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Toll-like receptor 3 activation modulates hippocampal network excitability, via glial production of interferon-β

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ABSTRACT

The family of toll-like receptors (TLR) plays a major role in innate immunity due to their pathogen-recognition abilities. TLR3 is a sensor for double-stranded RNA, and regulates host-defense responses to several viruses, via the production of type I interferons. Interferon-β (IFNβ) is a primary product of TLR3 activation, and its transcription is elevated in the CNS response to the synthetic TLR3 ligand, polyinosinic-polycytidylic acid (poly(I:C)). Peripheral infections, along with TLR-induced inflammatory mediators, are known to have detrimental effects on brain function, exerting a negative impact on cognitive impairment and enhancing seizure susceptibility. In the current study, we assessed hippocampal function *in vitro*, in response to systemic delivery of a TLR3 agonist. Unlike agonists of other TLRs, intraperitoneal injection of poly(I:C) did not adversely affect evoked short- and long-term synaptic plasticity in mouse hippocampal slices. However, sustained and interictal-like spontaneous activity was observed in CA1 pyramidal cells in response to poly(I:C) and this was associated with alterations in the expression of phosphorylated NR2B subunit-containing NMDA receptors and an astrocyte-specific glutamate/aspartate transporter (GLAST) which impact on extracellular glutamate concentration and contribute to the genesis of epileptiform activity. We provide evidence that the production of IFNβ from microglia and astrocytes, and using mice deficient in the type I IFN receptor α 1 (IFNAR1), demonstrate that its subsequent activation is likely to underlie the TLR3-mediated modulation of hippocampal excitability.

INTRODUCTION

Toll-like receptors (TLRs) are a family of pathogen- and damage-sensing receptors, expressed in cells of the innate immune system (for review see: Lehnardt, 2010; Moynagh, 2005; O'Neill, 2008). Within the CNS, TLR expression has been identified in abundance on glial cells, primarily on microglia and astrocytes (Jack *et al.*, 2005; Olson *et al.*, 2004). Some evidence suggests the presence of TLRs on neurons, albeit to a comparably lesser extent (Hanke and Kielian, 2011; Lafon et al., 2006; Tang et al., 2007), where they are known to play a significant role in regulation of neurogenesis (Okun *et al.*, 2011; Rolls *et al.*, 2007). To date, TLR2- and TLR4-mediated inflammatory responses in the brain have been most extensively investigated, due to their ability to recognise infections of bacterial origin (Lehnardt, 2010). Activation of TLR2 and 4 has detrimental effects on brain function; specifically impairments in synaptic and cognitive abilities have been described (Costello *et al.*, 2011a; Costello *et al.*, 2011b; Mazarati *et al.*, 2011). Receptor activation is also associated with epilepsy and seizure susceptibility (Maroso et al., 2010; Vezzani et al., 2011), bacterial meningitis (Hanke and Kielian, 2011; Klein et al., 2008) and age-associated pathologies such as Alzheimer's disease (Balistreri et al., 2009; Richard et al., 2008; Walter et al., 2007).

Unlike TLR2 and 4, TLR3 is primarily expressed intracellularly, where it acts as a sensor for double-stranded RNA, a product of replicating viruses (Alexopoulou et al., 2001; Matsumoto et al., 2002). As such, recent evidence has ascribed a central role to TLR3 in

the regulation of host-defense responses to certain (Daffis et al., 2008; Edelmann et al., 2004; Wang et al., 2004; Zhang et al., 2007), although not all (Edelmann et al., 2004) viral infection. Engagement of the receptor with the synthetic double-stranded RNA polyinosinic-polycytidylic acid (poly(I:C)), which has been shown to mimic the effects of systemic viral infection (Cunningham et al., 2007), leads to the production of type I IFNs, IFNα and IFNβ (Alexopoulou *et al.*, 2001) via activation of the transcription factors interferon regulatory factor (IRF)-3 and -7 (Moynagh, 2005). In microglia and astrocytes, poly(I:C)-mediated TLR3 activation has been shown to augment IFNβ mRNA, in addition to a range of inflammatory mediators including tumor necrosis factor (TNF)α and interleukin (IL)-6 (Jack et al., 2005; Olson and Miller, 2004). The actions of IFNα and β are predominantly mediated through activation of the type I IFN receptor, which is composed of two chains, IFNAR1 and IFNAR2, and the subsequent activation of the Janus kinase (JAK)-Signal transducers and activators of transcription (STAT) signaling pathway (Pestka, 2007). Among other inflammatory cytokines, type I IFNs are known to mediate the response to viral infection in mammalian cells (Guidotti and Chisari, 2001; Pestka, 2007). Evidence to date suggests that type I IFNs can also elicit profound neuromodulatory effects. IFNα/β, applied to organotypic hippocampal slice cultures, was reported to indirectly enhance excitability of CA3 pyramidal neurons (Muller *et al.*, 1993). Similarly, augmented neuronal activity has been reported in subsequent studies in ventromedial hypothalamus, amygdala, hippocampus and somatosensory cortex in response to IFNα (Dafny *et al.*, 1996) and in neocortical neurons following application of IFNβ (Beyer *et al.*, 2009; Hadjilambreva *et al.*, 2005).

With the exception of their role in neurogenesis and neuronal development, the effects of TLR activation on neuronal function have been poorly explored. Among others, evidence from our own laboratory has highlighted the detrimental effects of TLR2 and 4 activation on hippocampal long-term potentiation (LTP) and hippocampal-dependent learning (Costello *et al.*, 2011a; Costello *et al.*, 2011b). A recent investigation carried out in TLR3-deficient mice has also identified a role for TLR3 as a constraint to hippocampal-dependent memory retention, and the cellular processes underlying neuronal plasticity (Okun et al., 2010). Additionally, activation of TLR3 by intracerebroventricular administration of poly(I:C) impaired working and contextual memory (Galic et al., 2009; Okun et al., 2010), and enhanced seizure susceptibility (Galic et al., 2009). The effects of viral infection on CNS function are widely reported, and include encephalitis and associated epileptic activity (Getts et al., 2008; Sellner and Trinka, 2012; Wang et al., 2004; Zhang et al., 2007). While the mechanisms remain undefined, activation of TLR3 has been implicated in regulating the pathogenic responses to at least some viruses, including West Nile virus (Daffis *et al.*, 2008; Wang *et al.*, 2004) and herpes simplex virus (Zhang *et al.*, 2007). However, no direct investigation of the functional consequences of a systemically-administered TLR3 agonist on CNS physiology has been reported. The current study assesses hippocampal synaptic function and network excitability in response to peripheral challenge with poly(I:C). We have investigated the direct association between systemic TLR3 activation and IFNβ-induced neuromodulation and provide evidence of enhanced hippocampal excitability in response to TLR3 activation, which is mediated by IFNβ.

MATRIALS AND METHODS

Animals

Male and female C57BL/6 mice (7-11 months; Harlan, UK; see: Lyons et al., 2012) and two cohorts of IFNAR1$^{-/-}$ mice (Hwang et al., 1995; Swann et al., 2007; 3-4 months and 8-9 months; a gift from Prof. K. Mills, School of Biochemistry and Immunology, Trinity College Dublin), bred on a C57BL/6 background, were maintained in the Bioresources Unit, Trinity College Dublin. Animals of mixed gender were used based on previous evidence that inflammatory-associated brain dysfunction was not gender-specific (Costello et al., 2011b). In certain experiments, mice received an intraperitoneal (i.p.) injection of either poly(I:C) (12mg/kg; Amersham Biosciences, UK) or the equivalent volume of saline (0.9%) 4h prior to experimentation. This concentration of poly(I:C) was chosen based on the previous report that systemic administration can induce a robust inflammatory response in the CNS within 4h (Field *et al.*, 2010). Experiments were performed under licence from the Department of Health and Children (Ireland) and with local ethical approval.

Electrophysiology

Hippocampal slices were obtained from male and female C57BL/6 and IFNAR1$^{-/-}$ mice as previously described (Costello *et al.*, 2011b). In brief, hippocampal slices (400μm) were prepared using a McIlwain tissue chopper, and maintained at room temperature for ≥1h prior to experimentation. Slices were transferred to a submerged recording chamber and continually perfused (2-3ml/min) with oxygenated artificial cerebrospinal fluid

(aCSF) containing (in mM): 125 NaCl, 1.25 KCl, 2 $CaCl_2$, 1.5 $MgCl_2$, 1.25 KH_2PO_4, 25 $NaHCO_3$, and 10 D-glucose, at room temperature (22-23°C).

To record field excitatory postsynaptic potentials (EPSPs), the Schaffer collateral-commissural pathway was stimulated at 0.033Hz (0.1ms duration) using a bipolar tungsten stimulating electrode (Advent Materials, UK). Extracellular EPSPs were recorded from the CA1 stratum radiatum using a monopolar recording electrode. Recording electrodes (~2 MΩ) were pulled from borosilicate glass capillary tubes (Harvard Apparatus, US) and filled with aCSF. The stimulus intensity was adjusted to produce a response 40-50% of maximal EPSP amplitude as determined from an input–output curve for each experiment. Paired-pulse facilitation (PPF) was recorded in response to two consecutive stimuli (50ms inter-pulse interval), and determined as the ratio of the slope of EPSP2/EPSP1. A stable baseline of at least 10-20 min was recorded prior to application of theta-burst stimulation (TBS), which consisted of 10 trains (4 pulses at 100Hz) repeated at 5Hz. TBS is routinely used to induce LTP (Hess et al., 1996), which has been shown in hippocampus to be adversely affected by inflammatory stimuli (Costello et al., 2011a; Costello et al., 2011b). Evoked EPSPs were normalised to the slope recorded in the 5 min period prior to LTP induction, and LTP was measured as a mean value of the final 5 min of recording (55-60 min post-TBS). Data are presented as mean percentage EPSP slope ± SEM, and for clarity of illustration, error bars are included to correspond with every 2 min of recording. Sample EPSP traces represent an average of 4 consecutive EPSPs, taken immediately prior to TBS, and 60 min following LTP induction.

Spontaneous activity was recorded extracellularly from the CA1 stratum pyramidale in the absence of evoked stimulation. Slices were equilibrated in the recording chamber for 20-30 min prior to recording. Baseline activity was measured for 15-20 min (in sweeps of 1-minute duration). To facilitate spontaneous activity, slices were perfused with aCSF excluding $MgCl_2$ (0-Mg^{2+}) for 15 min. Wash-out of Mg^{2+} was confirmed by an increase in the amplitude of the evoked population spike, and the appearance of multiple spikes (Coan and Collingridge, 1985; Mody et al., 1987). Spontaneous activity was then monitored for a further 15-20 min in the absence of Mg^{2+}. To assess the effect of IFNβ on spontaneous activity, an additional set of experiments was carried out in the presence of bovine serum albumin (BSA, 0.04%; Sigma-Aldrich, UK) to facilitate perfusion. Recombinant mouse IFNβ (1kU/ml; PBL InterferonSource, US) was added to the perfusate for 15 min, and spontaneous activity was recorded subsequently for 15-20 min. The spontaneous activity defined as epileptiform-like events included bursts of population discharges (≥3 consecutive spikes), substantial shifts from baseline of >1s duration, sustained synaptic depolarisations of >1s duration, and synaptic depolarisations reaching threshold for burst population discharges (Jensen and Yaari, 1988; Xiong and Stringer, 2001). Event frequency was determined as the number of events per minute of recording. All data were acquired using WinWCP v4.0.7 software (Dr J. Dempster, Strathclyde, UK). Sample traces illustrate examples of typical activity observed in slices from poly(I:C)-treated animals in 0-Mg^{2+} conditions, or in the presence and absence of IFNβ.

Preparation and treatment of primary glial and neuronal cultures

Glial cell cultures were prepared from 1-day-old (P1) C57BL/6 mice as previously described (Nolan *et al.*, 2005). Tissue was taken from the cerebral cortex, following removal of the cerebellum, brainstem and olfactory bulb. All cortical, subcortical and neocortical structures, including hippocampus, were maintained and for simplicity, these cells are referred to as 'cortical' cultures. Briefly, tissue from 5-7 mice was dissected, roughly chopped and added to pre-warmed Dulbecco's modified Eagle's medium containing fetal bovine serum (FBS), penicillin and streptomycin (100U/ml) (cDMEM; Invitrogen, UK). Tissue was triturated, the suspension was filtered through a sterile mesh filter (40μm) and centrifuged (2000 rpm, 3 min, 20°C). The resulting pellet was resuspended in warmed cDMEM and cells seeded onto 25cm^2 flasks. After 24h, media was replaced with cDMEM containing granulocyte macrophage-colony stimulating factor (GM-CSF; 10ng/ml) and macrophage-colony stimulating factor (M-CSF; 20ng/ml) and cells were grown at 37 °C in a 5% CO_2 humidified environment for 12 days, with medium replaced every 3-4 days. Non-adherent microglial cells were isolated by shaking (110 rpm, 2h, room temperature), tapping and centrifuging (2000 rpm, 5 min) (Costello *et al.*, 2011a; Watson *et al.*, 2010). The pellet was resuspended in cDMEM and the microglia were plated onto 6-well plates at a density of 0.6x10^5 cells/cm^2 and maintained at 37°C in a 5% CO_2 humidified atmosphere for 2 days prior to treatment. In the absence of microglia, adherent astrocytes were incubated in trypsin-ethylenediaminetetraacetic acid (EDTA) (3ml/flask, 37ºC; Invitrogen, UK) for 3 min, and the digestion was inactivated by the further addition of cDMEM (6ml/flask). Cells were centrifuged (2000 rpm, 3 min) and pellet resuspended in cDMEM. Astrocytes were plated onto 6-well

plates at a density of $0.3x10^5$ cells/cm^2 and allowed to continue proliferating for 2 days at 37°C in a 5% CO_2 humidified environment. Astrocytes prepared using this protocol typically contain less than 10% microglial contamination (Cowley et al., 2012).

Neuronal cell cultures were prepared from cerebral cortex of P1 C57BL/6 mice. Tissue from 5-7 mice was dissected as described above, and incubated in trypsin-EDTA (2ml, 37ºC) for 2 min, followed by the addition of cDMEM (4ml) to inactivate the trypsin. Cells were centrifuged (960xg, 3 min, 20°C), and the pellet was resuspended in cDMEM. The cell suspension was gently triturated, and sediment allowed to settle for 2-3 min. The cellular suspension was removed and centrifuged (960xg, 3 min, 20°C), and the pellet was resuspended in warm neurobasal medium, supplemented with glutamax which acts as a stable form of L-glutamine, penicillin and streptomycin (100U/ml) and the serum substitute B27 (1%) (cNBM; Invitrogen, UK). Cells were plated onto coverslips coated with poly-D-lysine (50µg/ml; Invitrogen, UK) in 24-well plates at a density of $0.6x10^5$ cells/cm^2 and maintained (37°C, 5% CO_2) for 5-6 days prior to treatment.

Two days following cell isolation, microglia and astrocytes were treated with cDMEM in the presence or absence of poly(I:C) (25µg/ml; (Carpenter et al., 2011; Scumpia et al., 2005) for incremental periods from 0-6h (fig. 3). Following 5-6 days *in vitro*, cultured neurons were incubated with cNBM in the presence or absence of poly(I:C) (25µg/ml) for 4h or IFNβ (0.5kU/ml; (Hadjilambreva et al., 2005; Sweeney et al., 2011) for 1-4h. Supernatants were harvested on ice and stored at -20°C for later analysis of IFNβ. Cells

were removed using cell scrapers and prepared for Western immunoblot or quantitative PCR analysis, as described below.

Western immunoblotting

Hippocampal slices not used for electrophysiological recording and cultured microglia, astrocytes and neurons, were stored at -80ºC in lysis buffer (100µl; composition in mM: Tris-HCl 10, NaCl 50, $Na_4P_2O_7.H_2O$ 10, NaF 50, 1% Igepal, phosphatase inhibitor cocktail I and II, protease inhibitor cocktail; Sigma-Aldrich, UK), for later assessment of protein expression (Costello *et al.*, 2011a; Costello *et al.*, 2011b). We assessed GLAST/excitatory amino acid transporter (EAAT)1 expression as a marker of astrocytic glutamate regulation and pSTAT1 and pIRF3 as indicators of signalling events associated with IFNβ production. We also assessed expression of the NMDA receptor subunit, NR2B phosphorylated at tyrosine 1472, because it has been suggested to correlate with migration of the receptor from the synaptic to the extrasynaptic location, a feature of the brain during epileptogenesis (Frasca et al., 2011). For analysis, samples were added to 2x or 4x sodium dodecyl sulphate (SDS) sample buffer (composition: Tris-HCl 100mM, pH 6.8, 4% SDS, 2% bromophenol blue, 20% glycerol; Sigma, UK) and heated to 70°C for 5 min. Equal quantities of protein samples (5, 10 or 20µg) were separated on 10% standard SDS gels. Proteins were transferred to nitrocellulose membrane (Schleicher and Schuell, Germany) and blocked for 1h in Tris-buffered-saline-0.05% Tween® 20 (TBS-T) and 5% non-fat dried milk/TBS-T at room temperature. Membranes were incubated overnight at 4ºC with anti-pNR2B (Tyr^{1472}; 1:1000; Sigma-Aldrich, UK), anti-NR2B (1:500; Santa Cruz Biotechnology, US), anti-GLAST/anti-EAAT1 (1:1000; Abcam, UK) and anti-

pSTAT1 (1:1000; Cell Signaling, US) antibodies in 2% non-fat dried milk/TBS-T, and anti-pIRF3 (1:1500; Cell Signaling, US) in 2% BSA/TBS-T, washed and incubated with a secondary anti-rabbit (1:5000; Jackson Immunoresearch, US) antibody in 2% non-fat dried milk/TBS-T for 1h. Immunoreactive bands were detected using Immobilon Western chemiluminescent substrate (Millipore, US) and blots were stripped (Re-blot Plus; Chemicon, US) and reprobed using anti-β-actin (1:10,000; Sigma, UK) in 2% non-fat dried milk/TBS-T and a peroxidase-conjugated secondary anti-mouse antibody (1:5000; Jackson Immunoresearch, US) in 2% non-fat dried milk/TBS-T. Images were captured using the Fujifilm LAS-4000 imager. To quantify expression of the proteins, densitometric analysis was carried out using ImageJ (http://rsb.info.nih.gov/). Values are presented as mean ± S.E.M., normalised to β-actin.

Real-time PCR

Total RNA was extracted from hippocampal tissue, cortical tissue and cultured microglia, astrocytes and neurons using a NucleoSpin® RNAII isolation kit (Macherey-Nagel Inc., Germany) and cDNA synthesis was performed on 250ng (cells) or 1.5µg (tissue) total RNA using a High Capacity cDNA RT kit (Applied Biosystems, Germany). Real-time PCR was performed as described previously (Lyons *et al.*, 2007) using an ABI Prism 7300 instrument (Applied Biosystems, Germany). The following primers were used TLR3: Mm00628112_m1, IFNα4: Mm00439544_m1, IFNβ1: Mm00439546_s1, IFNAR1: Mm00833443_s1 and Slcla3 (GLAST): Mm00600697_m1. Gene expression was calculated relative to the endogenous control samples (β-actin or 18S) to give a relative quantity (RQ) value (2^{-DDCt}, where CT is the threshold cycle).

Assessment of supernatant concentrations of IFNβ

IFNβ concentration from cultured cell supernatant was assessed by a custom enzyme-linked immunosorbent assay (ELISA), modified from a previously described protocol (Roberts *et al.*, 2007). Briefly, 96-well plates (Nunc, Denmark) were coated with rat anti-mouse IFNβ antibody (1:1000; 50µl/well; Santa Cruz Biotechnology, US) overnight at 4ºC, and blocked with PBS containing 10%FBS for 2h at room temperature. Supernatant samples and recombinant mouse IFNβ standard (0-0.5kU/ml; 40µl/well; PBL InterferonSource, US) were incubated overnight at 4ºC. Plates were washed (0.05% Tween20/PBS, 3 times) and incubated in rabbit anti-mouse IFNβ antibody (1:2000; 50µl/well; PBL InterferonSource, US) overnight at 4ºC. Plates were washed incubated in the presence of anti-rabbit horseradish peroxidase antibody (1:2000; 50µl/well; Jackson Immunoresearch, US) for 3h at room temperature. Substrate solution (50µl/well; 1:1 mixture of H_2O_2 and tetramethylbenzidine; Sigma-Aldrich, UK) was applied and incubated at room temperature in the dark for 20 min, and the reaction was stopped using 1M H_2SO_4 (50µl/well). Absorbance was read at 450nm using a BioTek Synergy HT microplate reader and data is presented as U/ml.

Statistical analysis

Data were assessed using two-tailed Student's t-tests for independent means (paired or unpaired as appropriate), one-way analysis of variance (ANOVA) followed by post-hoc Newman-Keuls test to determine significant differences between multiple groups, ANOVA by repeated measures followed by post-hoc Newman-Keuls test to assess

temporal changes between groups, or two-way ANOVA with post-hoc Bonferroni test to assess progressive changes between groups.

RESULTS

Systemic poly(I:C) administration induces spontaneous interictal-like activity and altered glutamatergic transmission

To activate TLR3, C57BL/6 mice were injected intraperitoneally with poly(I:C). We examined synaptic efficacy in these animals, by evoking short- and long-term plasticity of Schaffer-collateral synapses in the hippocampus *in vitro*. No alteration in the probability of neurotransmitter release, as assessed by paired-pulse facilitation (Zucker and Regehr, 2002), was identified in poly(I:C)-treated animals when compared with saline-treated animals (Figure 1a). Interestingly, unlike previous reports of TLR2 and TLR4 activation (Costello *et al.*, 2011a; Costello *et al.*, 2011b; Nolan *et al.*, 2005), TLR3 activation did not impair hippocampal LTP induced by TBS (Figure 1b), or indeed high-frequency tetanic stimulation (data not shown). However, while evoked synaptic activity was not altered, sustained spontaneous population activity was observed in the CA1 pyramidal cell layer of hippocampal slices prepared from poly(I:C)-treated animals. This interictal-like activity included prolonged sub-threshold synaptic activity persisting for 1s or more, bursts of population discharges, and synaptic activation reaching threshold for induction of population burst firing (Figure 1c,d). The frequency of these events was significantly higher in hippocampal slices from poly(I:C)-treated mice than the activity

recorded in saline-treated tissue (Figure 1c; F=14.70, **p<0.01, 2-way ANOVA). Additionally, in the absence of Mg^{2+}, 2-way ANOVA revealed a further enhancement in the event frequency in poly(I:C)-treated slices, and also when compared with slices from saline-treated animals under 0-Mg^{2+} conditions (Figure 1c; F=4.61, *p<0.01).

A role for altered glutamatergic neurotransmission, mediated by the NR2B subunit-containing forms of the N-Methyl-D-aspartate (NMDA) receptor, has been implicated in the mechanisms of inflammatory-induced epileptiform activity (Balosso *et al.*, 2008; Viviani *et al.*, 2003). A recent report indicated that a reduction in expression of NR2B, phosphorylated at tyrosine 1472 (pNR2B(Tyr^{1472})), was associated with receptor translocation from a post-synaptic to an extra-synaptic locus during epileptogenesis. In response to status epilepticus however, phosphorylation was increased (Frasca *et al.*, 2011). Here, we identified a significant reduction in pNR2B(Tyr^{1472})/NR2B in hippocampus of poly(I:C)-treated animals (Figure 1e; t=2.84, *p<0.05); whereas pNR2B(Tyr^{1472}) was significantly reduced, there was no change in total NR2B expression as illustrated in the sample immunoblot. The association between phosphorylation of NR2B and the spontaneous hyperexcitability reported in this study (Figure 1c,d) parallels the changes observed by Frasca and colleagues during epileptogenesis. Unlike synaptic NMDA receptors, those located extra-synaptically are not activated directly by synaptically-released glutamate, but by excess glutamate due to impaired re-uptake or indeed release by astrocytes (Jourdain *et al.*, 2007). In light of this evidence, and reports of altered astrocytic function in epilepsy (Aronica *et al.*, 2012; Jabs *et al.*, 2008), we assessed levels of the astrocyte-specific glutamate/aspartate transporter GLAST.

Although no alteration in GLAST mRNA was observed (data not shown), levels of protein expression were significantly reduced in tissue from poly(I:C)-treated animals, compared with hippocampus from saline-treated animals (Figure 1f; t=3.66, **p<0.01). This result suggests that dysregulation of glutamate homeostasis may be a consequence of TLR3 activation as previously suggested (Scumpia *et al.*, 2005), potentially enabling activation of extra-synaptic NMDA receptors. Taken together, these findings highlight two poly(I:C)-induced alterations in hippocampus which mimic cellular features of the epileptic brain.

Poly(I:C) induces IFNβ production and activation of IFNAR1

To investigate the cellular mechanisms underlying this interictal-like activity, we first confirmed the response to poly(I:C) in the hippocampus, by assessing transcription of inflammatory mediators in tissue from saline- and poly(I:C)-treated mice. While IFNα mRNA was unchanged (Figure 2a), a significant increase in transcription of IFNβ was identified in poly(I:C)-treated tissue (Figure 2b; t=2.52, *p<0.05, Student's t-test). Transcription of IFNAR1 was also enhanced (Figure 2c; t=2.50, *p<0.05, Student's t-test). Consistent with previous findings (Cunningham *et al.*, 2007; Field *et al.*, 2010), poly(I:C) increased transcription of TLR3 in hippocampus within 4h (Figure 2d; t=3.08, * p<0.05, Student's t-test). To address whether TLR3-mediated responses in the brain may be region-specific, cortical tissue from saline- and poly(I:C)-treated animals was also assessed. In a similar manner to the results obtained in hippocampal tissue, a significant increase in the expression of TLR3 mRNA (t=7.44, p<0.0001, Student's t-test) and IFNβ mRNA (t=2.51, p<0.05, Student's t-test), but not IFNα mRNA was observed in

cortical tissue from poly(I:C)-treated animals (n=7) relative to saline-treated controls (n=6; data not shown). Along with the findings in hippocampal and hypothalamic tissue reported previously (Field et al., 2010), these data illustrate that the production of IFNβ in response to poly(I:C) is unlikely to be restricted to certain brain regions.

To identify the cell types which respond to poly(I:C), we incubated cultured microglia, astrocytes and neurons in the presence or absence of poly(I:C) (25μg/ml; 4h) and show a significant increase in transcription of IFNβ in both microglia and astrocytes (Figure 3a; Interaction: $F=5.36$, $p<0.05$, 2-way ANOVA; $F=7.981$, $^{**}p<0.01$, $^{*}p<0.05$, 1-way ANOVA). Additionally, analysis of supernatant revealed significant release of IFNβ from poly(I:C)-treated microglia and astrocytes (Figure 3b; Interaction: $F=13.34$, $p<0.0001$, 2-way ANOVA; $F=19.34$, $^{***}p<0.001$, 1-way ANOVA). Interestingly, no alteration in the transcription or release of IFNβ was observed in neurons in response to poly(I:C) (Figure 3a,b). We confirmed the presence of the IFNβ receptor IFNAR1 in all cell types, with significantly higher levels in microglia relative to astrocytes and neurons (Figure 3c; $F=46.14$, $^{***}p<0.001$, 1-way ANOVA). Higher expression of TLR3 mRNA was also identified in microglia and astrocytes relative to neurons ($F=70.48$, $^{***}p<0.001$, Figure 3d). Due to the lack of effect of poly(I:C) on neurons, further analysis of its effects was undertaken in glia and the data revealed that it increased phosphorylation of IRF3 and STAT1 within 2h (Figure 3e).

The data obtained in hippocampus (Figure 2d) and cortex (described above) suggests that TLR3 activation auto-regulates its expression. We confirm this finding in glia, illustrating

a significant increase in TLR3 mRNA in both microglia (Figure 3f(i); t=7.19, **p<0.01, Student's t-test) and astrocytes (Figure 3f(ii); t=15.89, **p<0.01, Student's t-test) following 4h exposure to poly(I:C), consistent with the previous observation in human glial cells (Jack *et al.*, 2005).

IFNβ mimics the cellular and physiological effects of TLR3 activation

We considered that the effect of TLR3 activation on neuronal activity might be a consequence of release of IFNβ from glia. To investigate this possibility, cultured neurons were treated with recombinant mouse IFNβ (0.5kU/ml) for 1-4h. The data show that phosphorylation of STAT1 was significantly increased within 1h of IFNβ exposure (Figure 4a; F=5.91, *p<0.05, repeated measures ANOVA). IFNAR1 activation was associated with a significant and persistent reduction in pNR2B expression which was observed within 2h (Figure 4b; F=7.99, *p<0.05, repeated measures ANOVA).

To evaluate the effects of IFNβ on spontaneous synaptic activity, recordings were made from hippocampal slices prepared from C57BL/6 mice. Spontaneous activity was facilitated under 0-Mg^{2+} conditions (Figure 4c; F=282.0, **p<0.01, repeated measures ANOVA) and the frequency of sustained spontaneous events was further enhanced, when recording began 15 min following the addition of mouse recombinant IFNβ (1kU/ml; Figure 4c,d; F=282.0, ***p<0.001, repeated measures ANOVA). In the presence of Mg^{2+}, increased spontaneous activity was also observed, 2.5-3h following application of IFNβ (1kU/ml; data not shown).

Poly(I:C)-induced changes in neurotransmission are prevented in IFNAR1-deficient mice

While these findings confirm that poly(I:C) and recombinant IFNβ produce similar effects on neuronal excitability, they do not conclusively determine whether endogenous IFNβ mediates the effects of poly(I:C). To address this question, the effects of poly(I:C) were assessed on hippocampal slices prepared from IFNAR1-deficient mice ($IFNAR1^{-/-}$; 3-4 month-old), 4h following injection. The frequency of sustained spontaneous events was not altered between saline- and poly(I:C)-treated animals, either under control or 0-Mg^{2+} conditions (Figure 5a,b). Additionally, while IFNβ mRNA was significantly enhanced in hippocampus from poly(I:C)-treated $IFNAR1^{-/-}$ mice (Figure 5c; $t=2.73$, $*p<0.05$, Student's t-test), no reduction in the expression of pNR2B (Figure 5d) or GLAST (Figure 5e) was observed. To investigate the possibility that the lack of enhancement in spontaneous activity in poly(I:C)-treated $IFNAR1^{-/-}$ animals was age-associated, 8-9 month-old $IFNAR1^{-/-}$ mice were evaluated. No significant age-related change in the frequency of sustained spontaneous events was observed in hippocampal slices following treatment with poly(I:C) (Figure 5a,b). Removal of Mg^{2+} did not alter the response to poly(I:C) (data not shown). Taken together these finding illustrate that the poly(I:C)-induced changes in hippocampal network activity, and associated alteration in glutamate transmission require activation of IFNAR1.

In contrast with that seen in wildtype animals (Figure 2d, 3f), and despite the poly(I:C)-induced increase in IFNβ, no alteration in the transcription of TLR3 was observed in hippocampal tissue from poly(I:C)-treated IFNAR1-deficient mice (Figure 5f). In

addition to the evidence presented in the current study, auto-regulation of TLR3 has been widely reported in hippocampus (Cunningham *et al.*, 2007; Field *et al.*, 2010) and glial cells (Jack *et al.*, 2005; Scumpia *et al.*, 2005). This interesting finding proposes that the enhanced expression of TLR3, in response to poly(I:C), may be a consequence of the actions of IFNβ.

DISCUSSION

The evidence presented in the current study illustrates that systemic administration of a TLR3 agonist can modulate intrinsic neuronal network excitability in the brain. At a cellular level, we have identified several similarities between the changes induced by poly(I:C) treatment and changes that have been described in the epileptic brain. In particular, we describe spontaneous interictal-like activity, coupled with alterations in glutamatergic neurotransmission, which are known features of epileptogenesis (Frasca *et al.*, 2011).

Alterations in glutamate neurotransmision have long been implicated in the excitotoxicity associated with epilepsy (see review: (Casillas-Espinosa et al., 2012). Of particular interest has been conflicting information regarding expression of NMDA receptors containing the NR2B subunit; with reduced expression reported following kainate-induced seizure (Wyneken et al., 2003), in patients with temporal lobe epilepsy (Mathern et al., 1998) and neocortical epilepsy (Wyneken et al., 2003), but increased levels observed in hippocampus of non-sclerotic epileptic patients (Mathern et al., 1998). The

thorough investigation carried out by Frasca and colleagues, in order to investigate these discrepancies, has highlighted the migration of NR2B-containing receptors to the extrasynaptic space, facilitated by reduced phosphorylation at tyrosine 1472, as a pivotal stage of epileptogenesis (Frasca et al., 2011). This finding helps to clarify the previously conflicting reports by differentiating between receptor expression and localization during seizures and intervening periods. The evidence presented in the current study illustrates activity which is interictal in nature induced by poly(I:C) administration. Our further finding of reduced pNR2B(Tyr^{1472}) supports the previous report (Frasca et al., 2011), suggesting that migration of NR2B-containing receptors may underlie this epileptogenic phenotype.

The role of inflammatory mediators in the development of epilepsy and in seizure susceptibility has been widely reported. Of note are roles for proinflammatory cytokines including IL-1β (Balosso et al., 2008; Galic et al., 2009; Vezzani et al., 2011), TNFα (Riazi *et al.*, 2008) and the endogenous TLR2 and 4 agonist, high mobility group box (HMGB)1 (Maroso et al., 2010; Vezzani et al., 2011). Interestingly, direct application of poly(I:C) to the brain of neonatal rats has been reported to increase the susceptibility to chemically-induced seizures, and is associated with the increased production of IL-1β (Galic et al., 2009). Here, we report elevated IFNβ in the brain following TLR3 activation and provide evidence that glial cells are the primary source of its production. Additionally, we demonstrate that both poly(I:C) and IFNβ can potentially modulate neuro-glial communication by impairing the maintenance of glutamate homeostasis mechanisms by astrocytes and regulating neuronal receptor activation. While previous

studies have reported the effects of exogenous IFNβ on the modulation of intrinsic neuronal excitability (Beyer *et al.*, 2009; Hadjilambreva *et al.*, 2005), to our knowledge the current study is the first to highlight a causal link between endogenously produced IFNβ and the development of network dysfunction. Although an array of proinflammatory cytokines are likely to be produced in the brain in response to poly(I:C) (Cunningham et al., 2007; Field et al., 2010; Galic et al., 2009), the evidence we present from IFNAR1-deficient animals indicates that activation of the type I IFN receptor is essential for the observed modulation of excitability.

The current manuscript does not address the question of whether the effects of systemically-administered poly(I:C) on hippocampal activity are mediated by direct TLR3 stimulation in the CNS, or indirectly via a peripheral inflammatory response. It has been shown that poly(I:C) can compromise the integrity of the blood brain barrier (Wang et al., 2004) which offers the possibility of a direct route through which poly(I:C) might access the brain. Another possibility is that poly(I:C) induces cytokine release from peripheral cells which ultimately enter the CNS. However our present data, and data from others (Field et al., 2010), indicate that systemically-administered poly(I:C) increases IFNβ, which is a primary product of the TLR3-specific response, in brain tissue and this suggests that direct stimulation of TLR3 is likely.

A large body of evidence, including our own, describes the detrimental effects of exogenously-applied proinflammatory cytokines such as IL-1β (O'Connor and Coogan, 1999), TNFα (Costello *et al.*, 2011a) and HMGB1 (Costello *et al.*, 2011b) on

hippocampal synaptic plasticity. In light of the previous evidence of increased proinflammatory cytokine mRNA in hippocampus following TLR3 activation (Cunningham *et al.*, 2007; Field *et al.*, 2010), along with the known role of TLR3 as a memory constraint (Okun et al., 2010), it was somewhat surprising that administration of poly(I:C) did not impair LTP. It is possible that while cytokine transcription is increased following peripheral poly(I:C) administration, translation and subsequent release of cytokines occurs later, or in insufficient concentrations to impair LTP, or indeed requires an additional stimulus to trigger endogenous release. An alternative explanation rests with preparation used. Recording from *in vitro* hippocampal slices requires a period of incubation to allow for recovery following preparation. It is possible that washout or degradation of soluble endogenous inflammatory mediators occurs prior to recording. Indeed analysis of cytokines in hippocampal slices prepared from poly(I:C)-treated mice following several hours of incubation revealed no alterations in expression of proinflammatory cytokines including IL-1β, TNFα and HMGB1 (data not shown). This evidence highlights the robust nature of the IFNβ-mediated response, and further supports the hypothesis of IFNβ as the primary mediator of the TLR3-associated changes in hippocampal excitability.

Initial evidence suggested that, unlike some other members of the TLR family, activation of TLR3 did not induce its own transcription, at least in microglia (Olson and Miller, 2004). However, subsequent reports have identified increased TLR3 in response to poly(I:C) in microglia and astrocytes (Jack *et al.*, 2005; Scumpia *et al.*, 2005) as well as brain tissue from poly(I:C)-treated mice (Field *et al.*, 2010). Our data support these

findings, illustrating increased TLR3 transcription in hippocampal tissue and glial cells obtained from poly(I:C)-treated mice. An interesting additional finding of the current study is that TLR3 mRNA is not altered in hippocampus of IFNAR1$^{-/-}$ mice in response to poly(I:C), indicating that IFNAR1 is necessary to facilitate the poly(I:C)-mediated effect. This suggests that auto-regulation of TLR3 may not be a direct result of engagement by double-stranded RNA, but a consequence of INFβ production. As up-regulating TLR3 expression is likely to augment the TLR3-mediated immune response, it is tempting to speculate that this may play a role in the seizure susceptibility and development of epilepsy experienced by survivors of viral-encephalitis (Getts *et al.*, 2008). Taken together, the evidence presented here identifies IFNβ and IFNAR1 as potential targets for therapeutic intervention against certain cases of viral-associated epileptogenesis.

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Conflict of Interest

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1

Systemic application of poly(I:C) does not alter evoked synaptic function, but modulates spontaneous excitability and expression of proteins responsible for homeostasis of

glutamatergic neurotransmission in hippocampus, 4h following treatment. (a) Paired-pulse facilitation at CA1 synapses was not altered in poly(I:C)-treated (12mg/kg, i.p.) C57BL/6 mice (n=5 slices, from 5 mice) compared with saline-treated controls (n=5 slices, from 4 mice). (b) TBS induced a consistent LTP at CA1 synapses of hippocampal slices from saline-treated animals, which persisted for at least 60 min following induction (n=5 slices, from 4 mice). The LTP recorded in slices from poly(I:C)-treated animals under the same recording conditions, when measured 60 min following induction, showed no significant alteration (n=5 slices, from 5 mice) compared with saline-treated controls. Inset displays sample EPSP traces taken from a single experiment immediately prior to TBS, and 60 min following LTP induction (average of 4 consecutive traces). (c) Extracellular recordings in the CA1 pyramidal cell layer of hippocampal slices from poly(I:C)-treated mice displayed sustained (≥1s) spontaneous population activity, both sub-threshold and above the threshold for induction of burst population discharges. The frequency of the event occurrence was significantly higher in slices from poly(I:C)-treated mice (n=6 slices, from 6 mice), compared with saline-treated animals (n=6 slices, from 6 animals; **$p<0.01$). The frequency of spontaneous events was increased in both cases under Mg^{2+}-free (0-Mg^{2+}) conditions (*$p<0.05$), yet the significant difference between the activity in slices from poly(I:C)-treated mice (n=5 slices, from 5 mice) compared with slices from saline-treated mice (n=6 slices, from 6 mice) was maintained (**$p<0.01$). (d) Sample traces (1s duration) illustrating examples of the spontaneous activity recorded in slices from saline- and poly(I:C)-treated C57BL/6 mice under control and 0-Mg^{2+} conditions. (e) Treatment with poly(I:C) (12mg/kg, 4h) was associated with a significant reduction in the phosphorylation of NR2B in hippocampus when compared

with tissue from saline-treated mice (n=6; *p<0.05). (f) Expression of the GLAST was significantly reduced in hippocampus, 4h following poly(I:C) administration, compared with control tissue (n=5-6; **p<0.01). (e, f) Protein expression was determined by Western immunoblot, and values were normalised to total NR2B (e) and β-actin (f). Insets illustrate representative blots of pNR2B, NR2B and GLAST, along with respective β-actin blots.

Figure 2

Poly(I:C) increases IFNβ, IFNAR1 and TLR3 expression in hippocampus, 4h following administration. Hippocampal tissue from poly(I:C)-treated (12mg/kg, i.p.) C57BL/6 mice showed no alteration in (a) IFNα mRNA expression, but a significant increase in (b) IFNβ mRNA (n=5) compared with tissue from saline-treated animals (n=5; *p<0.05). Significant increases in (c) IFNAR1 mRNA and (d) TLR3 mRNA were also observed in response to poly(I:C) treatment, compared to saline-treated hippocampus (n=5-6, *p<0.05).

Figure3

Poly(I:C) induces production of IFNβ and IFNAR1 activation, and increases transcription of TLR3 in microglia and astrocytes. Treatment with poly(I:C) (25μg/ml, 4h) induced a significant increase in (a) mRNA expression and (b) supernatant concentrations of IFNβ from primary microglia and astrocytes cultured from C57BL/6 mice (n=3-4; ***p<0.001, **p<0.01, *p<0.05) compared with respective control values. However, no significant alteration in either (a) IFNβ mRNA or (b) IFNβ release was identified from poly(I:C)-

treated neurons. (c) Expression IFNAR1 mRNA was detected in isolated microglia, astrocytes and neurons. However, significantly higher levels of IFNAR1 mRNA were detected in microglia relative to values obtained from either astrocytes or neurons (n=3-4; ***p<0.001). (d) PCR analysis revealed that cultured microglia and astrocytes express similar levels of TLR3 mRNA, which in both cases were significantly greater than values obtained from neuronal cultures (n=3-4; ***p<0.001). (e) In light of the substantial expression of TLR3 (c), isolated microglia and astrocytes were treated with poly(I:C) (25μg/ml) for 0-6h. In both cell types, increased expression of pIRF3 was identified within 2h of treatment, suggesting that production of IFNβ was stimulated. Increased levels of pSTAT1 were identified in microglia and astrocytes, with highest levels apparent after 4h exposure to poly(I:C), indicative of maximal IFNAR1 activation during that period. Protein expression was determined by Western immunoblot. Insets illustrate expression of pIRF3 and pSTAT1, along with respective β-actin blots. (f) Treatment with poly(I:C) for 4h significantly increased expression of TLR3 mRNA in both (i) isolated microglia and (ii) isolated astrocytes, compared with respective control values (n=3-4; **p<0.01).

Figure 4

IFNβ reduces pNR2B expression in neurons following IFNAR1 activation, and induces spontaneous interictal-like activity in hippocampus. Primary cultured neurons, prepared from C57BL/6 mice, were treated with mouse recombinant IFNβ (0.5kU/ml) for 1, 2 and 4h. (a) Expression of pSTAT1 was significantly increased following 1h of exposure to IFNβ, indicative of IFNAR1 activation (n=3; *p<0.05). (b) Subsequent to the increase in

pSTAT1, levels of pNR2B expression were significantly reduced, reaching significance 2h following IFNβ treatment (n=3; *p<0.05), compared to untreated cells. Protein expression was determined by Western immunoblot, and values were normalised to β-actin. Insets illustrate representative blots of pSTAT1 and pNR2B, along with respective β-actin blots. (c) Spontaneous activity was recorded extracellularly from CA1 pyramidal cells of hippocampal slices, prepared for C57BL/6 mice. The frequency of sustained (≥1s) spontaneous population activity was significantly increased under Mg^{2+}-free conditions, compared to control levels, and further facilitated when recorded 15 min following application of recombinant IFNβ (1kU/ml) treatment (n=3 slices, from 3 mice; ***p<0.001, **p<0.01). (d) Sample traces (1s duration) illustrating examples of the sustained spontaneous population events recorded in slices under control conditions, in the absence of Mg^{2+}, and following further application of IFNβ.

Figure 5

Systemic poly(I:C) treatment does not alter excitability and glutamate neurotransmission, or increase TLR3 expression in hippocampus of IFNAR1-deficient mice, 4h following administration. (a) 2-way ANOVA revealed no significant alteration in the frequency of sustained spontaneous events recorded in poly(I:C)-treated (12mg./kg, i.p.) 3-4 month-old $IFNAR1^{-/-}$ mice (n=6, from 3 mice) or 8-9 month-old (n=8 slices, from 4 mice) relative to values obtained in slices from saline-treated $IFNAR1^{-/-}$ animals (3-4 month-old: n=6, from 3 mice; 8-9 month-old: n=7 slices, from 8 mice). (b) Sample traces (1s duration) illustrating examples of the population recordings in slices from saline- and poly(I:C)-treated $IFNAR1^{-/-}$ mice. (c) Expression of IFNβ mRNA was significantly

increased in hippocampal tissue from IFNAR1$^{-/-}$ mice 4h following poly(I:C) treatment (3-4 month-old; n=4), compared with values obtained from saline-treated IFNAR1$^{-/-}$ animals (n=6; *$p<0.05$). No significant differences in the expression of either (d) pNR2B (n=6) or (e) GLAST (n=6) were identified in hippocampus between saline-treated and poly(I:C)-treated IFNAR1$^{-/-}$ mice (3-4 month-old). Protein expression was determined by Western immunoblot, and values were normalised to β-actin. Insets illustrate representative expression of pNR2B and GLAST, along with respective β-actin blots. (f) Poly(I:C) did not induce a change in the expression of TLR3 mRNA in hippocampus of IFNAR1$^{-/-}$ mice (3-4 month-old; n=4), compared with values obtained in tissue from saline-treated IFNAR1$^{-/-}$ animals (n=6).

Figure 1

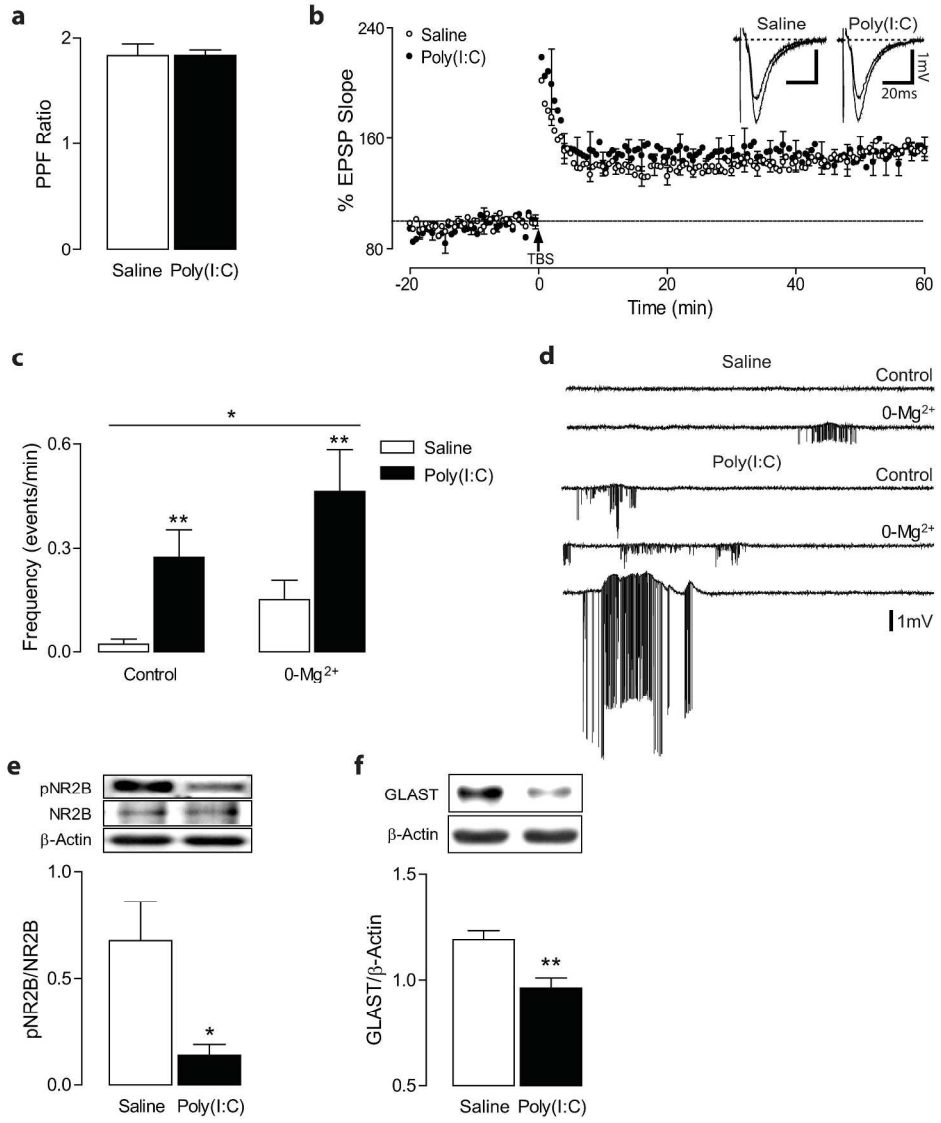

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Figure 2

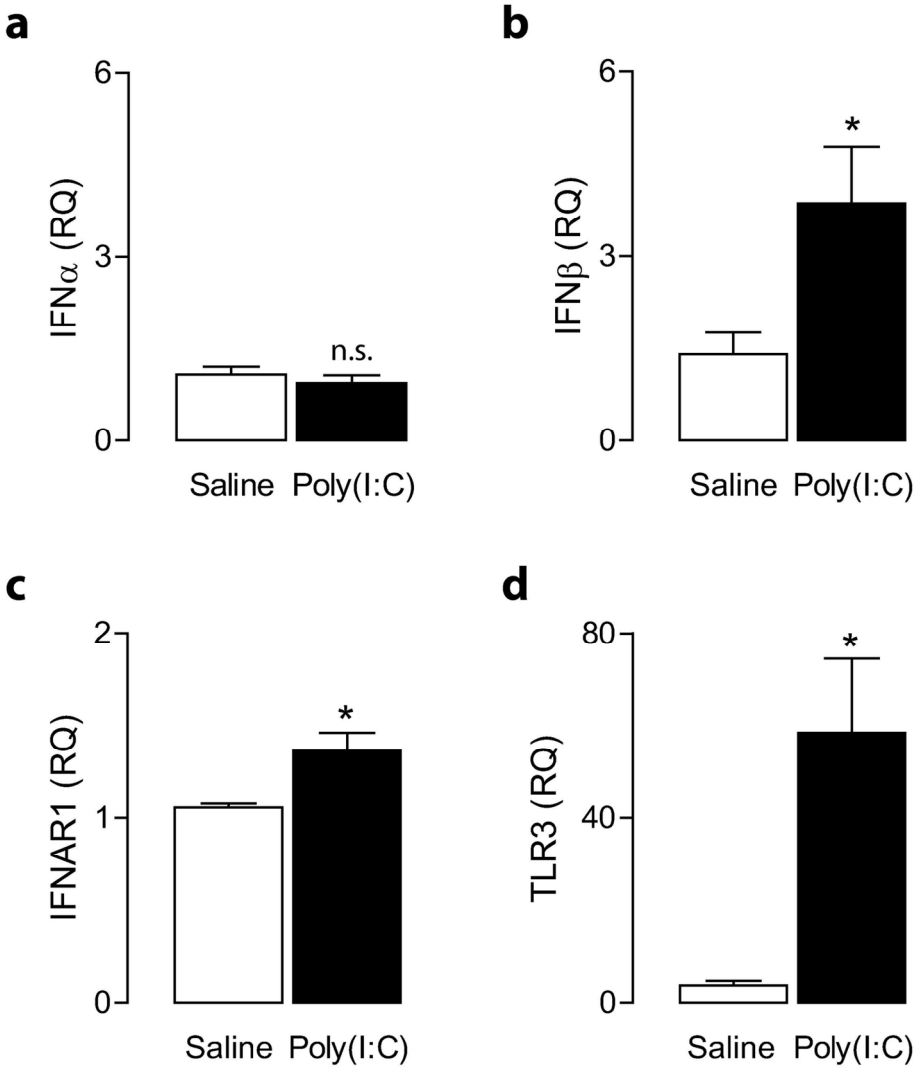

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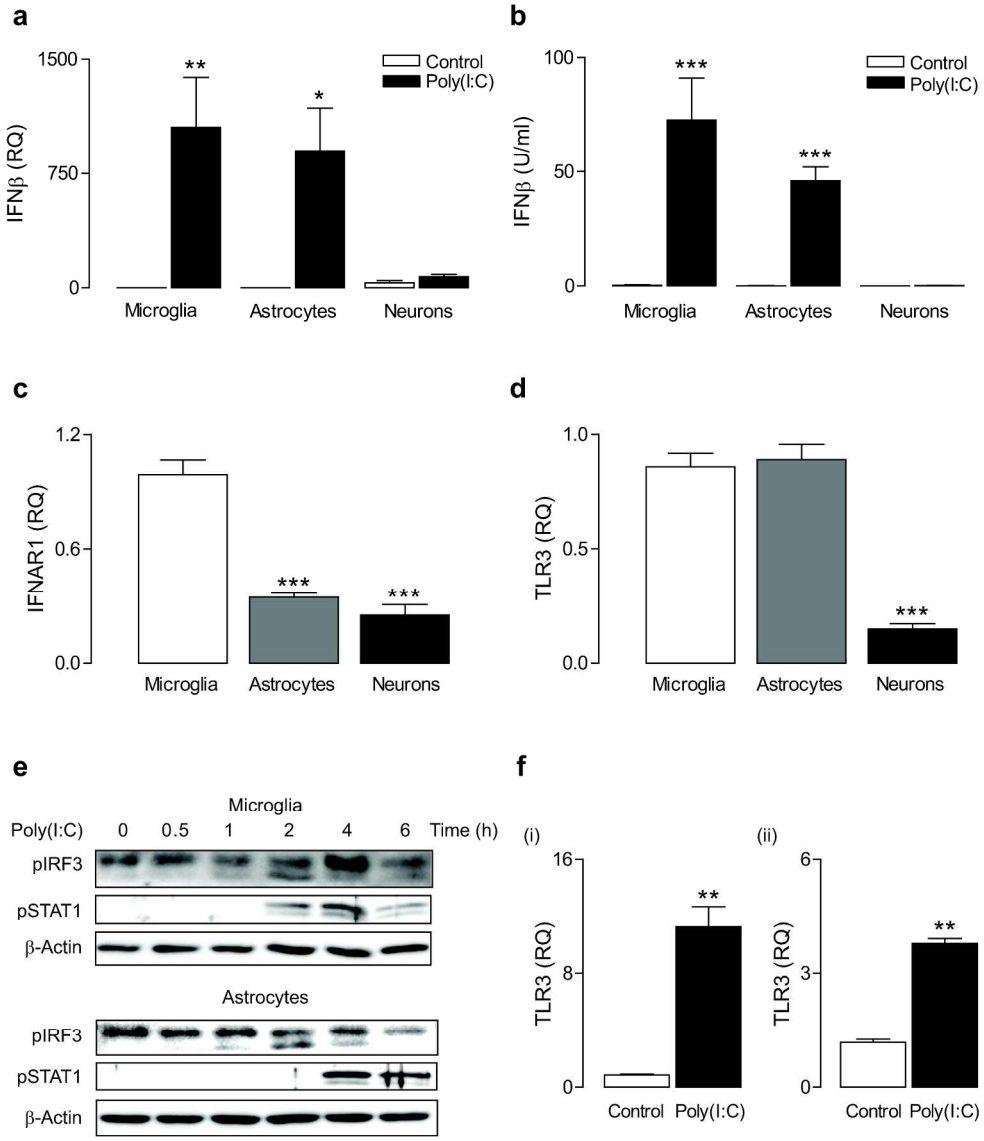
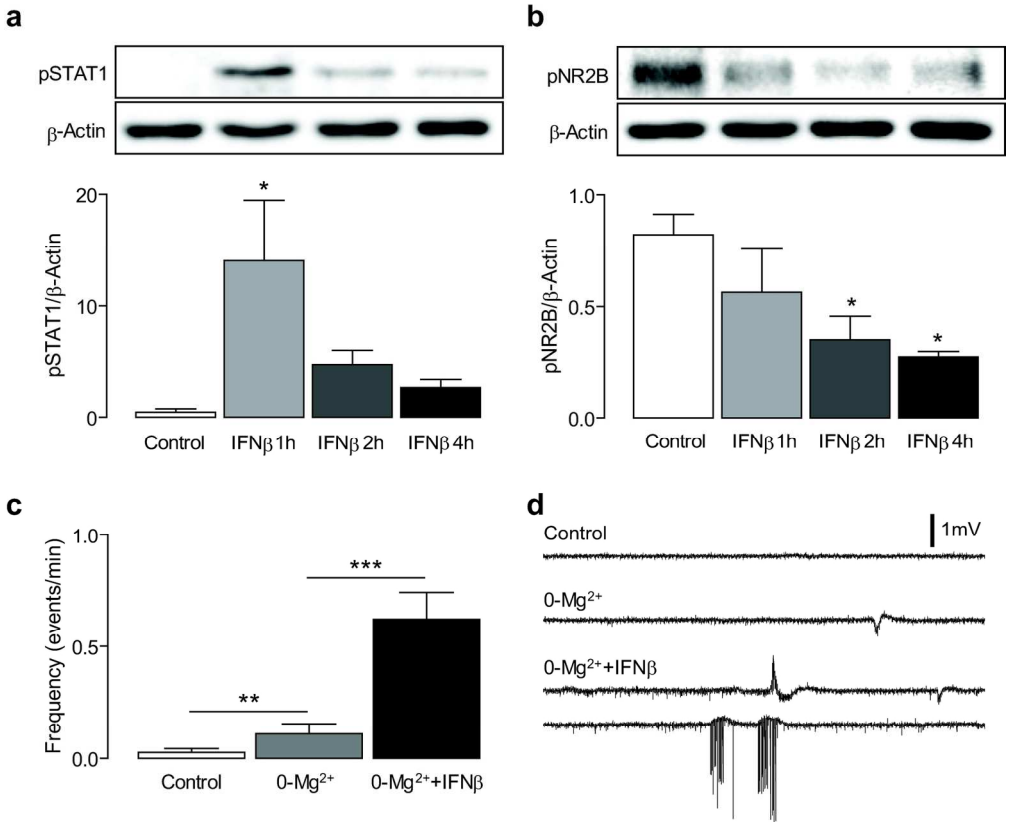
Figure 3



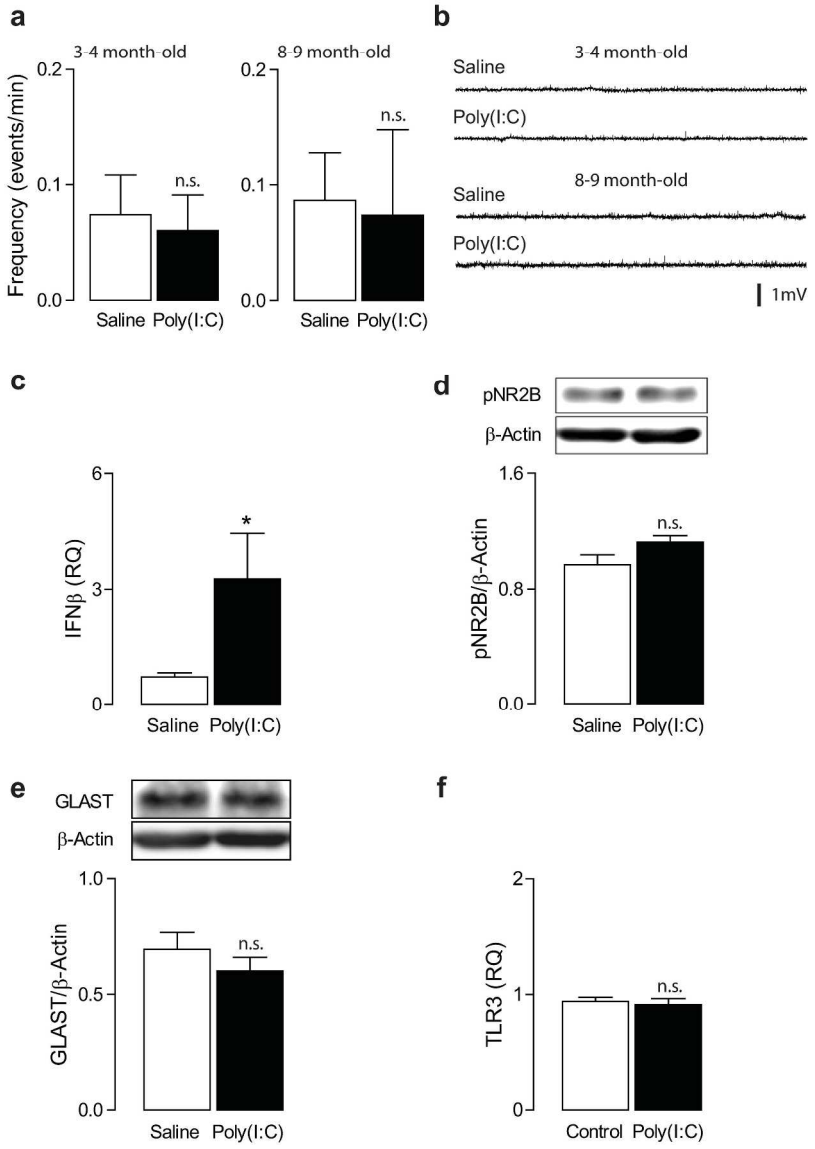
240x296mm (300 x 300 DPI)

Figure 4



162x139mm (300 x 300 DPI)

Figure 5



236x318mm (300 x 300 DPI)