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# NEUROPATHOGENESIS OF NOONAN SYNDROME IS MEDIATED BY INFLAMMATORY MICROGLIA

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**Abstract**

Microglia are resident hematopoietic cells that play important roles in the damaged or degenerating adult nervous system. Microglia are involved in neuropathogenesis of various diseases. Microglia are also essential for neuroprotection and comprise an essential component of the neural stem cell niche. The activation of microglia is an important phenomenon associated with several neurological disorders that arise from infections to developmental abnormalities and behavioral pathologies. Noonan syndrome (NS) is associated with mutations in the *PTPN11* gene and also accounts for mental retardation in children. Interestingly, in mouse models of NS, mutations in the *PTPN11* gene resulted in dysregulation of neural progenitors. The present study describes the activation of microglia in the NS mouse model, which results in an inflammatory phenotype with expression of IL-1β and defective phagocytosis. To test whether microglia from NS mice are important for neural precursor maintenance or self-renewal, embryonic neural precursors from the cortex of WT mice were cultured. Microglia from NS and WT mice were then added to cortical precursor cells which showed that microglia from NS mice inhibited astrogenesis. Together, these results demonstrate that microglia can dysregulate neural precursor development in NS, and suggest that alterations in microglial number as a consequence of genetic or pathological events may perturb neural development by directly affecting embryonic neural precursors.

**Keywords**

• Microglia • Cortical precursors • Neural stem cells • Gliogenesis • Inflammation • Noonan syndrome

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## 1. Introduction

The resident tissue macrophages in the central nervous system (CNS), microglia and perivascular macrophages, are highly specialized cells that influence their local microenvironment. Microglia rapidly change their morphology and their expression of secreted and membrane-bound factors in response to changes in homeostasis and insults to the brain [1]. Inflammation alters microglial phenotype, which is evident on histological analysis-a downregulated phenotype with long processes conferring a ramified morphology and an activated phenotype characterized by shortened and extensively branched processes and hypertrophy of the cell body [2]. However, cells in the non-activated state are not 'resting' but rather are in a constant state of surveillance [3].

There is interesting and novel evidence that microglia play a crucial role in monitoring and maintaining synapses in the uninjured brain [4] as well as synaptic pruning [5]. These functions are complementary to the traditional role of phagocytosis that microglia perform and serves to highlight the important biology of these cells. However, microglia also mediate several pathogenic processes and particularly in the developing brain, where microglia become activated following maternal infections, this can lead to profound neurodevelopmental disorders. In the fetal brain from mothers diagnosed with schizophrenia, microglia are in an activated state [6,7]. Interestingly, in Down's syndrome, microglia are ramified and more numerous compared to normal fetal CNS [8]. Activated and inflammatory microglia express enhanced levels of MHC class I and II proteins conferring on these cells the ability to recognize and respond to antigens. In the adult CNS, microglia play a key role in neuroinflammation [9]. Interestingly, microglia are also found adjacent to adult neural stem cells [10] suggesting that these cells might alter the cellular environment of neural precursors. Indeed, microglia clear dying neurons, as well as promote programmed cell death of Purkinje neurons [11]. While it is known that activated microglia affect neuronal differentiation and several other functions during maternal infections [12], there is very little evidence for genetic mutations involving microglia affecting neurodevelopmental disorders.

The present study investigated the role of microglia in the development of a neurological disorder called Noonan syndrome (NS). Germline mutations in *PTPN11* (*SHP-2*) cause the autosomal dominant disorder, NS in humans, characterized by short stature, facial dysmorphia, hypertelorism and cardiac defects [13]. Shp-2 is a nonreceptor protein tyrosine phosphatase (PTP) which is required for full activation of the Ras/ERK pathway and for multiple receptor-evoked functions, including cell proliferation, differentiation and migration [14]. The molecular, cellular and developmental effects of disease-associated *SHP-2* mutations are slowly being unraveled. Previously, a knock-in mice expressing the NS-associated

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mutation D61G demonstrated significant craniofacial abnormalities [15]. Examination of neural progenitors expressing NS *SHP-2* allele demonstrated decreased astrogenesis and enhanced neurogenesis [16]. However, it was not known as to whether microglia also regulate neural precursor development in this mouse model. This study demonstrates for the first time that microglia are activated in NS and the inflammatory microglia also suppresses astrogenesis. Defective microglia might be unable to fulfill their normal biological roles during brain development and thus targeting microglia might be a therapeutically valid approach for neurodevelopmental disorders.

## 2. Experimental Procedures

### 2.1 Animals

Generation of mice with D61G mutation has been previously described [15], and these mice were maintained and genotyped as previously described [15]. Postnatal (P2) Fragile-X mice were obtained from Dr Min Zhuo [17]. Time-pregnant CD1 mice were obtained from Charles River Laboratories. All animal use was approved by the Hospital for Sick Children Animal Care Committee, and use was in accordance with the Canadian Council on Animal Care guidelines.

### 2.2 Cell culture and reagents

Cortical precursor cells were cultured as previously described [16,18-20]. Briefly, cortices were dissected from embryonic day 12 (E12) to E13 mouse embryos in ice-cold HBSS (Invitrogen, Gaithersburg, MD) and transferred to Neurobasal medium (Invitrogen) containing 500 µm L-glutamine (Cambrex Biosciences, Hopkinton, MA), 2% B27 supplement (Invitrogen), and 1% penicillin-streptomycin (Invitrogen). The medium was supplemented with 40 ng/ml FGF2 (Promega, Madison, WI). The tissue was mechanically triturated with a plastic pipette into single cells and cells were plated on four-well chamber slides (Nunc, Naperville, IL). Chamber slides were previously coated with 2% laminin and 1% poly-D-lysine (BD Biosciences, Bedford, MA) and cell density was 120,000 cells/well. Culture medium was changed every two days for the duration of the experiment. Embryonic microglia were isolated from mice cortices by the Percoll gradient method [21] with minor modifications. The tissue was mechanically triturated with a plastic pipette into small clusters of cells, which were then passed through a 40 µM filter (Nunc). For Percoll (Sigma) gradients, stock isotonic Percoll (SIP) was prepared by mixing nine parts of Percoll with one part 10x HBSS. The cells were suspended in 4 ml of 37% SIP. This was transferred to 15 ml conical tubes and was underlaid with 4 mL of 70% SIP. Then on top of the 37% layer, 4 mL of 30% SIP was pipetted, followed by 2 mL of HBSS. The gradient was centrifuged for 40 min at 200*g* (18°C) with no brakes so that the interphase was not disturbed. Using a transfer pipette, the layer of debris was removed and the 70%-37% interphase was collected into a clean tube and washed with HBSS three times by centrifuging the contents for 7 min at 500*g* at 4°C. The cells were cultured in DMEM (Invitrogen) supplemented with 1% penicillin-streptomycin (Invitrogen) and 10% fetal bovine serum (FBS, Invitrogen). For experiments that involved adding microglia to cortical precursor cultures, microglia isolated using the Percoll gradient were harvested and resuspended into cortical precursor culture medium, triturated, counted and added at 0.6% of the cortical precursor cell density. Bacterial LPS was purchased from Sigma and used at 100 ng/mL.

### 2.3 Transfections

For transfections, 24 h after plating, 1 µg of DNA and 1.5 µl of Fugene 6 (Roche) were mixed with 100 µl of Opti-MEM (Invitrogen) and were incubated at room temperature for 45 min and then added to each well. Microglia were transfected with D61G-HA (a kind gift from Dr Benjamin Neel) or a previously-described plasmid encoding EGFP under the CMV-promoter (pEGFP) [20]. For transductions, primary mouse microglia or macrophage cell line (RAW, a kind gift from Dr Sergio Grinstein) were infected with varying titers (1:50 or 1:200) of Adenovirus-GFP or Adenovirus-D61G (a kind gift from Dr Benjamin Neel).

### 2.4 Fluorescence activated cell sorting

Cortical tissue from E12-13 *PTPN11*$^{D61G/+}$ (NS) and age-matched wild type (WT) litter mate embryos was mechanically triturated and filtered to obtain a single cell suspension in 1 mL of HBSS supplemented with growth factors and antibiotics (HBSS-GA), as for cortical precursor cultures. The cells were blocked with 1 µL normal goat serum (Jackson Labs) for 20 min with gentle shaking at 4°C, and 1 µg/mL of the Iba-1 antibody (Wako) was added to the cells with gentle shaking for 20 min at 4°C. Cells were then centrifuged at 200*g* for 5 min at 4°C, and the pellet was resuspended in HBSS with growth factors and antibiotics. The secondary antibody, Alexa 488-conjugated goat-anti-rabbit (1:1000, Jackson ImmunoResearch) was added and the cells were incubated with gentle shaking for 20 min at 4°C. Cells were then washed 2 times by centrifuging at 200*g* for 5 min at 4°C and resuspended in 200 µL of HBSS-GA. Propidium iodide (PI, 1:10,000, Chemicon) was added to the cell suspension and cells were stored on ice until sorted. Cells were sorted using a Mo-Flo (DAKO) cell sorter, and cells were gated based on the intensity of the fluorescent secondary antibody (to select Iba-1-positive microglia) and propidium iodide (to assay for cell viability). The live, gated cell populations were sorted into DMEM supplemented with FBS (10%), and cell numbers were determined using a hemocytometer.

### 2.5 RT-PCR

Total cellular RNA was isolated from cultured microglia or mouse brain tissue as previously described [22] using Trizol reagent (Gibco, Burlington, ON, Canada), DNAse treated for 1 hour at 37°C, and the absence of cellular DNA was confirmed by polymerase chain reaction (PCR) using the conditions and primers described below. cDNA was prepared from 100ng of treated RNA using a first strand cDNA Synthesis kit (Boehringer-Mannheim, Quebec City, PQ, Canada) with a poly-(dT) primer in combination with a gene-specific 3′ primer. Template (300-400 ng) was amplified by 1 cycle of 95°C for 1 minute; 30 cycles of 95°C for 1 minute, 58°C (GAPDH) or 55°C (IL-1β) for 1 minute, 72 °C for 2 minutes; and 1 cycle of 72°C for 10 minutes. The primer sequences are GAPDH (5′- GCA TGG CCT TCC GTG TTC CTA CCC-3′ and 5′- GGC CGC CTG CTT CAC CAC CTT CT-3′; amplified product size-110 bp) and IL-1β (5′- GCA CCT TCT TTT CCT TCA TC-3′ and 5′- CTG ATG TAC CAG TTG GGG AA-3′; amplified product size-448 bp).

### 2.6 Immunocytochemistry

Immunocytochemistry of cultured cells and tissue sections was performed as described [16,19,20]. For immunocytochemistry of cultured cells, cells were washed with HEPES-buffered saline (HBS) and fixed with 4% PFA for 15 minutes, permeabilized with 0.2% NP-40 (USB Corporation, Cleveland, OH) in HBS, and blocked with buffer containing 6% normal goat serum (NGS) (Jackson ImmunoResearch, West Grove, PA) and 0.5% bovine serum albumin (BSA) (Jackson ImmunoResearch) for 1-2 hours at room temperature. Cells were then incubated with primary antibodies in HBS containing 3% NGS and 0.25% BSA at 4ºC overnight. After washing with HBS, cells were incubated with secondary antibodies prepared in HBS containing 3% NGS and 0.25% BSA at room temperature for 1 hour. Samples were then washed with HBS, counterstained with Hoechst33258 (1:1000; Sigma, St-Louis, MO) for 2 minutes, and mounted with GelTol (Fisher Scientific, Houston, TX). For immunocytochemistry of tissue sections, sections were dried at 37ºC for 15 minutes, washed in phosphate buffer solution (PBS) (Hyclone, Logan, UT), and postfixed with 4% PFA for 10-15 minutes. They were then blocked and permeabilized with 10% BSA and 0.3% Triton X-100 (EMD Chemicals Inc., Gibbstown, NJ) for 1 hour. The M.O.M. blocking kit (Vector Laboratories, Burlingame, CA) was then used according to the manufacturer's protocol. Sections were incubated with primary antibodies at 4ºC overnight, washed with PBS, and incubated with secondary antibodies at room temperature for 1 hour. They were then counterstained with Hoechst33258 for 2 minutes and mounted with GelTol. The primary antibodies were rabbit anti-Iba-1 (1:1000; Wako), goat-anti-CD14 (clone M20, 1:50, Santa Cruz Biotech. Inc, USA), mouse anti-Ki67 (1:200; PharMingen), mouse anti-βIII-tubulin (1:800; Covance), mouse-anti-HA (1 mg/mL; Sigma) and rabbit anti-GFAP (1:1000; DAKO). The secondary antibodies (all from Jackson ImmunoResearch) were Alexa 488-conjugated goat-anti-rabbit (1:1000), indocarbocyanine (Cy3)-conjugated goat anti-mouse and anti-rabbit IgG (1:400), FITC conjugated anti-mouse and anti-rabbit IgG (1:200), dichlorotriazinyl amino fluorescein-conjugated streptavidin (1:1000), and Cy3-conjugated streptavidin (1:1000).

### 2.7 Microglia phagocytosis assay and time-lapse photography

Microglia from embryonic mice were isolated as described above and cultured in DMEM with 10% FBS in four-well glass chamber slides (Nunc, Naperville, IL). After an overnight incubation, medium was replaced with $CO_2$-independent medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS. The plates were transferred to a Zeiss Axioscope inverted microscope equipped with a heated stage. Five minutes after the addition of yeast, time-lapse images (×63 magnification) were captured with an AxioCam HR camera, using AxioVision, release 5, software (Zeiss) to configure image acquisition. Images were acquired every 1.0 min by dual channels set for exposure times of 10 to 30 ms (differential interference contrast) during a 2h period. Nomarski images as Zeiss (.zvi) formatted files were normalized for brightness and contrast using AxioVision software.

### 2.8 Quantification and microscopy

Microscopy of cultured cortical precursors was performed using a Zeiss Axioplan2 upright microscope equipped with fluorescence optics. Analysis of cultured cortical precursors was performed by scoring all labeled cells per condition per experiment in at least six randomly selected fields spanning the culture well. Images were acquired with Northern Eclipse software (Empix) using a Sony XC-75CE CCD video camera. For all analyses, at least three independent culture experiments were performed at different times with comparable results. Error bars indicate SEM, and statistics were performed using Student's *t* test.

## 3. Results

### 3.1 Noonan syndrome (NS) mice show increased number of microglia in the brain

To ask whether microglia are activated in Noonan syndrome (NS), age-matched wild-type (WT) and *PTPN11*$^{D61G/+}$ (hereafter referred to as NS) postnatal day 2 (P2) mouse brains were examined by immunohistochemistry. When homozygous, the D61G mutant is embryonic lethal, whereas heterozygotes have decreased viability. Since surviving *PTPN11*$^{D61G/+}$ embryos have short stature and craniofacial abnormalities similar to those in NS [15], the *PTPN11*$^{D61G/+}$ mice were used for the present studies. Initially, microglial localization was characterized in the hippocampus and cortex of NS and WT mice (Figure 1). Specifically, the expression of Iba-1, a microglial marker, in P2 brain sections was examined which revealed that Iba-1-positive microglia were scattered throughout the hippocampus (Figure 1A,B,C) and cortex (Figure 1E,F,G). However, the number of microglia was significantly increased in the hippocampus of NS mouse compared to that of the WT (Figure 1D). Indeed, earlier during development when embryonic day 18.5 (E18.5) embryos were examined, there was again a significant increase in the hippocampus (Figure 1I) but this increase in microglia number in the NS mouse brain was not significant in the cortex at E18.5 (Figure 1H) and later on in the adult hippocampus (Figure 1J).

### 3.2 Increased microglia numbers in NS mouse suggests higher proliferation

These data suggest that microglial numbers are higher in the brain of postnatal NS mouse compared to WT controls. To determine if this increase in microglial number is due to microglial proliferation due to genetic perturbation, *in vitro* experiments were performed. Firstly, fluorescent activated cell sorting (FACS) was used to isolate Iba-1-positive microglia from embryonic mouse brain. Whole brain tissues from WT and NS mice were processed and FACS performed. Figure 2A (box R4) shows that the number of Iba-1-positive microglia was 2.39% in WT vs 4.8% in NS. Secondly, cortical tissues from E14.5 embryonic mice were removed and cortical precursor cultures were performed as described previously [23]. Specifically, microglia within E14.5 cortical precursor cultures were immunostained with the Iba-1 antibody which revealed a significant increase in the number of microglia within cortical tissues (Figure 2B). Having determined that microglia numbers

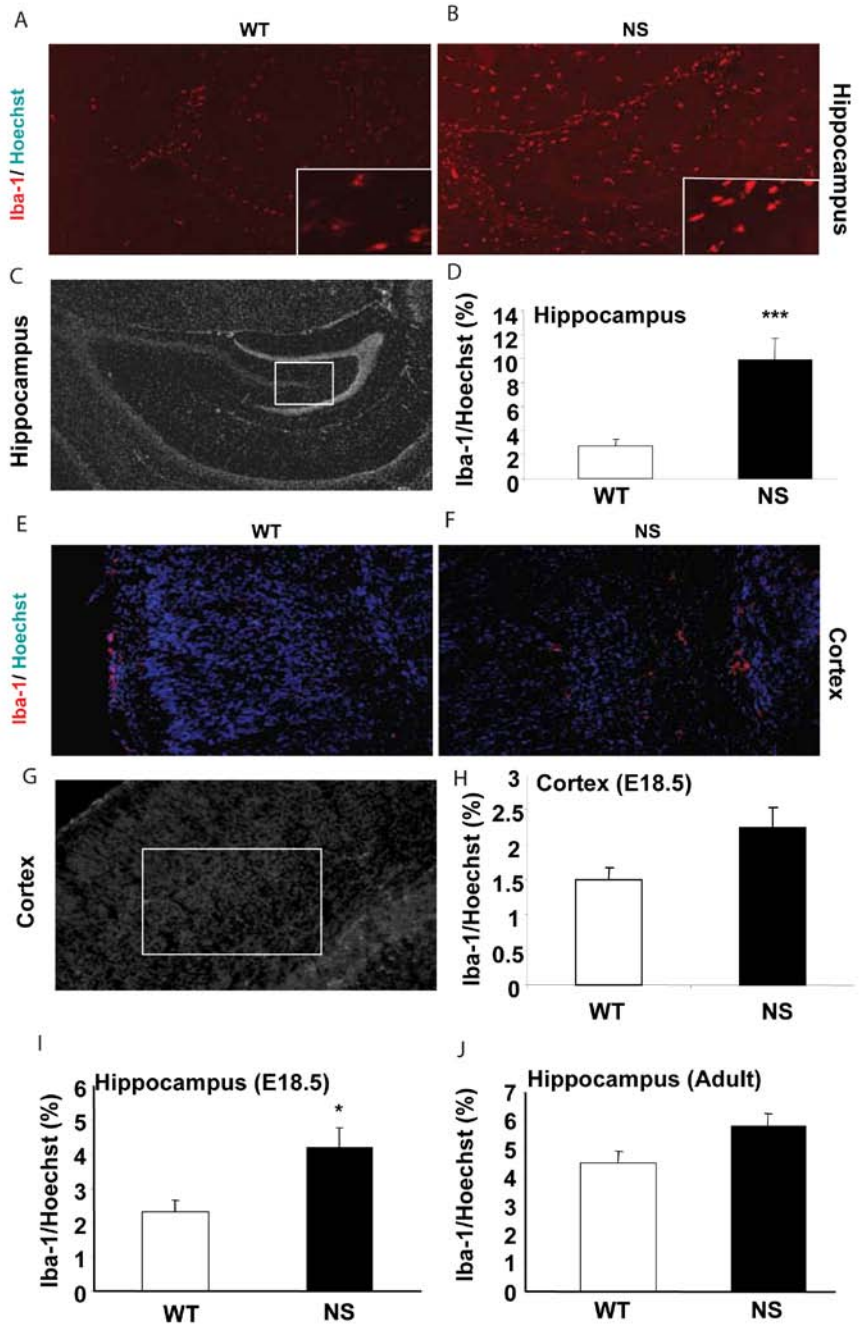


Figure 1. ***Noonan syndrome (NS) mice show increased number of microglia in the brain.***
(A-B) Immunocytochemical analysis of coronal sections of WT and NS brains at P2 labeled for the microglia marker Iba-1 (in red) show the presence of a large number of short, stubby and activated microglia in NS brain (inset, B) compared to fewer and ramified microglia in WT brain (inset, A) (inset, x63 mag; A-B, x5 mag.) (C) Location (marked by a rectangle) in the hippocampus where microglia was quantified (x5 mag.) (D) Quantification shows a significant increase in the number of microglia in the hippocampus of NS mouse (n=3) compared to WT (n=3). (E-F) Immunocytochemical analysis of coronal sections of WT and NS cerebral cortices at P2 labeled for the microglia marker Iba-1 (in red) and the nuclear stain Hoechst33258 (x20 mag.) (G) Location (marked by a rectangle) in the cortex where microglia was quantified (x5 mag.) (H) Quantification of microglia in the cortex of E18.5 NS mouse (n=3) compared to WT (n=3). (I) Quantification shows a significant increase in the number of microglia in the hippocampus of E18.5 NS mouse (n=3) compared to WT (n=3). (J) Quantification of microglia in the hippocampus of adult NS mouse (n=3) compared to WT (n=3) mouse. Error bars denote s.e.m. * $p<0.001$ *$p<0.05$ compared to control cultures. The graphs represent combined data (n = 3 each).

were elevated in mouse brain and cell cultures, microglia was then isolated using the Percoll gradient protocol as described previously [23]. Microglia were cultured overnight, and then the cells were transfected with D61G or GFP plasmids. This was followed by immunostaining the cultures for Ki-67, a marker of cell proliferation (Figure 2C). Complementing this approach, microglia were also infected with adenovirus expressing D61G or GFP at multiplicity of infection (MOI) of 1:5 (Figure 2D) and of 1:200 (Figure 2E). These experiments showed that D61G induced proliferation in pure microglia cultures. To address whether cortical precursor differentiation was affected, cortical progenitors from WT and NS mouse were cultured as described previously [23]. Cells were immunostained for βIII-tubulin to determine neurogenesis and GFAP to determine astrogenesis. Analysis of the percentage of GFAP-positive cells at 6 days demonstrated that there was a slight decrease in the number of GFAP-positive astrocytes in culture (data not shown). Thus, D61G expression in microglia induces proliferation which could account for the increase in microglia in the brain of NS mouse.

### 3.3 Microglia in NS mouse brain are inflammatory and defective in phagocytosis

Microglia proliferation is a key response to damage in the brain [24]. Cytokines such as IL-1β and TNF-α which are produced in a variety of CNS pathologies, have numerous functions including stimulation of microglia proliferation [25]. Activated microglia express several molecules and in particular, a robust immune response requires expression of pattern recognition receptors such as CD14, a surface molecule of monocytoid cells which is up-regulated after monocyte stimulation and is involved in cellular activation [26]. In order to determine whether microglia in NS mouse is activated and inflammatory, immunohistochemistry was performed which revealed a significant number of CD14-positive inflammatory microglia/macrophages in the cortex of NS (Figure 3B) mouse compared to that of WT (Figure 3A). In order to examine the cytokine gene expression pattern involved in

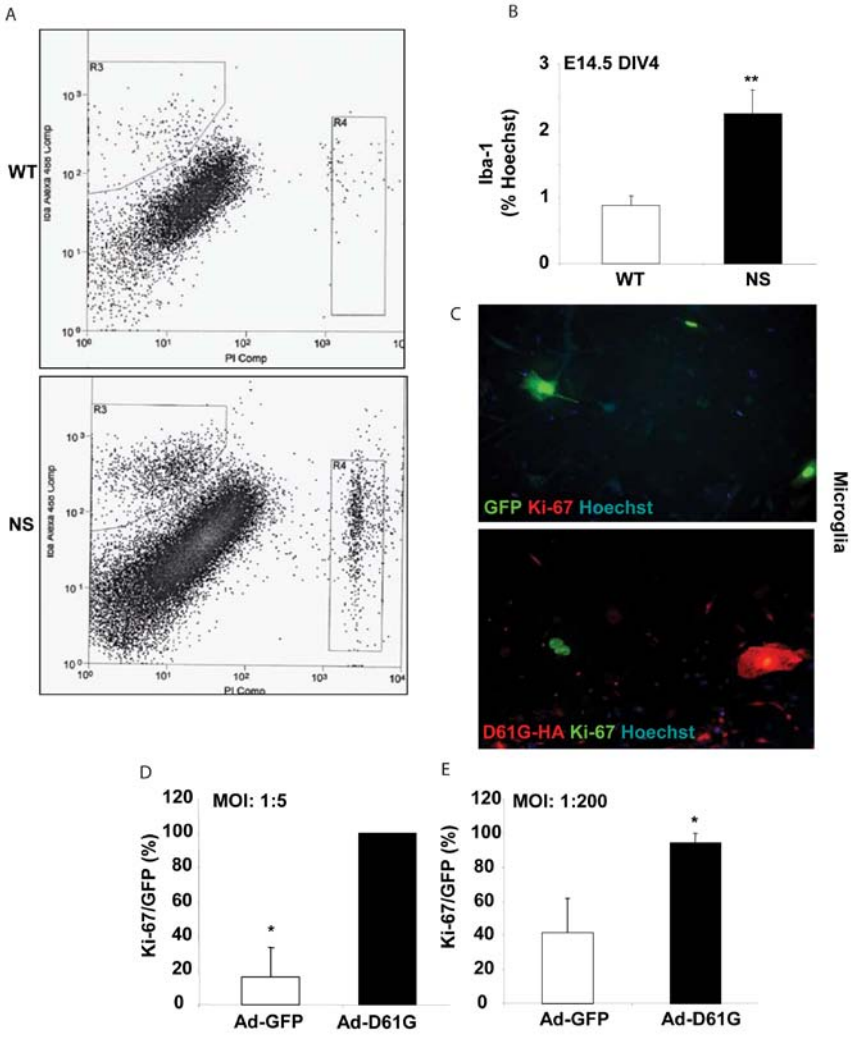


Figure 2. ***Increased microglia numbers in NS mouse suggests higher proliferation.***
(A) Single cell suspensions from brain tissue of WT or NS mice were labeled with Iba-1 and analyzed by FACS. While NS mouse had 4.8% Iba-1-positive microglia, the WT mouse had only 2.39% Iba-1-positive microglia. (B) Cortical tissues from E14.5 WT and NS mice were isolated, dissociated and cultured in the presence of FGF-2 overnight, fixed and immunostained for Iba-1. Quantification revealed a significant increase in the number of Iba-1-positive microglia in NS mouse brain compared to WT. (C) Fluorescent photomicrographs of primary microglia cultures transfected with GFP (green, top panel) or D61G-HA (red, bottom panel) and immunostained for Ki-67 (red, top panel or green, bottom panel), and counterstained with Hoechst33258 to show nuclei (in blue) (x20 mag.) (D) Quantification of Ki-67+ microglia expressing GFP or D61G showed a significant increase in the number of Ki-67+ microglia expressing D61G compared to microglia expressing GFP at two different MOIs of adenovirus infections, 1:5 (D) and 1:200 (E). Error bars denote s.e.m. ** $p<0.01$ *$p<0.05$ compared to control cultures. The graphs represent combined data (n = 3 each).

neuropathogenesis of NS, a macrophage cell line, RAW, was infected with Ad-GFP (Figure 3D) or Ad-D61G (Figure 3C) at 1:5 MOI, followed by RT-PCR analysis for IL-1β gene expression. Interestingly, macrophages expressing D61G showed higher levels of IL-1β than the corresponding cells expressing GFP (Figure 3E top panel). Indeed, treatment of cells with LPS, which binds CD14 receptor on macrophages, did not have an effect on the level of IL-1β in cells expressing D61G (Figure 3E top panel). To determine if NS mice show similar increase in IL-1β expression, cortices and whole brain tissue from WT and NS mice were obtained and subject to RT-PCR analysis. Interestingly both the cortex (Figure 3E middle panel) and the whole brain (Figure 3 bottom panel) tissues from NS mice show increased IL-1β expression compared to WT mice. Expression of IL-18, a cytokine associated with neurodevelopmental disorders [27] did not show any differences between WT and NS mice (data not shown). These results show that D61G mutant microglia have an inflammatory phenotype. In a further attempt to understand the biology of microglia in Noonan syndrome, a phagocytosis assay was performed. Microglia are specialized cells that perform phagocytosis in the brain. This activity is essential to maintaining homeostasis of the brain and it allows optimal neuronal and axonal growth. Indeed, microglia are also known to actively promote the death of developing neurons [11]. To determine whether microglia from NS mice are capable of phagocytosis, the ability of microglia to ingest yeast was determined by time-lapse photography. Interestingly, when fed yeast particles, microglia expressing GFP were able to ingest yeast as shown in the time-lapse image (Figure 3F top). However, microglia expressing D61G were unable to ingest yeast particles (Figure 3F bottom). These results show that microglia from NS mice are activated, inflammatory and defective in phagocytosis.

### 3.4 Increased microglia perturb cortical precursor development

Increased microglial numbers in the adult brain have been associated with many biological effects, both beneficial and pathological [28]. In the embryonic brain, increased microglial numbers might occur in response to pathological conditions such as maternal infections [29,30]. Since a previous study has shown that endogenous microglia can regulate neural precursor biology [23], it became imperative to ask whether an increase in the proportion of activated and inflammatory microglia might adversely affect cortical precursors. To do this, embryonic microglia were isolated from WT or NS (P2) mice by FACS and then added to E12-E13 cortical precursors from CD1 mice, until the relative microglial density was 0.6%. Immunostaining and subsequently quantifying the number of Ki-67+ cells in these cultures at 2 days demonstrated that the activated and

inflammatory microglia from NS mouse had no effect on cell proliferation compared to that of the WT (Figure 4A). Astrogenesis was examined in these cultures by immunostaining for GFAP at 6 days, which demonstrated that activated and inflammatory microglia from NS mouse decreased astrogenesis (Figure 4B). In parallel experiments, control microglia from CD1 mice were added to cortical precursors which showed an increase in astrogenesis, as expected [23] (Figure 4C). Interestingly, microglia from a mouse with Fragile-X, a different neurodevelopmental disorder, also induced astrogenesis (Figure 4D). These results suggest that microglia in NS mouse brain exhibit a specific pathological phenotype that affects cortical progenitor development, particularly dysregulating astrogenesis, and that the effect of D61G is dependent on the phenotype of the cells being studied.

## 4. Discussion

Based on the results presented here, two major conclusions can be made. First, the data indicate that activated and inflammatory microglia are present in the brain of postnatal NS mice, particularly in the hippocampus. Second, microglia in NS mice are activated and inflammatory and likely participate in regulating the differentiation of embryonic cortical precursors. Specifically, activated and inflammatory microglia reduced astrogenesis in cortical precursor cultures. These effects might be due to the phenotype of microglia in NS mice, which is characterized by high levels of IL-1β. Together, these results demonstrate that microglia in NS mouse are inflammatory and can dysregulate neural precursor development, and suggest that alterations in microglial phenotype as a consequence of genetic mutations might perturb neural development by directly affecting neural precursors.

Microglia play a key role in phagocytosis in the embryonic brain [31], and also directly regulate programmed cell death in neurons [11]. Exogenous microglia and/or their conditioned medium have been shown to induce astrogenesis [32,33], in addition to other effects including neuronal migration and differentiation [12,34]. Thus, microglia in the developing brain fulfill multiple roles [35], and, like other non-neural cells within the embryonic neural tissue [10], play an important role (s) in regulating the neural precursor environment.

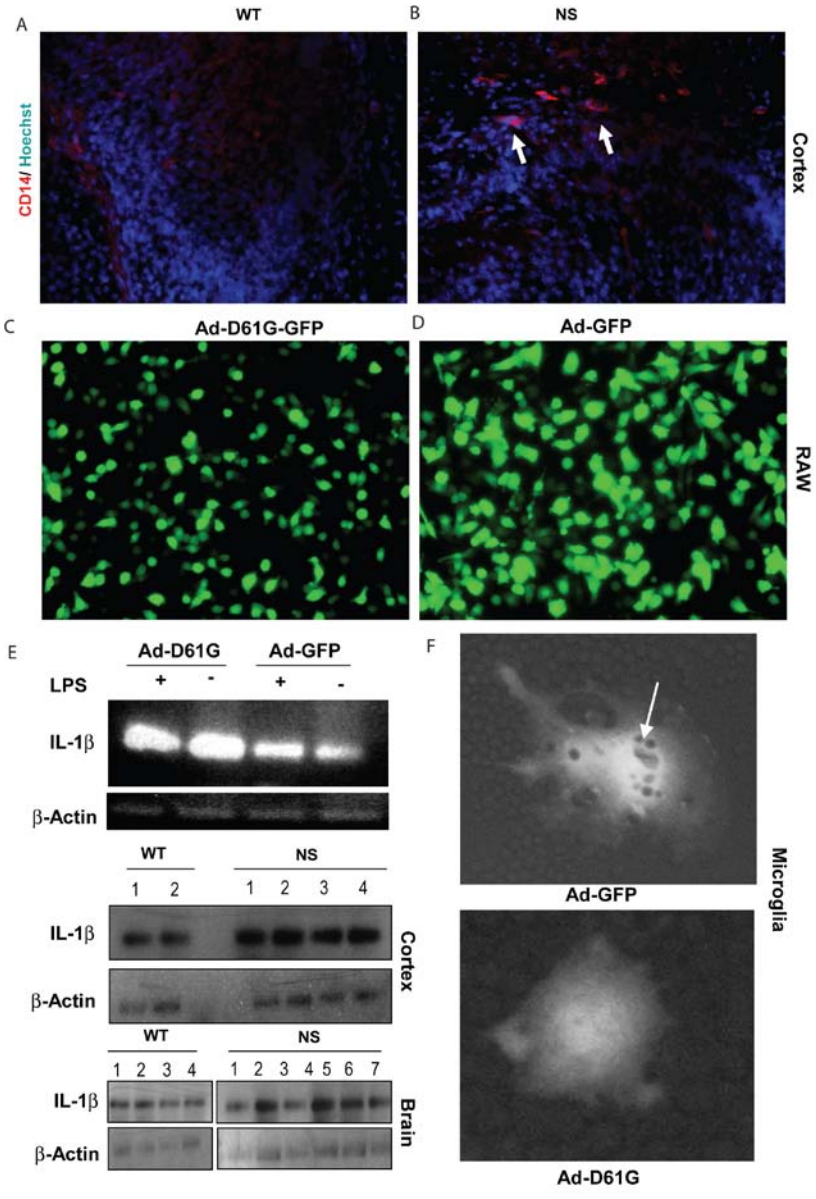


Figure 3. ***Microglia in NS mouse brain are inflammatory and defective in phagocytosis***. Immunocytochemical analysis of coronal sections of postnatal day2 WT (A) and NS (B) brains labeled for the microglia marker Iba-1 (in red) and the nuclear stain Hoechst33258, shows an increase in the number of CD14+ cells (arrows) in the cortex of NS mouse compared to WT (x40 mag.). RAW macrophage cell line was infected with Ad-D61G (C) or Ad-GFP (D). RT-PCR was performed to detect IL-1β gene expression in GFP- or D61G- expressing RAW macrophages which showed increased IL-1β expression in Ad-D61G macrophages compared to Ad-GFP macrophages even in the absence of bacterial LPS, a known activator of macrophages (E, top panel). RT-PCR of cortex tissue (E, middle panel) and whole brain tissue (E, bottom panel) also showed a significant increase in the expression of IL-1β in NS mouse compared to WT. (F) A phagocytosis assay using time-lapse imaging was performed to determine the ability of microglia to ingest yeast particles. Results show that microglia expressing GFP showed a number of yeast particles within the cell body (F, top, white arrow) whereas microglia expressing D61G did not ingest yeast particles (F, bottom).

Microglia regulate neural precursor development due to their ability to synthesize and secrete several cytokines and growth factors, including but not limited to,

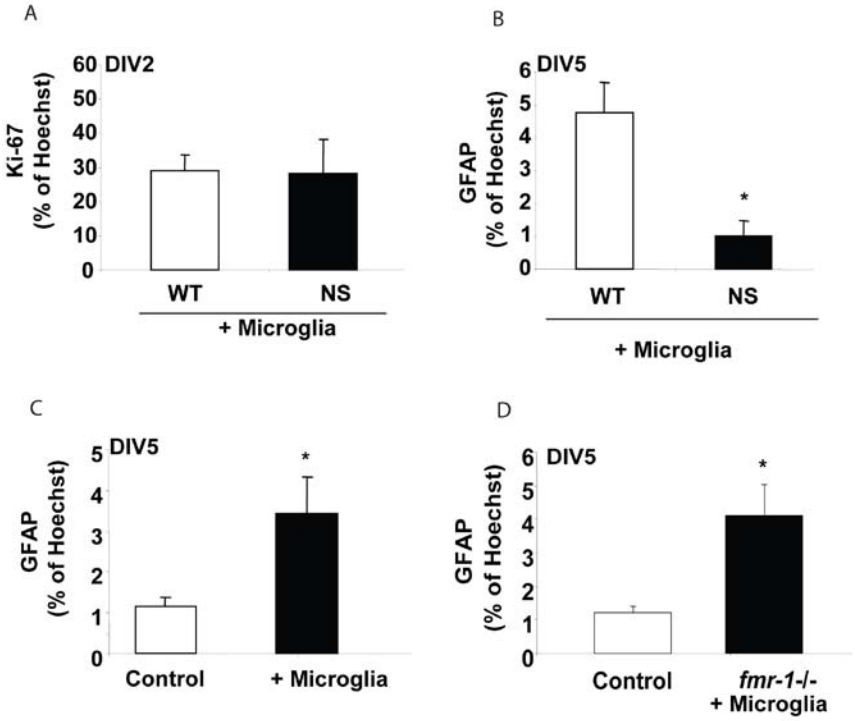


Figure 4. ***Increased microglia perturb cortical precursor development***
Cortical precursor cells from E12/13 CD1 mice were cultured for 2 to 6 days and analyzed for cell proliferation and differentiation in the presence of added microglia from WT or NS mouse. (A) Quantification of the percentage of Ki-67+ cells at day 2 showed no change in the number of proliferating precursors in the presence of microglia from either WT or NS mouse (B) Quantification of the percentage of GFAP-positive astrocytes show a significant decrease in the number of astrocytes when cortical precursors were cultured with microglia from NS mouse compared to WT mouse at 5 days of culture. (C) As a positive control, normal healthy microglia added at 0.2-1.0% of the cortical precursor cells induced astrogenesis. (D) Similarly, microglia from a different mouse model (Fragile X) also induced astrogenesis in cortical precursor cells at 5 days. Error bars denote s.e.m. n = 3. *p<0.05.

BDNF [36], FGF [37], and IGF [38], all of which are known to regulate cortical precursor biology [39]. Activated microglia might block astrogenesis in the developing brain, either due to the inflammatory response or the lack of specific growth factors associated with astrogenesis. Indeed, NS mice exhibited a dysregulated growth factor expression profile (data not shown). Interestingly, a transgenic mouse model exhibiting sustained expression of IL-1β in the hippocampus showed hypertrophied astrocytes and activated microglia along with deficits in spatial memory [40]. This suggests that in NS, cytokines and growth factors might be acting in synergy to block astrogenesis, which corroborates the finding that the D61G mutation in a key signaling protein, SHP-2, instruct multipotent precursor cells to generate one cell type versus another [16].

The data presented here, together with previous work, demonstrating that exogenous microglia (or their conditioned medium) added to developing neural precursors [12,33,34] can induce astrogenesis [23], suggested that embryonic perturbations which enhance microglial number or activation state will alter neural precursor development. The increase in microglial proliferation due to D61G mutation is similar to the increase in the number of cytokine-independent myeloid colonies when D61G was expressed in murine bone-marrow cells [41]. Indeed, the NS mouse model is a good example of a neurological disorder where microglia are inflammatory on account of a genetic mutation. In fact, the NS mouse shows deficits in hippocampus-dependent learning (Araki T, personal communication).

In addition to inducing an inflammatory phenotype in microglia, D61G expression also dysregulated growth factor expression (data not shown) and abrogated phagocytic functions. Importantly, reduced astrogenesis observed in response to inflammatory microglia is similar to the phenotype shown in NS mouse which exhibits reduced astrogenesis at the expense of increased neurogenesis [16]. Thus, a signaling protein, Shp-2, plays a key role in allowing environmental cues such as cytokines to affect differentiation of multipotent precursor cells in a particular lineage. Supporting the present findings is the recent identification that in the *Hoxb8*-null mouse model, microglia were the principal cells involved in obsessive-compulsive disorder (OCD) [42] and it is postulated that microglia might be having an effect on grooming behavior through a paracrine effect on neurons [43]. These results provide certain clues as to how genetic perturbations lead to mental retardation. Thus, perturbations in neural precursor development cause cognitive dysfunction in neurodevelopmental disorders where microglia are aberrantly activated or increased in number as a consequence of genetic and/or environmental abnormalities.

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