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# Neuroinflammation Is Associated with Changes in Glial mGluR5 Expression and the Development of Neonatal Excitotoxic Lesions

**JANELLE DROUIN-OUELLET,[1] ANNA-LIISA BROWNELL,[2] MARTINE SAINT-PIERRE,[1] CAROLINE FASANO,[3] VINCENT EMOND,[1] LOUIS-ERIC TRUDEAU,[3] DANIEL LÉVESQUE,[4] AND FRANCESCA CICCHETTI[1,5]***

[1]*Centre de Recherche du CHUL (CHUQ), Axe Neurosciences, RC-9800, 2705 Boulevard Laurier, Québec, Qc, Cananda, G1V 4G2*
[2]*Department of Radiology, Experimental PET Laboratory, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA, 02114*
[3]*Département de Pharmacologie, Groupe de Recherche sur le Système Nerveux Central, Faculté de Médecine, Université de Montréal, Montréal, Qc, Canada, H3C 3J7*
[4]*Faculté de Pharmacie, Université de Montréal, Montréal, Qc, Canada, H3C 3J7*
[5]*Département de Psychiatrie et Neurosciences, Université Laval, Québec, Qc, Canada, G1K 0A6*

*KEY WORDS*
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*ABSTRACT*
It has been hypothesized that neuroinflammation triggered during brain development can alter brain functions later in life. We investigated the contribution of inflammation to the alteration of normal brain circuitries in the context of neuroexcitotoxicity following neonatal ventral hippocampal lesions in rats with ibotenic acid, an NMDA glutamate receptor agonist. Excitotoxic ibotenic acid lesions led to a significant and persistent astrogliosis and microglial activation, associated with the production of inflammatory mediators. This response was accompanied by a significant increase in metabotropic glutamate receptor type 5 (mGluR5) expression within two distinct neuroinflammatory cell types; astrocytes and microglia. The participation of inflammation to the neurotoxin-induced lesion was further supported by the prevention of hippocampal neuronal loss, glial mGluR5 expression and some of the behavioral perturbations associated to the excitotoxic lesion by concurrent anti-inflammatory treatment with minocycline. These results indicate that neuroinflammation significantly contributes to long-lasting excitotoxic effects of the neurotoxin and to some behavioral phenotypes associated with this model. Thus, the control of the inflammatory response may prevent the deleterious effects of excitotoxic processes that are triggered during brain development, limiting the risk to develop some of the behavioral manifestations related to these processes in adulthood. 

## INTRODUCTION

Some of the theories concerning the pathogenesis of neuropsychiatric disorders, such as schizophrenia, suggest that many of these illnesses are the result of neurodevelopmental perturbations, leading to clinical manifestation later in adult life. However, this relationship has not been clearly established. These developmental alterations could cascade into abnormal connectivity or accentuated brain vulnerability and exacerbate the effects of other insults occurring in adulthood.

The idea that neuroinflammation, at a critical time point during brain development, can be responsible for misconnection of brain circuits has emerged from recent studies on psychotic disorders in both animal models and humans. For example, immune challenges in animals (Borrell et al., 2002; Meyer et al., 2005; Shi et al., 2003) induce increased brain cytokines (interleukine (IL)-6, IL-1β, TNF-α, INF and cyclooxygenase (Cox)-2) in pups originating from mothers *a priori* exposed to the toxin (Cunningham et al., 2007) and lead to abnormal behaviors, namely cognitive deficits, further supporting the neurodevelopmental hypothesis of such disorders. Very few attempts have been made to identify a causal link between inflammatory processes and abnormal behaviors nor to establish the molecular and cellular neural substrates involved. Insights into these mechanisms would offer additional therapeutic target sites applicable to several disease conditions, and more specifically cognitive-related disorders.

It is also now well-established that in the context of any traumatic brain injury, excessive amount of glutamate can lead to excitotoxicity and cell death in response to the activation of a number of glutamate receptors, as reported in several pathologies, including schizophrenia, Parkinson's, Alzheimer's and Huntington's diseases, epilepsy, and brain ischemia. Glutamatergic neurotransmission is complex and involves both ionotropic and metabotropic receptors. The mGluR5 receptor

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*Correspondence to: Francesca Cicchetti, Ph.D., Centre de Recherche du CHUL (CHUQ), Axe Neurosciences, RC-9800, 2705, Boulevard Laurier, Québec, QC, G1V 4G2, Canada. E-mail: Francesca.Cicchetti@crchul.ulaval.ca

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subtype is widely distributed in the striatum, nucleus accumbens, hippocampus, and the prefrontal cortex (Abe et al., 1992), structures which show, to various degrees, perturbations in disorders such as schizophrenia. In addition to its established neuronal expression, mGluR5 can be located onto glial cells. In primary microglial cultures, its activation negatively regulates the release of microglial inflammatory factors and related neurotoxicity (Byrnes et al., 2009a). *In vivo*, the link between inflammation and mGluR5 modulation has been described, especially under pathological conditions such as neuropathic pain (Neugebauer and Carlton, 2002), amyotrophic lateral sclerosis (Aronica et al., 2001), spinal cord injury (Byrnes et al., 2009b), epilepsy (Aronica et al., 2000), and multiple sclerosis (Geurts et al., 2003).

Neonatal lesion of the ventral hippocampus (nVH) in rats using ibotenic acid, an *N*-methyl-D-aspartate (NMDA) glutamate receptor agonist, at a critical period of development (post-natal day 7 (p7)) has been used as a neurodevelopmental model of schizophrenia but provides a valuable setting in which to study the contribution of brain developmental inflammation to neuronal impairment (see review Tseng et al., 2008). This procedure is associated with a plethora of behavioral alterations, of which the majority have a strong relevance to neuropsychiatric-related symptoms, including psychostimulant-induced hypersensitivity, impaired working memory and social deficits (Tseng et al., 2008). In this study, we used this model to investigate the contribution of inflammation to the excitotoxicity engendered by nVH ibotenic acid lesions in rats.

## MATERIALS AND METHODS

### Neonatal Ventral Hippocampal Lesions

All animal experiments were performed in accordance with the Canadian Guide for the Care and Use of Laboratory animals, and all procedures were approved by the Institutional Animal Care Committee of Laval University. Pregnant Sprague-Dawley rats were obtained at gestational Day 14 (Charles River Laboratories, Montreal, QC, Canada). At p7, males were assigned to sham operated/vehicle, ibotenic acid/vehicle, sham operated/minocycline or ibotenic acid/minocycline groups. Animals were deeply anesthetized under isoflurane 2.5% in the presence of oxygen (1.5 L/min flow) and placed on a platform fixed to a stereotaxic frame (David Kopf Instruments, Tujunga, CA). A 30-gauge needle attached to a Hamilton syringe (Hamilton Company, Reno, NV) was lowered into the ventral hippocampus (Coordinates: AP: −3.5 mm, ML: ± 4.5 mm to bregma and VD: −4.0 mm from dura). For each side, 0.3 μl of either ibotenic acid (10 μg/μl; Sigma, St. Louis, MO) or saline were injected via an infusion pump (World Precision Instrument Inc., Sarasota, FL) at a rate of 0.15 μl/min. This procedure was repeated in the contralateral ventral hippocampus. In total, 254 rats (sham operated/vehicle $n = 69$, ibotenic acid/vehicle $n = 56$, sham operated/minocycline $n = 70$ and ibotenic acid/minocycline $n = 59$) were used in this study (see Supp. Info. Table 1).

### Anti-Inflammatory Drug Treatment

Minocycline (45 mg/kg; Sigma, St. Louis, MO) was administered intraperitoneally (i.p.) to the male pups 12 h prior to the surgery, 30 min before the surgery and once a day for three days following the procedure, at 24-h intervals (Cai et al., 2006; Fan et al., 2005a; Fan et al., 2005b).

### PET Ligand and Imaging

Positron emission tomography (PET) imaging studies of mGluR5 were approved by the Harvard Medical School Institutional Animal Care and Use Committee (IACUC) and were conducted according to a previously published protocol (Wang et al., 2007) using 3-[$^{18}$F]fluoro-5-(2-pyridinylethynyl(benzonitrile ([$^{18}$F]FPEB) as radiolabeled ligand that we developed. Catheterization of tail vein was performed for the administration of the radiolabeled ligand ([$^{18}$F]FPEB; 0.3 mCi, i.v. specific activity of 1000-2000 mCi/μmol). The average accumulation of [$^{18}$F]FPEB in different brain areas were calculated at the time interval from 30 to 60 min after administration of the radioligand. The accumulation of mGluR5 expression in specific brain areas was divided by the accumulation in the cerebellum, displaying only negligible mGluR5 expression (Shigemoto and Mizuno, 2000).

### Behavioral Testing

Behavioral measures were performed on p35 (prepuberty) and p56 (adulthood). These measures included three distinct tests to decipher various behavioral alterations related to the nVH ibotenic acid model. The locomotor behavioral assessment test required the injection of amphetamine. To avoid potential amphetamine interaction with the expression of mGluR5 (Parelkar and Wang, 2004), groups were divided so that the same animal would not undergo a drug-induced behavioral test and mGluR5-related *post-mortem* analysis (see Supp. Info. Table 1).

Amphetamine-induced locomotor activity was assessed by open filled as previously described (Gibrat et al., 2009). Rats were first acclimated for 30 min to the plexiglas enclosures of an automated activity monitor and were then injected with amphetamine (1.5 mg/kg; Sigma, St. Louis, MO) and tested for an additional 60 min. Recorded parameters were scored on total distance traveled over the testing period. Statistical analyses were performed using a one-way ANOVA followed by a Newman-Keuls post-hoc test. The significance of interaction between the anti-inflammatory treatment (minocycline) and lesioning was evaluated by a two-way analysis of variance (ANOVA).

### Social Interactions

Social interactions were assessed according to a previously published protocol (Lipska et al., 2002). Evaluation of investigation episodes (sniffing, nosing, following, climbing over or going under) was assessed in a 10 min session.

Each session was filmed and two investigators blind to treatment status scored and timed the duration of each of these behaviors during 1-min intervals. Statistical analyses were performed using Kruskal-Wallis test and multiple comparisons based on the Conover method (Conover, 1999).

### T-Maze Reward Alternation Task

Spatial working memory was evaluated in adulthood with a reward alternation task paradigm using an elevated T-maze, according to previously published protocol (Deacon and Rawlins, 2006). Length of testing period required a 4-day trial extending from p56 to p59. Prior to the habituation phase, rats were put on food restriction to maintain their weight at 90–95% of their original unrestricted-feeding weight. Each rat received a total of 10 trials and the percentage of alternation was calculated. Given the particularly high variability in lesion size for animals that underwent this behavioral test, only animals that presented an hippocampal volume equivalent to 75% of sham operated rats (as assessed by volumetric stereology) were included in the final data analysis.

### Sample Preparation and Lesion Assessment

Animals were sacrificed at p14, p35, and p56 under deep anesthesia with ketamine/xylazine (75/10 mg/kg, i.p. [0.1 ml/100 g]) and perfused via transcardiac infusion with saline (0.9%) followed by 4% paraformaldehyde (PFA) in 0.1 M PBS, pH 7.4. Brains were collected and post-fixed in 4% PFA for 6 h and transferred to 20% sucrose in 0.1 M PBS for cryoprotection. Coronal brain sections of 35 μm thickness were cut using a freezing microtome (Leica Microsystems, Montreal, QC, Canada). The remaining animals were perfused via transcardiac infusion with PBS 1X (BioShop, Burlington, ON, Canada) containing protease inhibitors (Sigma, St. Louis, MO). The brain was rapidly removed, and one hemisphere was immediately frozen in isopentane (Sigma, St. Louis, MO) and stored at −80°C. The other hemisphere was quickly dissected into three structures (hippocampus, ventral striatum, frontal cortex) and frozen on dry ice, then stored at −80°C. Coronal brain sections of 12 μm thickness were cut using a cryostat (Leica Microsystems, Montreal, QC, Canada). For assessment of lesion size, serial sections at the level of hippocampus were stained with cresyl violet (Sigma, St. Louis, MO), dehydrated, and coverslipped. Sections were then observed under a E800 Nikon microscope (Nikon Canada Inc, Mississauga, ON, Canada).

### Total Hippocampal Volume Assessment

3D reconstruction was performed using the *Serial Section Reconstruction* method provided by the Neurolucida software, version 6.0 (Microbrightfield, Colchester, VT) in order to calculate the total hippocampal volume of the various experimental groups. The total volume calculation was based on the selection of three representative sections interspersed by 19 sections each, and taking into account section thickness (12 μm).

### [$^3$H]PK11195 Autoradiography

Microglial activation was evaluated with [N-Methyl-$^3$H]PK11195 (PerkinElmer, Boston, MA, USA; 84.8 Ci/mmol). Cryostat tissue sections were preincubated at room temperature (RT) for 30 min in a Tris buffer (50 mM, pH 7.4). After sections were dried, they were incubated 30 min at RT with 4 nM [N-Methyl-$^3$H]PK11195. Non-specific binding was determined in the presence of 10 μM PK11195 (Sigma, St. Louis, MO). Sections were apposed against BiomaxMR radioactive sensitive films for 33 days.

### Quantification of [$^3$H]PK11195 Autoradiography

Digitized brain images of the ventral hippocampus were obtained with a CCD camera model XC-77 (Sony Electronics Inc., New York, NY) equipped with a 60 mm f/2.8D magnification lens (Nikon Canada Inc., Mississauga, ON, Canada). The optical density of [$^{\text{N-Methyl-}3}$H]PK11195 specific binding was analyzed on a MacIntosh computer using Image J (NIH, http://rsbweb.nih.gov/ij/) software. The average labeling for each area was calculated from three adjacent brain sections of the same animal at the ventral hippocampal level (AP levels: −5.16 mm to −5.64 mm) (Paxinos and Watson, 2005). Non-specific binding was subtracted from every measurement.

### Cytokine ELISA Immunoassays

The production of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α was determined by ELISA kits (R&D Systems, Minneapolis, MN).

### Post-Mortem Histological Evaluation

For the mGluR5 and microglia co-localization analysis, immunofluorescence was assessed utilizing Fab-fragments. Standard immunofluorescence technique were performed on fixed tissue sections, which were incubated overnight at 4°C with 0.4% Triton X-100 and either of the following antibodies: mouse anti-ED1 (1:2500; Serotech Inc., Raleigh, NC), rabbit anti-Iba1 (ionized calcium binding adaptor molecule 1; 1:1000; Waco Pure Chemicals Industries, Richmond, VA), rabbit anti-mGluR5 (1:500; Millipore, Temecula, CA), chicken anti-GFAP (1:500; Millipore, Temecula, CA) or mouse anti-NeuN (1:2500; Millipore, Temecula, CA). For NeuN immunohistochemistry, sections were post-fixed in PFA 4% for 5 h and immunohistochemistry was performed according to previously published protocol (Cicchetti et al., 2009). Briefly, sections were then incubated overnight at 4°C with mouse anti-NeuN (1:5000; Millipore, Temecula, CA). After incubation with this primary antibody, sections were incubated for 1 h at RT with biotinylated goat anti-mouse IgG (1:1500; Vector Laboratories, Burlington, ON, Canada).

### Quantification of NeuN-Immunoreactive Neurons

The extent of the lesion was determined by counting NeuN-immunoreactive neurons under bright-field illumination. Three sections representing every 20th section from the lesioned area were sampled at higher magnification (20X objective) using Stereo Investigator software (MicroBrightfield, Colchester, VT) attached to an E800 Nikon microscope (Nikon Canada Inc, Mississauga, ON). Section sampling was performed based on the achievement of the desired coefficient of error (0.12) (Glaser and Glaser, 2000; Slomianka and West, 2005), which insured the correct representations of cell counts on three sections of the same brain (AP levels: −5.16 mm to −5.64 mm) (Paxinos and Watson, 2005). Cell counts were performed by two investigators blind to experimental conditions.

### *In Situ* Hybridization for mGluR5 Oligoprobes

Rat probes were generated according to known rat cDNA sequences. The specificity of the probes was determined using a BLAST database search. The mGluR5 receptor exists in two splice variants, mGluR5a and mGluR5b. The latter consists of an insert of 96 bp in the carboxyl-terminal intracellular region of mGluR5. Two oligonucleotide probes were used in parallel, mGluR5a (probe 1), expressed in neurons, astrocytes and microglia and mGluR5b (probe 2), which is not present in microglia (Biber et al., 1999). A third probe (probe 3) was also synthesized to discriminate microglial mGluR5 mRNA, but the targeted sequence was restricted and the probe signal could not be detected with this sequence (see schematic drawing below). Antisense 5′ CAGGCCCCCGGTTCTCT GTGCTCTTGGGAAAGGGTTT GATGACCGCCG 3′ was used for mGluR5a (probe 1), and 5′ TTTCCGACTTGTGCTGGGC CAGTCTCCTGTCTTTG TACCTTAGG GTTTCC 3′ was used for mGluR5b (probe 2). Oligonucleotides were labeled with $^{35}$S-dATP using a 3′-terminal deoxynucleotidyl transferase enzyme kit. The reaction was carried out at 37°C for 60 min and labeled oligonucleotides were purified using a nucleotide removal kit (Qiagen, Mississauga, ON, Canada). Hybridization buffer contained 50% deionized formamide, 10% dextran sulfate, 1X denhardt's solution, 0.25 mg/ml yeast tRNA, 0.5 mg/ml denaturated salmon sperm DNA, and 4X SSC. A 2000-fold excess of cold oligonucleotides in the hybridization buffer was added to determine nonspecific signal.


*In situ* hybridization was performed as described (Julien et al., 2009) at 40°C for the mGluR5a probe and at 37°C for the mGluR5b probe for approximately 18 h in a humid chamber (2X SSC) with each slide covered with a glass coverslip. Slides were exposed to Kodak BIOMAX MR film at RT for 17 days in the case of the mGluR5a probe and 10 days for the mGluR5b probe.

The same quantification method as for the [$^3$H]PK11195 autoradiography analyses was used. The relative optical density of the hippocampal CA2 region was quantified for each hybridization, and the measurement of neuronal and astroglial mGluR5 mRNA expression (mGluR5b) was subtracted from the measurement of total mGluR5 mRNA expression (mGluR5a), to obtain specific microglial mGluR5 mRNA expression.

### Statistical Analysis

Statistical analyses were performed using JMP software 6.0.2 (SAS Institute Inc., Cary, IL) and PRISM 4 (Graphpad Software, San Diego, CA). Unless otherwise stated, all data derived from the animal experiment analyses are expressed as group mean ± SEM, and statistical analyses were performed using a one-way ANOVA assessing the p14, p35, and p56 groups independently. The significance of interaction between anti-inflammatory treatment (minocycline) effect and lesioning was evaluated by a two-way ANOVA.

### Primary Cell Culture

Primary neuronal cultures on glial monolayers were prepared from hippocampus of p1 Sprague-Dawley rat pups following a protocol adapted from Fasano et al., (2008). All procedures regarding the handling of experimental animals were approved by the University of Montreal animal ethics committee. For astrocyte cultures, glial cells were grown for six days whereas for astrocyte cultures enriched with microglia, glial cells were grown during 18 days to allow microglial proliferation. Dissociated hippocampal neurons were plated at a density of 240,000 living cells per mL, on hippocampal astrocyte cultures enriched or not with microglia. Drug treatments were performed after 7 days in culture as follows: 1 μM minocycline (Sigma, St. Louis, MO) or vehicle was added to the cultures for 12.5 h, then 150 μM ibotenic acid (Sigma, St. Louis, MO) or vehicle was added for a total period of 3 h. Cells were then fixed before being processed for immunocytochemistry.

### Immunocytochemistry

Cells were incubated overnight at 4°C with primary antibodies: rabbit polyclonal anti-Iba1 (1:1,000, Wako Pure Chemical Industries, Richmond, VA) and mouse monoclonal anti-MAP2 (microtubule associated protein 2 antibody; 1:250, Sigma, St. Louis, MO). Cells were then incubated for 1 h at RT with mouse Alexa-fluor 488 and rabbit Alexa-fluor 546 conjugated secondary antibodies (1:500, Molecular Probes Inc., USA). Nuclei were labelled with 5 μM DAPI (Fluka Chemical Corp., Milwaukee, WI) for 15 min. Cover-

slips were mounted with Vectashield (Vector laboratories, Burlington, Canada).

### Cell Counts in Neuronal Cultures

A neuron was considered to be intact when it met the following criteria: undamaged plasma membrane around the cell body, absence of MAP2 labeling inside the nucleus and presence of at least two neuronal processes longer than twice the cell body diameter. The significance of an interaction between the effects of drugs (minocycline and ibotenic acid) was evaluated by a two-way ANOVA. When appropriate, we performed a one-way ANOVA followed by a Tukey post hoc test to identify significant differences between experimental groups.

## RESULTS

### Neuroinflammatory Response to Ibotenic Acid Excitotoxicity

To investigate the contribution of an exacerbated neuroinflammatory response to brain development, the ventral hippocampal area was bilaterally injected with ibotenic acid in p7 rat pups (Fig. 1A). Post-mortem autoradiographic binding studies using the [$^3$H]PK11195 ligand showed activated microglial activity at p35 ($P < 0.05$) corresponding to prepuberty in the rat (28 days following the surgery), but not in adulthood (p56) (Fig. 1B–F). Cytokine release was further correlated with post-mortem evidence of activated microglia, revealing an increased expression of the cytokine IL-1β ($P < 0.05$) in ibotenic acid/vehicle animals at p14 (Fig. 1G), a sign of the emergence of the inflammatory response early after the lesion. Minocycline treatment prevented the change of IL-1β levels ($P < 0.05$) (Fig. 1G). The lack of variation of IL-6 levels (Fig. 1H) and the non-detection of TNF-α levels (data not shown) implicates the pro-inflammatory cytokine IL-1β in long-term inflammatory response in the ibotenic nVH lesion model.

Post-mortem immunofluorescence imaging of Iba1 staining, a selective marker of microglia, revealed abundant expression of Iba1 in the ventral hippocampus of ibotenic acid/vehicle animals at p14, seven days following the lesion (Fig. 2A), in comparison to homogenously dispersed resting microglial cells in sham operated/vehicle animals (Fig. 2I). This was not observed in other groups at this time point (Fig. 2D). ED1 labeling, identifying microglia, monocytes and macrophages was also present in ibotenic acid/vehicle animals at p14 (Fig. 2A) but not in sham operated/vehicle animals of the same age (Fig. 2I). Overall, the microglial marker Iba1 was less prominent at p35 or p56, and ED1 virtually absent at these time points, suggesting that the inflammatory response was transitory in nature (Fig. 2A–C). Inflammatory cells expressing both Iba1 and ED1 were characterized by a typical ameboid reactive state in the acute inflammation that was present early after the lesion (Fig. 2G) as opposed to microglial cells observed in later stages following the lesion, which displayed a more ramified, resting state and which did not express ED1 (Fig. 2H). The pronounced inflammatory response present early following ibotenic acid lesion was completely prevented by minocycline treatment (Fig. 2D–F).

### Neuroinflammation Modulates mGluR5 Expression in Non-Neuronal Cells

The binding of mGluR5, using positron emission tomography (PET) imaging, showed increased levels only in ibotenic acid/vehicle animals in adulthood (Fig. 3A). Binding quantification was specifically performed in the ventral hippocampus of lesioned or sham operated animals treated with minocycline (Fig. 3B) and revealed a significant augmentation of mGluR5 binding in ibotenic acid lesioned animals, which was prevented by minocycline treatment ($P < 0.05$). We also developed oligoprobes and quantitatively demonstrated a significant increase in mRNA levels for the microglial form of mGluR5 within the Ammon's horn fields 2 (CA2) region of lesioned animals (Fig. 3C). The relationship between mGluR5 expression and the inflammatory response following nVH lesions in rat pups was additionally supported by immunofluorescence studies. In the CA2 area where NeuN labeling is lost (Fig. 3E), intense mGluR5 immunoreactivity was observed (Fig. 3F), which could be superimposed with the astrocytic marker GFAP (Fig. 3G). Notably, mGluR5 expression was prominently detected within Iba1-immunoreactive microglial cells at the lesion site (Fig. 3J) and not under physiological conditions (Supp. Info. Fig. 1). This expression pattern is seemingly a specific feature of mGluR5, as other metabotropic glutamate receptors, such as mGluR2/3 are expressed onto microglia in both resting and activated conditions. These observations demonstrate selective induction of mGluR5 expression in the cells participating to the inflammatory response including both astrocytes and microglia.

### Anti-Inflammatory Treatment Prevents the Appearance of Behavioral Phenotypes Related to the Ibotenic Acid nVH Lesion

To probe the contribution of the neuroinflammatory response to the development of various behavioral phenotypes related to the ibotenic acid nVH lesion, we tested whether the blockade of the inflammatory response by minocycline interrupts the development of some of the characteristics triggered by the nVH lesion model. Animals were tested at prepuberty (p35) and adulthood (p56) for amphetamine-induced hyperlocomotion (Fig. 4A) and potential impairments in social interactions (Fig. 4C), while spatial working memory deficits were assessed at p56 exclusively (Fig. 4B), which represent conventional measures of behavioral abnormalities previously observed in this lesion model. Overall, ibotenic acid lesioned animals did not display behavioral abnormalities at prepuberty, except for a slight tendency for hypolocomotion in young lesioned animals treated with minocycline (Fig. 4A). In adulthood, ibotenic acid lesioned animals showed hyperlocomotion induced by

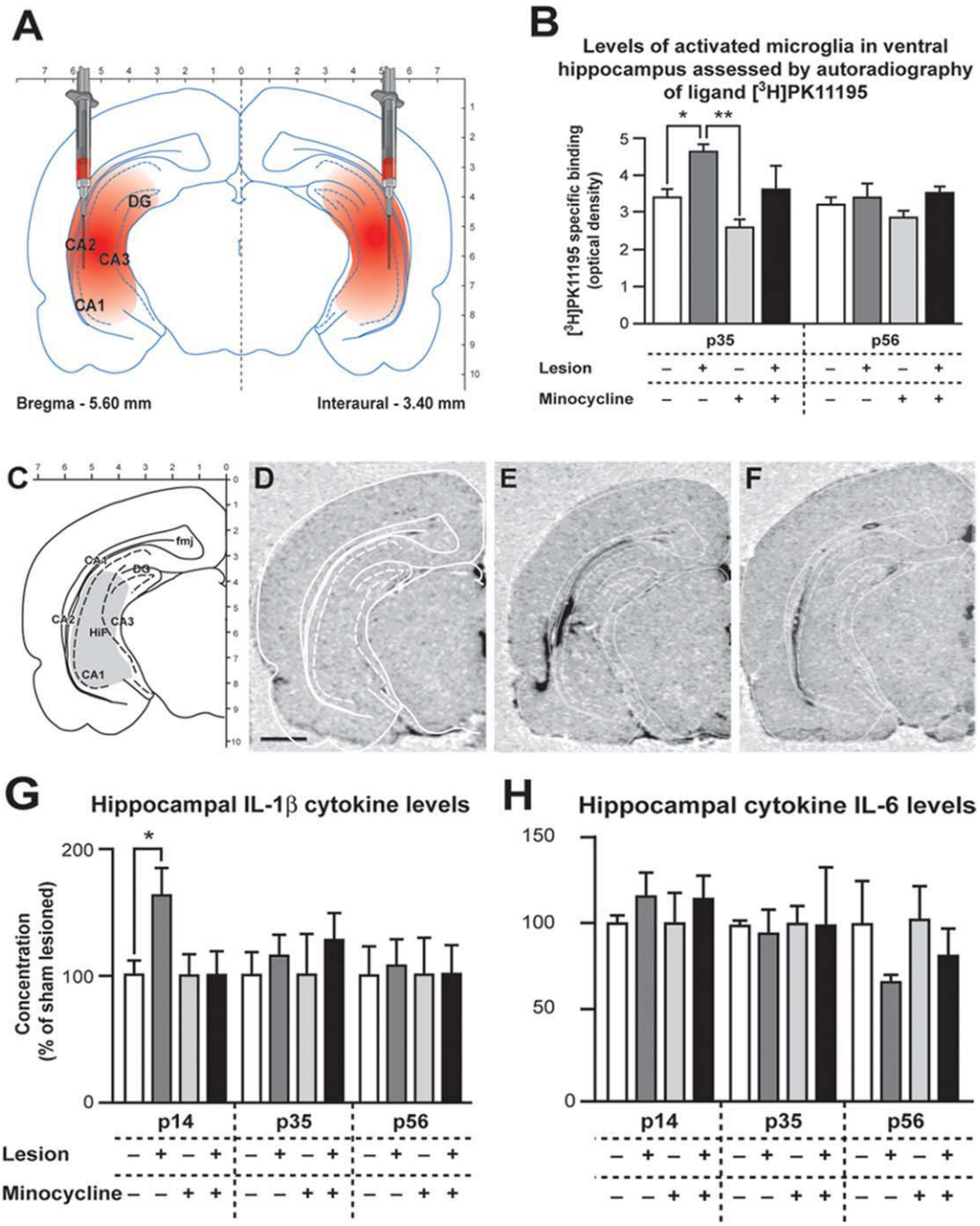


Fig. 1. Brain inflammation following ibotenic acid lesions. **A**: Schematic atlas drawing (Paxinos and Watson, 2005) showing the level used to ensure a successful lesioning procedure. **B**: The quantification of [$^3$H]PK11195 specific autoradiographic binding activity demonstrates a significant increase ($P < 0.05$) in prepubertal (p35) ibotenic acid/vehicle as compared with prepubertal sham operated/vehicle and sham operated/minocycline animals. Data are illustrated as mean ± SEM and were analyzed using a one-way ANOVA followed by a Tukey post hoc test. By a two-way ANOVA, data analysis revealed a significant effect of treatment and lesion, only at the prepuberty time point. **D–F:** Autoradiograms of coronal brain hemisections illustrate increased [$^3$H]PK11195 binding in the ventral hippocampus of prepubertal (p35) ibotenic acid/vehicle (E) as compared with prebubertal sham operated/vehicle animals (D) and ibotenic acid/minocycline animals (F). **G,H**: ELISA assays of hippocampal pro-inflammatory cytokine levels in rats at p14, p35 and p56 revealed a significant increase in IL-1β concentrations (G) ($P < 0.05$) at p14 in ibotenic acid/vehicle in comparison with the sham operated/vehicle group. Histogram bars illustrates means ± SEM (*$P < 0.05$ and **$P < 0.01$ vs. untreated lesioned group). CA, Ammon's horn field; DG, dentate gyrus; HiF, hippocampal fissure; fmj, forceps major corpus callosum. Scale bar D (applies to E,F) = 1 mm.

amphetamine ($P < 0.05$), as reported (Lipska et al., 1993). Remarkably, ibotenic acid lesioned animals treated with minocycline did not develop locomotor hypersensitivity following amphetamine administration ($P < 0.05$) (Fig. 4A). Spatial working memory deficits ($P < 0.05$) were also prevented by minocycline treatment (Fig. 4B). Lastly, analysis of investigation episodes revealed a strong propensity for social behavioral

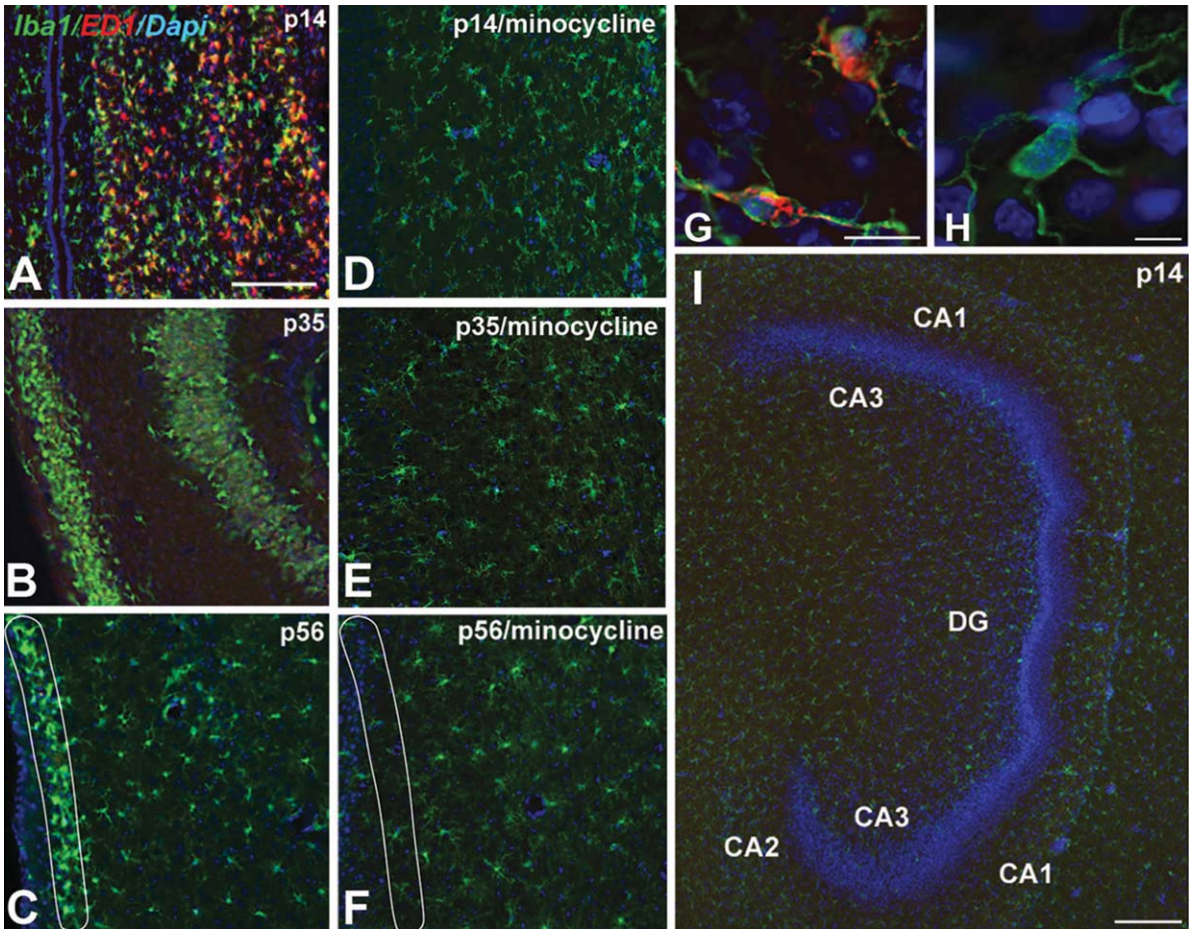


Fig. 2. Minocycline prevents hippocampal microgliosis induced by ibotenic acid. **A–C:** A very strong microgliosis (Iba1)/macrophage (ED1) response was observed in ibotenic acid/vehicle animals at p14 (A), with a time-dependent decrease at p35 (prepuberty) (B), and p56 (adulthood) (C). No inflammatory response was observed in minocycline treated animals at any of the time points assessed (**D–F**). **G,H:** High power photomicrographs of double-stained (G; macrophages; yellow) or single-stained microglia (H). For comparative purposes, (**I**) illustrates a representative low power photomicrograph of double staining for Iba1 (microglial cells; green) and ED1 (macrophages; red) in a sagittal section of the ventral hippocampal area showing the baseline expression of microglia in a sham operated/vehicle animal one week post surgery. CA, Ammon's horn field; DG, dentate gyrus. Scale bars A = 150 μm (applies to B–F); G = 10 μm; H = 8 μm; I = 200 μm.

impairments at adulthood (p56) in lesioned animals ($P < 0.1$) (Fig. 4C), which was not discernible following subchronic minocycline treatment during the developmental period (early postnatal days). These behavioral evaluations emphasize the strong link existing between inflammation and the appearance of postpubertal symptoms related to the nVH lesioned animals.

### Neuroprotective Effects of Minocycline Against Ibotenic Acid Excitotoxicity

Finally, to evaluate the repercussion of inflammation on the anatomical integrity of the hippocampus, various hippocampal volume quantification and neuronal density measurements were conducted. Measures of the total volume of ventral hippocampi at p35 and p56 using digital 3D reconstructions revealed a significant volume loss in ibotenic acid/vehicle as compared with sham operated/vehicle animals ($P < 0.05$) (Supp. Info. Fig. 2B). We further determined that the total volumes of CAs (CA1, CA2, CA3) and dentate gyrus (DG) were significantly diminished in ibotenic acid animals at prepuberty (p35; $P < 0.05$) and adulthood (p56; $P < 0.05$) as compared with sham-operated rats ($P < 0.001$) (Supp. Info. Fig. 2D). This volume reduction resulted from a loss of neuronal cells, as illustrated by the almost complete disappearance of NeuN labeling at the site of the ibotenic acid injection in the CA2 area (Supp. Info. Fig. 2F,G). This significant CA volume loss, as assessed by stereology, was the result of a NeuN-immunoreactive cell loss, as the NeuN cell density remained unchanged across groups (Supp. Info. Fig. 2E). Minocycline regime led to complete preservation of the ventral hippocampus morphology in ibotenic acid lesioned animals ($P < 0.05$). Minocycline treatment did not prevent the loss of CA volumes in ibotenic acid injected animals, but rather prevented the loss of neurons specifically in the CA2 region (Supp. Info. Fig. 2H,I), as compared with sham-operated animals treated with vehicle (Supp. Info. Fig. 2L,M).

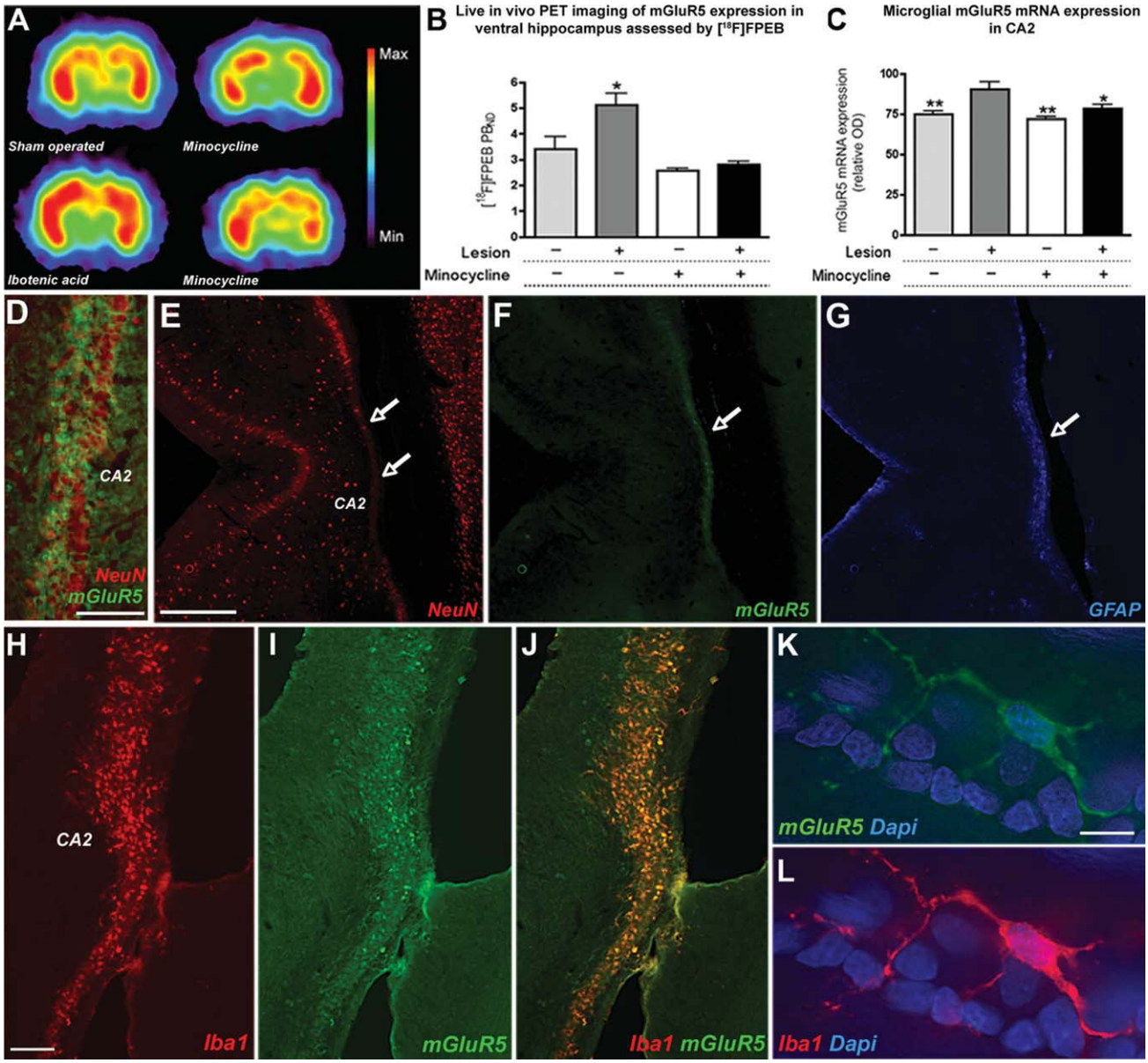


Fig. 3. Relationship between neuroinflammation and mGluR5 expression. **A**: In ibotenic acid/vehicle rat, coronal slices at the hippocampal level show moderately enhanced binding of [$^{18}$F]FPEB compared with the corresponding brain regions in the sham operated/vehicle rat ($P < 0.05$). Minocycline treatment diminished mGluR5 binding levels in the hippocampus ($P < 0.05$) (**B**). Quantitative analysis of mGluR5 mRNA also revealed elevated levels of microglial mGluR5 mRNA in the CA2 region of lesioned animals only (**C**). Data are illustrated as mean ± SEM and were analyzed using a one-way ANOVA followed by a Tukey post hoc test. Data analysis using a two-way ANOVA did not reveal a significant effect of age (p35 and p56), and thus the two time points were pooled. **D**: Representative photomicrograph of double immunofluorescent staining of NeuN (neuronal nuclei in red) and mGluR5 (membranous/perinuclear immunoreactivity in green) at the level of the ventral hippocampus in a sham operated/vehicle animal. **E–G**: Low power photomicrographs of NeuN immunofluorescent staining (E), mGluR5 (F) and GFAP (G; astrocytes) in an ibotenic acid/vehicle animal. While a significant loss of NeuN-immunoreactive neurons is noticeable at the level of CA2 (E), mGluR5 is still visibly detectable in this particular region (F), which corresponds to an astrocytic response (G). **H–J**: Low power photomicrographs illustrating the significant microgliosis (H), as identified by Iba1 fluorescent staining, corresponding to mGluR5 expression (I) where a significant proportion of identified cells were doubly labeled for these markers (J). **K,L:** Higher magnification images exemplifying a microglial cell (L) expressing mGluR5 (K). CA2, Ammon's horn field 2. Scale bar D = 25 μm, E (applies to F,G) = 300 μm, H (applies for I,J) = 100 μm, K = 10 μm (applies to L).

## Implication of Microglia in the Neuroprotective Effects of Minocycline

To test the hypothesis that the neuroprotective mechanisms of minocycline in the present excitotoxic model implicates an action on microglia, we also used rat primary hippocampal neuronal cultures produced from p7 pups. We prepared primary cultures of neurons on an astrocyte layer, which was subsequently enriched with microglial cells to mimic the *in vivo* situation in lesioned animals. Ibotenic acid exposure had a similar (60–70% of neuronal cell loss) effect on both astroglial/neuronal and astroglial/neuronal/microglial cultures (Fig. 5 and Supp. Info. Fig. 3). Cell loss of hippocampal cultured

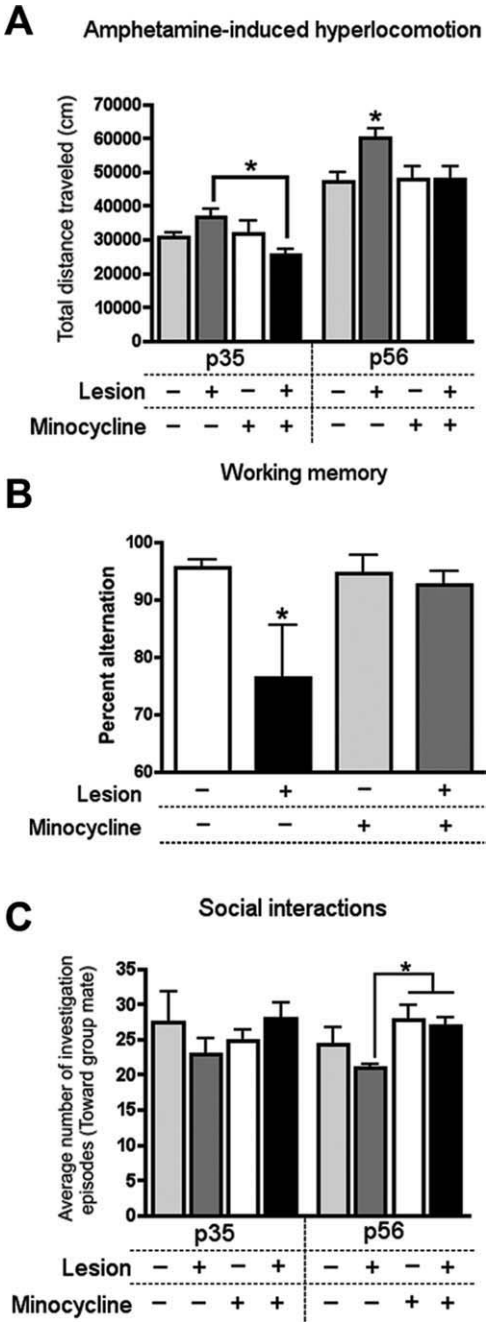


Fig. 4. Minocycline prevents ibotenic acid-induced behavioral phenotype. **A**: Analyses of total distance traveled show no difference between groups at p35, except for a slight hypolocomotion in ibotenic acid lesioned animals treated with minocycline compared with ibotenic acid lesioned/vehicle. In adulthood (p56), a significant amphetamine-induced hyperactivity is observed in nVH lesioned animals ($^*P < 0.05$ vs. sham-operated or lesioned minocycline-treated groups). **B:** Spatial working memory was only impaired in lesioned rats that did not receive minocycline treatment, while lesioned/minocycline animals showed normal alternating behaviors ($^*P < 0.05$ vs. untreated sham-operated group). **C**: Social interactions were comparable in all groups at prepuberty (p35). In adulthood (p56), investigation episodes were significantly decreased in ibotenic acid lesioned/vehicle animals compared with ibotenic acid lesioned/minocycline animals, of which values are similar to controls ($^*P < 0.05$ vs. untreated lesioned group). Data are illustrated as mean ± SEM and were analyzed using a one-way ANOVA. $^*P < 0.05$.

neurons exposed to ibotenic acid was significantly diminished when minocycline was added to cultures containing microglial cells ($P < 0.001$) (Fig. 5), whereas in the absence of microglia, minocycline failed to obstruct the toxic effects of ibotenic acid (Supp. Info. Fig. 3). These data provide evidence that the neuroprotective properties of minocycline require the presence of microglia.

## DISCUSSION

Our results demonstrate that the nVH ibotenic acid lesion, a well-characterized model of excitotoxicity, is associated with a strong and persistent inflammatory response. This is accompanied by a selective induction of mGluR5 expression in glial cells. Our study provides evidence that an anti-inflammatory treatment with minocycline prevents neuronal cell loss and behavioral phenotype normally associated with neurotoxin exposure in this model, while reducing all inflammatory signs. These results also afford the first *in vivo* evidence of mGluR5 localization onto activated microglial cells in the brain, suggesting the potential participation of this glutamate receptor to the brain inflammatory response.

Minocycline's neuroprotective actions have been demonstrated in several animal models of disorders such as Parkinson's disease (Du et al., 2001), Huntington's disease (Berger, 2000; Chen et al., 2000) and ischemic injury (Yrjanheikki et al., 1999) and are believed to act via anti-inflammatory actions by inhibiting microglial activation and proliferation, consequently decreasing the release of various inflammatory mediators including cytokines, chemokines, matrix metalloproteases, and nitric oxide (NO) (Stirling et al., 2005). Our results show that [$^3$H]PK11195 autoradiographic binding, as well as the presence of a significant microglial response, increase in ibotenic acid lesioned animals, and are reversed by minocycline treatment. More specifically, treatment with minocycline has been reported to inhibit microglial production of IL-1β (Seabrook et al., 2006; Wu et al., 2002), which is consistent with our results.

Minocycline confers neuroprotective effects against glutamate excitotoxicity through a p38-MAPK-dependent mechanism, which has been demonstrated *in vitro* to be mediated via inhibition of microglial activation (Tikka et al., 2001; Tikka and Koistinaho, 2001) or by the reduction of NO-induced death in rat cerebellar granule neuronal cultures (Lin et al., 2001). Our analyses also showed that minocycline treatment prevents the overall atrophy of the ventral hippocampus induced by ibotenic acid lesioning, but only partially prevents volume loss in the CA areas of lesioned animals. This suggests that the prevented CA2 neuronal loss, in combination with the preserved morphology, is sufficient to modify the behavioral phenotype of these animals (see discussion below). To evaluate whether minocycline has a direct activity on neuronal survival in ibotenic acid lesioned animals, we examined the effect of minocycline in primary cultures of rat hippocampal neurons exposed to ibotenic acid. Our *in vitro* data clearly indicate that

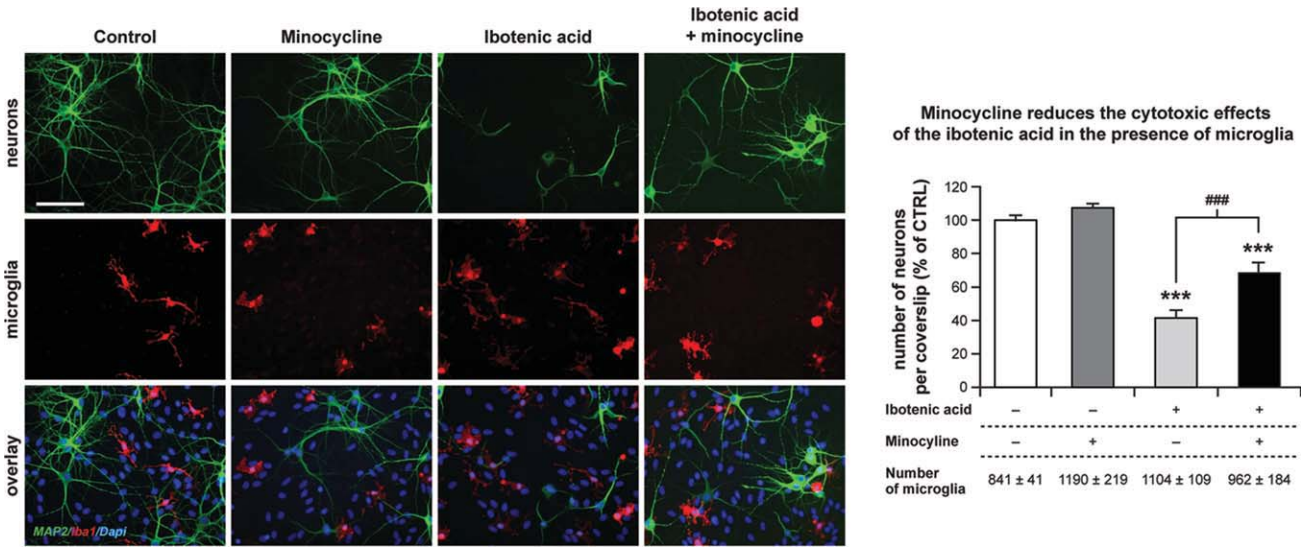


Fig. 5. Minocycline prevents ibotenic acid-induced neurotoxic effects in the presence of microglia. Fluorescence micrographs exemplifying neurons and microglia in primary hippocampal cultures prepared from rat pups. Neurons and microglia were identified by antibodies directed against MAP2 (green) and Iba1 (red), and cell nuclei by Dapi (blue). The number of microglial cells remained constant in all the culture conditions, as indicated under each histogram bar ($n$ = 4 for each group). Minocycline (1 μM) alone had no effect on neuron survival, whereas ibotenic acid (150 μM) induced a statistically significant decrease in the number of neurons. Application of minocycline prior to ibotenic acid significantly decreased neuronal loss compared with the effect of ibotenic acid alone. ***$P < 0.001$ vs. control (CRTL) condition, ###$P < 0.001$ vs. ibotenic acid condition. Scale bar = 100 μm.

minocycline acts through microglial inhibition, as opposed to a direct action on neurons, as seen in previous *in vitro* studies (Lin et al., 2001). This suggests that the neuroprotection observed in the ventral hippocampus in our animal model indeed results from an inhibition of microglial activation and associated glial mGluR5 binding levels.

Permanent inactivation of the rat ventral hippocampus caused by bilateral ibotenic acid lesion during its critical period of development (p7), when hippocampus-accumbens-prefrontal cortex connections are being established, leads to cellular, molecular and morphological changes in the brain. These changes are then translated to altered behaviors such as amphetamine-induced hyperlocomotion, impaired working memory, social interaction deficits, as well as other behavioral abnormalities, including sensorimotor deficits (Tseng et al., 2008). Here the amphetamine-induced hyperlocomotion observed in ibotenic acid lesioned animals in adulthood was reversed by early minocycline treatment. In addition, working memory deficits, evaluated by the reward alternation task, was precluded by the anti-inflammatory treatment as well. Lesioned animals also showed evidence of social interaction disability (investigation events), a tendency that was no longer observed when lesioned animals were treated with the anti-inflammatory drug. In the context of cognitive mental disorders, recent case reports (Miyaoka et al., 2007), which led to the first open-label trial (22 patients), have also highlighted the antipsychotic effects of minocycline on positive and negative symptoms in patients with schizophrenia (Miyaoka et al., 2008). Given the period of administration of minocycline (during development) and the complete blockade of the inflammatory response at this stage, the neuroprotective effect of minocycline demonstrated in this study appears to result from the prevention of abnormal cerebral development rather than a direct antipsychotic effect of the compound. The investigation of a more specific anti-inflammatory drug, such as dexamethasone for example, would exclude potential alternative mechanisms of action, although its use in this model is limited by its effect on brain development (Barrington, 2001; Uno et al., 1994; Yeh et al., 2004).

Our PET imaging study of mGluR5 binding is the first *in vivo* imaging report of this metabotropic glutamate receptor subtype, of which the results are supported by quantitative glial mGluR5 mRNA expression in the CA2 region. An important finding of this study is the demonstration of a relationship between the microglial and astroglial responses that follow an ibotenic acid lesion, and the specific expression of mGluR5 within activated microglia, likely conferring a specific role of mGluR5 in inflammation. This is the first demonstration of the co-localization of mGluR5 with microglial cells in the brain, further supporting very recent evidence of this type of co-localization in a spinal cord injury model (Byrnes et al., 2009b). One particular *in vitro* study identified a complex interaction between microglia and astrocytes during inflammation induced by LPS, which further influenced the glutamate-dependent response of astrocytes (Tilleux et al., 2007). Activation of mGluR5 receptors expressed in microglial cells, by the selective agonist (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG), can markedly reduce microglial activation induced by LPS (Byrnes et al., 2009a; Loane et al., 2009), as well as levels of inducible nitric-oxide synthase, NO, reactive oxygen species and TNF-α production, suggesting that

mGluR5 receptors can modulate the course of the inflammatory response, possibly via the inhibition of the enzymatic activity of NADPH oxidase (Loane et al., 2009). *In vivo*, a seven-day administration of CHPG in rats inflicted with spinal cord injury resulted in functional motor recovery, reduced lesion volume as well as sparing of white matter, 28 days following this injury. These results strongly suggest that selective activation of mGluR5 can generate an anti-inflammatory effect, as it could be the case in our model. Notably, the increasing interest for the development of therapeutic strategies targeting mGluR5 for mental and neurodegenerative disorders originates from the observations of neuronal mGluR5 expression (Conn et al., 2009; Lea and Faden, 2003). The present study suggests that the expression of mGluR5 within inflammatory mediator cells, may represent a new alternative mechanistic route by which mGluR5 can modulate, at least in part, the evolution of neurodevelopmental disorders.

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