enriched microglia were cultured for 24 h in the presence of various concentrations of LPS. CM was used as medium for E15 BF progenitors. ChAT activity was assessed after 6 d. Controls were cultured in N2 medium that had not been in contact with microglia. Data are expressed as CPM of acetylcholine synthesized and represent the average ± SEM of 3 cultures. Data were compared with a one-way ANOVA with a post hoc Bonferroni test for multiple comparisons. $*p < 0.05$ when compared with cells cultured in CM from untreated microglia. (*B*) LPS-CM was removed at the days indicated and replaced with N2 medium. ChAT activity was assessed at the end of 6 d.

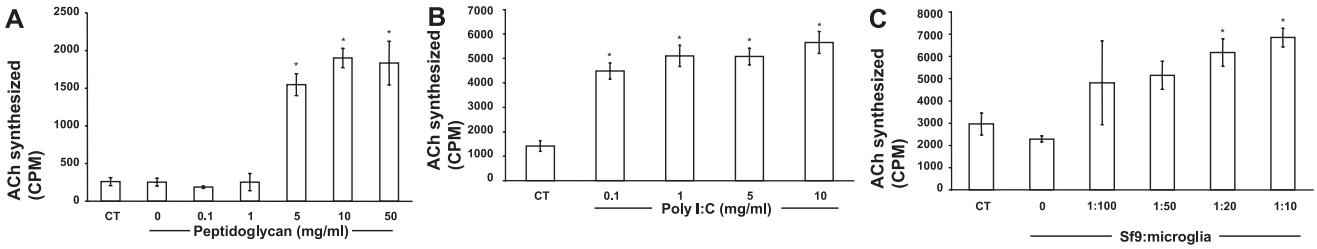

Figure 2. Enriched microglia were cultured for 24 h in the presence of various concentrations of PGN (*A*), Poly I:C (*B*) or with various densities of CD154-expressing Sf9 cells (*C*). CM were used to treat E15 BF cultures. ChAT activity was assessed after 6 d. Data were compared as in Fig. 1. In *A* and *C*, $p < 0.05$ when compared with cells cultured with CM from untreated microglia CM. In *B* comparisons are made to cells treated with N2 alone.

BF cultures (22). To determine whether other microglial stimuli produce the same effect, we treated cultures of enriched neonatal microglia with Toll-like receptor ligands LPS, PGN, Poly I:C, activators of Toll-like receptors 4, 2 and 3 respectively and used as mimics of the innate immune response to pathogenic stimulation. Stimulation was also accomplished using CD154 to mimic the stimulation occurring during adaptive immunity. Conditioned media (CM) were collected from treated microglia after 24 h and added to BF cultures prepared from E15 rat embryos. After 6 d *in vitro*, ChAT activity was assessed as the phenotypic hallmark for differentiated cholinergic neurons.

CM from untreated microglia caused an insignificant increase in ChAT activity. Of the several stimulators tested, LPS proved the most potent. In 15 separate experiments, LPS produced CM that resulted in an average 6.2 ± 0.8-fold increase in ChAT activity at maximal doses. In cultures prepared from E14 fetuses, the effect of LPS-CM was even more notable, producing an 8.2 ± 0.43-fold increase in ChAT activity. The effect of LPS was dose-dependent (Fig. 1*A*). Removal of the medium after 2 d did not change final levels of activity (Fig. 1*B*). The inclusion of the LPS inhibitor polymyxinB (10 μM) did not affect the increase in ChAT activity produced by the LPS-CM suggesting that the CM and not LPS itself was the active agent (data not shown).

PGN, Poly I:C and CD154 all stimulated microglia to produce CM that elevated ChAT activity over untreated controls (Fig. 2). All three effects were dose-dependent. The optimal ratio of CD154-expressing Sf9 cells to microglia was at 1:10.

To determine whether the effect on cholinergic neurons was specific, we measured mRNA levels for the glutamate/GABAergic synthetic enzymes glutamic acid decarboxylase (GAD) 65 and 67. Cells cultured in the absence of LPS-CM showed a steady, rapid rise in both GAD_{65} and GAD_{67} expression. By contrast, cells cultured with LPS-CM had a slower and negligible increase in these enzymes (Fig. 3*A, B*).

LPS-CM does not enhance precursor proliferation. At E15 neural precursors in the BF region are bi-potential (23,30) and retain their proliferative ability. To determine whether the increase in ChAT activity was the result of increased precursor proliferation, BrdU incorporation was assessed in cultures treated with and without LPS-CM. Cultures examined on each of 4 d showed no differences in cell proliferation (Fig. 4). FGF2, a well-known stimulator of progenitor proliferation, was included as a positive control.

LPS- and CD154-CM increase the proportion of cholinergic neurons. To determine whether the increase in ChAT activity reflected an increase in the number of cholinergic cells developing in the culture, cultures were stained immunocytochemically for ChAT and ChAT-positive neurons were counted as a percentage of the total number of neurons present in the culture. The percentage of ChAT+ cells in the LPS-CM-treated cultures more than doubled (Fig. 5) while in the

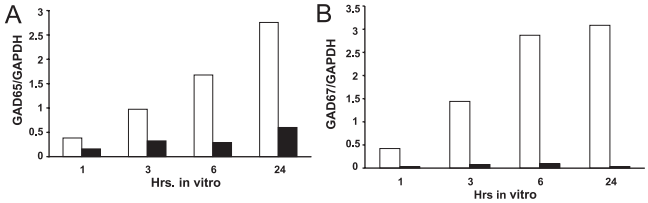

Figure 3. GAD_{65} (*A*) and GAD_{67} (*B*) mRNA were assessed by RT-PCR at various times in cells cultured with (□) and without (■) LPS-CM. Data are expressed in arbitrary units of specific RNA normalized to GAPDH as described in Methods section. Duplicates yielded virtually identical results.

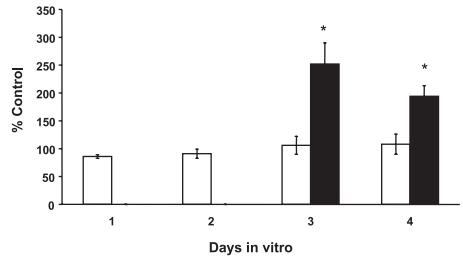

Figure 4. E15 BF cultures were grown with LPS-CM (□). BrdU incorporation was determined on each of the first 4 d in 3-4 individual cultures and compared with cultures grown without LPS-CM. FGF2 (20 ng/mL; ■) was included on days 3 and 4 as a positive control. *$p < 0.05$ when compared with control.

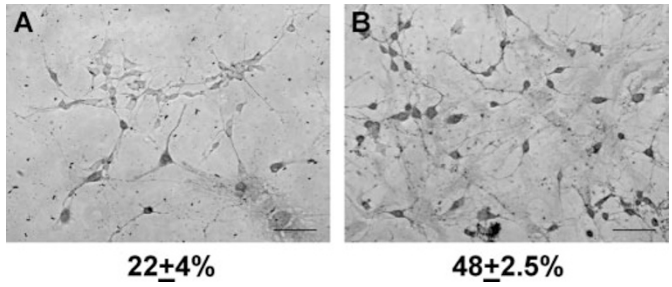

Figure 5. E15 BFs were cultured without (*A*) and with (*B*) LPS-CM for 6 d. Cultures were prepared for immunocytochemistry and quantified as described in Methods. The percentage of cholinergic neurons in the cultures is listed below each figure. Bars equal 40 μm.

CD154-CM-treated cultures the percentage increased from 16.2 ± 2.5% to 45 ± 0.5%.

LPS-CM increases the differentiation of undifferentiated precursors. Because younger cultures responded more vigorously than did older ones and because the action of the CM appeared to take place within the first 2 d *in vitro*, it seemed plausible that the action of the CM was on undifferentiated progenitors present in the cultures. However, because the LPS-CM was not mitogenic, this early action was not one of increasing the number of those progenitors. Furthermore, the increase in the percentage of cholinergic neurons in the treated cultures suggested that one mode by which the CM was acting was by prompting undifferentiated progenitors to generate postmitotic cholinergic neurons. To test this hypothesis, we used a LacZ-expressing retrovirus to label progenitors. Because the retrovirus integrates into the DNA of proliferating cells, the differentiated fate of progeny can be assessed. Individual clones were examined 6 d after retroviral labeling for the simultaneous expression of lacZ and ChAT (Fig. 6).

Consistent with the BrdU incorporation data, neither CM from untreated microglia nor from LPS-treated microglia affected the number of cells per clone (Table 2). Hence, increased cell division of individual progenitors could not account for increases in ChAT activity. Clones contained both neurons and astrocytes, attesting to the bipotential nature of the precursors (23,30). However, LPS-CM, but not untreated microglial CM, almost tripled the absolute number of ChAT+ cells within each clone and more than doubled the percentage of ChAT+ cells within each clone, suggesting that LPS-CM promoted cholinergic differentiation from undifferentiated progenitors.

LPS-CM up-regulates the expression of neural-specific differentiation factors while down-regulating glial-specific factors. Activation of neuronal-specific genes and the concomitant silencing of glial genes by vertebrate members of the atonal and Achaete-Scute families of basic helix-loop-helix (bHLH) transcription factors is intimately involved in terminal lineage decisions (31,32). Nevertheless, the role of these transcription factors – notably NeuroD1, Math 1, Mash1, and Neurogenin (NeuroD3) – in terminal cholinergic differentiation in the basal forebrain has gone largely unexamined. We, therefore, sought to determine whether CM that promotes cholinergic differentiation does so by the activation of these transcription factors. Neither NeuroD1 nor NeuroD3 showed significant change over the first 24 h with either treatment (Fig. 7*A, B*). MASH1 expression fell over the first 3 h in both untreated and LPS-CM-treated cultures, though the drop was rapid and sustained in the LPS-CM-treated cultures (Fig. 7*C*). The burst in MASH1 expression at 6 h in the untreated cultures and the lesser rise at 24 h in the LPS-CM cultures was a consistent, albeit puzzling, finding. The profile of MATH1 expression was the mirror image of MASH1 with levels rising in both the untreated and LPS-CM-treated cultures (Fig. 7*D*). However, the increase was rapid in the LPS-CM-treated cultures while untreated cultures were delayed in their ability to achieve levels coincident with LPS-CM-treated cultures.

By contrast, factors associated with glial differentiation achieved higher levels of expression in the untreated cultures than in the CM-treated ones. Levels of the Notch effector Hairy (H) Enhancer of Split(es)-5 declined within the first 6 hrs in both sets of cultures. Untreated cultures recovered substantially after 24 hrs, while cultures treated with LPS-CM did not. Inhibitors of neuronal differentiation (Id) 1, 2, and 3, showed a consistent pattern across all three factors: the expression increased over time in both sets of cultures, but the rise was consistently higher in the untreated cultures (Fig. 8).

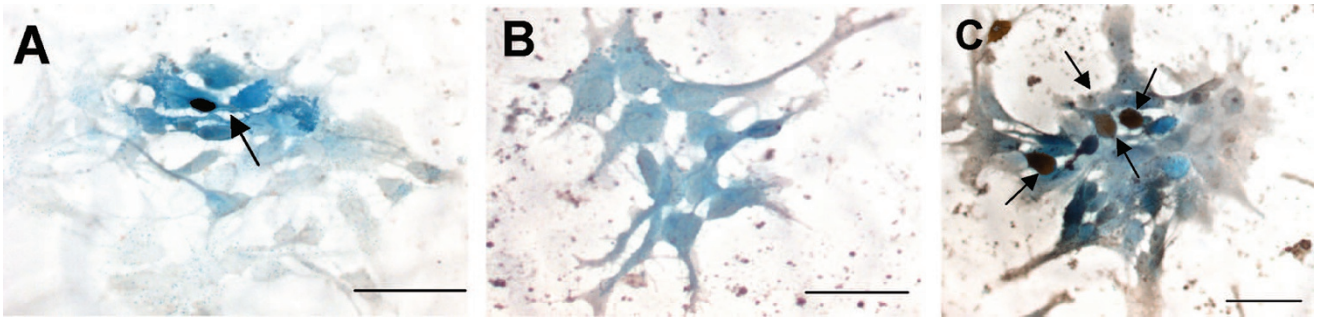

Figure 6. Histochemical and immunocytochemical detection of lacZ and ChAT in E14 BF that are untreated (*A*), treated for 6 d with untreated microglial-CM (*B*) or with LPS-CM (*C*). Cholinergic neurons labeled with ChAT antibody are brown while lacZ+ cells are blue. Arrows point to double-labeled cells. Bars = 40 μm.

Table 2. *Cultures established at E14*

Treatment	ChAT activity (CPM ± SE)	No. of clones counted	Cells/clone	ChAT + cells/clone	Perrcentage of ChAT + cells/clone
Control	316 ± 22	22	7.4 ± 1.2	1.3 ± 0.3	17.6 ± 4.9
Mic-CM	128 ± 4.7	30	5.8 ± 1.6	0.8 ± 0.3	14.4 ± 7.4
LPS-CM	2594 ± 135	27	9.8 ± 2.2	3.2 ± 0.6*	45.1 ± 6.3*

Untreated microglial-CM or LPS-CM was added at plating. A 6-hr period of retroviral labeling occurred 18 hrs after plating. After staining for both lac-Z and ChAT, the number of double-labeled cells was determined. Asterisks indicate groups significantly different from Control at the 0.05 level following an ANOVA with a Student-Newman-Keuls post hoc test for multiple comparisons.

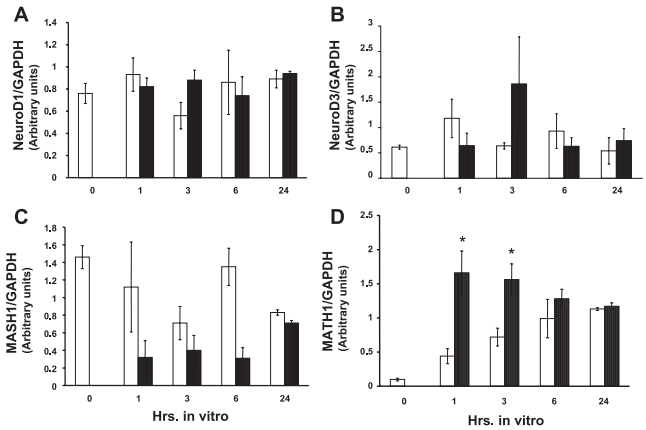

Figure 7. Expression of NeuroD1 (*A*), NeuroD3 (*B*), MASH1 (*C*) and MATH1 (*D*) in basal forebrain cultured with (■) or without (□) LPS-CM was assessed by RT-PCR in 3 independent experiments. Data are expressed in arbitrary units of specific RNA normalized to GAPDH. They were compared using an ANOVA with a posthoc Student-Newman-Keuls test for significance at the 0.05 level. *Difference when compared with samples freshly dissected and dissociated, but not cultured (Time 0).

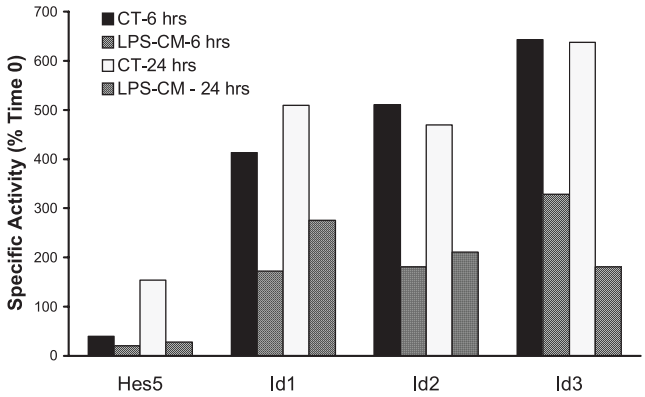

Figure 8. Expression of Hes-5, Id1, Id2 and Id3 in basal forebrain cultured with or without LPS-CM. Cells were harvested at 6 and 24 h and subjected to RT-PCR analysis. Data were expressed as specific RNA normalized to GAPDH and placed as a percentage to samples taken at Time 0. The experiment was performed three times with comparable results. CT-6 h (■); LPS-CM- 6 h (▨); CT- 24 h (□); LPS-CM – 24 h (▧)

DISCUSSION

Our data are consistent with the notion that microglial stimuli result in the production of a factor or cocktail of factors that increases ChAT activity in the developing BF. Because younger cultures respond to CM even more robustly than do older cultures and because the action of CM seems to occur in the first two days after plating, an action on bipotential neural progenitors seemed likely. Indeed, retroviral analysis of individually labeled cells showed that LPS-CM increased the proportion of cells acquiring a cholinergic phenotype. Thus, it appears that the mechanism by which LPS-CM increases ChAT activity is by prompting undifferentiated progenitors to assume a cholinergic fate. This is consistent with the analysis of transcription factors from the LPS-CM-treated cultures that show a rapid rise in the neural transcription factor MATH1 and the concomitant decline in the expression of the Notch effector Hes-5 and transcription factors Id1, Id2, and Id3. MASH1 is expressed in high levels in the ventral telencephalon during development (33–35). Its rapid fall in the face of the microglial differentiation factor(s) is somewhat puzzling but may suggest that its role there precedes that of terminal cholinergic differentiation. Because Hes-5 interferes with binding of transcription factors like MATH1 (36), its down-regulation in the face of MATH1 up-regulation is consistent with movement toward a neuronal phenotype. The rapid rise of the Id factors in untreated cultures is reminiscent of data from Andres-Barquin *et al.* who found a similar transient increase in Id expression before astrocyte differentiation (37). Moreover, recent data suggest that Id2 is a negative regulator of neurogenesis (38). Its rapid down-regulation in LPS-CM-treated cultures is again consistent with a move toward neuronal differentiation. It is, thus, tempting to suggest that the rapid rise of the Id factors in the untreated cultures bespeaks the "selection" of a glial lineage as opposed to a neuronal one, while the rapid decline in the LPS-CM-treated cultures is a prerequisite for neuronal/cholinergic differentiation.

Complicating this picture somewhat, however, is the fact that control cultures show a rapid and substantial increase in the expression of GAD. Thus, it may not be that a glial lineage is favored over a neuronal one; rather, a cholinergic phenotype is supplanted by a glial and a GABAergic (and/or glutamatergic) neuronal phenotype. Inclusion of the LPS-CM, then, alters this "decision," pushing the progenitors down an abnormal differentiation pathway.

Maternal inflammation leading to fetal brain inflammation has been linked to a number of developmental disorders including autism, schizophrenia and cerebral palsy (see Introduction). The current work shows a direct mechanism by which microglial activation leads to an aberrant developmental outcome – in this case, excessive numbers of cholinergic neurons. It is noteworthy in this context that autistic children have excess numbers of cholinergic neurons in the basal forebrain (39) and show neuropathological evidence of inflamed microglia and astrocytes (5). The conditions leading to microglial activation and why that activation results in various outcomes is still puzzling, but the work described here raises the possibility that activation of microglia during certain periods of embryogenesis could have significant consequences for basal forebrain development.

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