



Kynurenic Acid Protects Against Reactive Glial-associated Reductions in the Complexity of Primary Cortical Neurons

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Abstract

Brain glia produce neuroactive metabolites via tryptophan-kynurenine catabolism. A role for kynurenine pathway (KP) metabolites is proposed in reactive glial associated neurodegeneration. The aim of this investigation was to assess the role of KP induction and KP metabolites in driving reactive glial associated neuronal atrophy. Rat primary mixed glia, and enriched microglial and astroglial cultures were stimulated with IFN γ (10 ng/ml) for 24 hours. KP induction in mixed glial cells was confirmed by raised expression of the rate limiting KP enzyme indoleamine 2,3 dioxygenase (IDO) and raised concentrations of KP metabolites kynurenic acid (KYNA) and quinolinic acid (QUIN) in the conditioned media. Conditioned media was transferred onto immature (3 days) and mature (21 days) primary cortical neurons *in vitro* for 24 hours. IFN γ -stimulated mixed glial conditioned media reduced neurite outgrowth and complexity of both immature and mature neurons and co-localised expression of synaptic markers determined by immunocytochemistry. Pre-treatment of mixed glial cells with the IDO inhibitor, 1-methyltryptophan (1-MT) (L) prevented these effects of IFN γ -stimulated mixed glial conditioned media. KYNA increased complexity and synapse formation in mature cortical neurons and protected against reduced neuronal complexity and co-localised expression of synaptic markers induced by conditioned media from IFN γ -stimulated mixed glia and by treatment of neuronal cells with QUIN (1 μ M). Overall, this study supports a role for the KP in driving neuronal atrophy associated with reactive glia and indicates that inhibition of the KP in glia, or raising the concentration of the astrocytic metabolite KYNA, protects against reactive microglial and QUIN-associated neuronal atrophy.

Keywords Kynurenine pathway · Kynurenic acid · Glia · Primary cortical neurons

Introduction

Metabolism of the essential amino acid tryptophan routed through the kynurenine pathway (KP) yields multiple metabolites that influence neuronal viability, outgrowth, complexity and synapse formation *in vitro* (O'Farrell et al. 2017). KP metabolites are known to be pharmacodynamically active at the N-methyl-D-aspartate (NMDA) and metabotropic glutamate receptors, the aryl hydrocarbon receptor and specific G protein coupled receptors. Alterations in KP metabolite

concentrations have been reported in several neurological disorders that exhibit an underlying neuro-inflammatory phenotype, and in a variety of neuropsychiatric and neurodegenerative disorders [For review O'Farrell and Harkin (2017)].

The initial conversion of tryptophan to kynurenine requires induction of either of the rate limiting enzymes, tryptophan 2, 3-dioxygenase (TDO), or indoleamine 2, 3-dioxygenase (IDO). IDO is ubiquitous throughout the body and is known to be activated by inflammatory cytokines such as interferon gamma (IFN γ) whereas TDO expression is primarily restricted to the liver with limited expression in the brain, with this expression restricted to astrocytes. Kynurenine is converted to kynurenic acid (KYNA) by the kynurenine aminotransferase (KAT) enzymes, with KAT II primarily responsible for the production of KYNA in the brain due to the inability of KYNA to cross the blood brain barrier. It is expressed in both microglia and astrocytes. In microglia, kynurenine may be converted to the free radical producing metabolites, 3-hydroxykynurenine (3-HK) by the flavin adenine

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dinucleotide-dependent monooxygenase enzyme kynurenine monooxygenase (KMO), or to anthranilic acid by kynureninase (KYNU). 3-HK is further metabolised to 3-hydroxyanthranilic acid (3-HAA) by KYNU, or to xanthurenic acid by KAT. 3-HAA undergoes further conversion to the unstable intermediate, 2-amino-3-carboxymuconate by 3-hydroxyanthranilic acid 3, 4-dioxygenase (3-HAO). 3-HAO is enzymatically converted to picolinic acid, or non-enzymatically transformed to the NMDA receptor agonist and excitotoxin, quinolinic acid (QUIN).

Previously we have reported that conditioned media drawn from IFN γ -stimulated BV-2 microglial cells reduce neurite outgrowth and complexity with reductions in various parameters of neurite outgrowth. These reductions were subsequently prevented when BV-2 microglia were pre-treated with either the IDO inhibitor, 1-methyltryptophan (1-MT) (L), the KMO inhibitor, Ro 61-8048, the synthetic glucocorticoid dexamethasone, which suppresses IFN γ -induced IDO, and the N-methyl-D-aspartate (NMDA) receptor antagonist, MK801. These findings indicate that inhibition of the KP in microglia may be targeted to protect against reactive microglial-associated neuronal atrophy (O'Farrell et al. 2017).

In the current investigation, we aimed to extend these findings by assessing the role of KP induction and kynurenine metabolites in driving reactive glial-associated neuronal atrophy. First, the response of glial cells of primary origin to IFN γ was assessed. Second, primary cortical neuronal cells were assessed at both early, as reported by O'Farrell et al. (2017), and later stages of development, on account of differences in sensitivity of immature and mature neuronal cells. Finally, the effect of direct application of KP metabolites to rat mature primary neuronal cells was assessed to show the counteraction between glial derived metabolites KYNA, a reported NMDA-receptor antagonist, and QUIN, an NMDA receptor agonist, in mediating reactive glial associated neuronal atrophy.

Materials and Methods

Reagents

1-methyltryptophan (1-MT) and kynurenic acid were sourced from Sigma Aldrich UK. Recombinant rat interferon gamma (IFN γ) was sourced from Biologend UK. Gene expression assays for IDO, TDO, KMO, KYNU, KATII as well as β -actin and Taqman master mix were obtained from Applied Biosystems. Cell culture reagents were obtained from Invitrogen Ireland. Anti- β III tubulin [mouse; G7121] was sourced from Promega. Anti-MAP2 was obtained from Sigma Aldrich, UK. Alexa Fluor 546 goat anti-mouse secondary antibody was obtained from Biosciences. Anti-synaptophysin, anti-PSD-95, Alexa Fluor 546 goat anti-

rabbit and Alexa Fluor 488 [goat anti-mouse; A11001] secondary antibodies were obtained from Thermo Fisher Scientific. Vectashield mounting medium with DAPI was obtained from Vector laboratories UK.

Preparation of Mixed Glial Cultures

Cultures of primary cortical mixed glia were prepared from postnatal day 2–3 neonatal Wistar rat pups obtained from the Comparative Medicine Unit, Trinity College Dublin. Mixed glial cultures prepared in this way consist of approximately 70% astrocytes and 30% microglia (Day et al. 2014). Pups were decapitated and the brain was removed from the skull. The surrounding meninges and obvious blood vessels were removed. Cortical tissue from both hemispheres was removed from the rest of the brain and placed in a drop of pre-warmed Dulbecco's modified Eagle's medium (DMEM) F12 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin and 0.1% (v/v) fungizone (referred to as complete DMEM (cDMEM)). Cortical tissue was finely chopped and then placed in 6 mL pre-warmed cDMEM and placed in the incubator (37 °C in a humidified, 5% CO₂) for 20 minutes. The tissue was then triturated until homogenous, passed through a sterile cell strainer (40 μ m) into a new 50 mL falcon tube, and centrifuged (RT; 2,000 rpm; 3 minutes). The supernatant was removed and the pellet was re-suspended in cDMEM. Cells were then plated in a 24-well plate and allowed to seed. The day of plating was referred to as DIV 0. Cortical cultures were maintained at 37 °C, 5% CO₂ and media was replaced with fresh pre-warmed cDMEM every 4–6 days.

Preparation of Primary Cortical Neuronal Cultures

Cultures of primary cortical neurons were prepared in the same way as mixed glia from postnatal day 1 Wistar rats obtained from the Comparative Medicine Unit, Trinity College Dublin. Following dissection, the tissue was transferred to 0.25% trypsin–EDTA and incubated at 37 °C for 2 minutes. An equivalent volume of cDMEM was added to deactivate trypsin followed by trituration and then centrifugation (RT; 2,000 rpm; 3 minutes). The supernatant was discarded and the pellet was re-suspended in cDMEM followed by trituration, passage through a sterile mesh filter (40 μ m) and centrifugation (RT; 2,000 rpm; 3 minutes). The supernatant was then discarded and the cell pellet was re-suspended in pre-warmed NBM supplemented with 1% B27, 1% Glutamax, 1% Penicillin–Streptomycin and 0.1% Fungizone. The resulting neuronal cell suspension was counted using the trypan blue exclusion method. Following counting, the cell suspension was diluted with cNBM to give a final concentration of 3×10^5 cells/mL and plated onto 24-well plates on 13-mm poly-d-lysine (50 μ g/mL)-coated glass coverslips.

Cortical cultures were maintained at 37 °C, 5% CO₂ and media was replaced with fresh pre-warmed cNBM every 4–6 days. Immature primary cortical neurons (DIV 3) were treated for 24 hours before fixation for Sholl analysis. Mature primary cortical neurons (DIV 18–21) were treated for 24 hours before fixation for Sholl analysis or synaptogenesis.

Viability Assay

Immature (DIV 3) and mature (DIV 21) primary neurons were treated directly with IFN γ (10 ng/mL) for 24 hours. Neurons were also treated with conditioned media from mixed glia that had been treated with IFN γ for 24 hours. Cell viability was assessed using the Alamar blue test - a widely used fluorometric assay which is based on the ability of viable cells to convert resazurin to highly fluorescent resorufin via mitochondrial metabolic enzymes.

Cell Culture Treatments

Following preparation, stock solutions were filter-sterilised using a 0.2 μ m syringe filter. Aside from QUIN, which was dissolved in dimethyl sulfoxide, and recombinant IFN γ , which was prepared in 0.1% bovine serum albumin, all stock solutions were dissolved in cNBM. QUIN was prepared freshly before use.

IFN γ , is a potent inducer of IDO and was used as the stimulus for these experiments. The concentration of IFN γ was based on previous work undertaken in our laboratory demonstrating that IFN γ (10 ng/mL) has no effect on the viability of either primary cortical neurons (DIV 21) or primary cortical mixed glia (O'Farrell et al. 2017).

Mature primary cortical neurons were directly treated with KYNA (0.03, 0.1, 1, 10 μ M). Concentrations of kynurenic acid were chosen based on previous work investigating its effect on primary neurons and previous studies demonstrating that concentrations of KYNA up to 10 μ M do not affect the viability of either immature or mature neurons (Braidly et al. 2009). In cases where QUIN was applied, QUIN was applied to cultures alone or with KYNA simultaneously.

Reverse Transcription Polymerase Chain Reaction

RNA was extracted from primary cortical mixed glia using the Nucleospin® RNA II total RNA isolation kit (Machery Nagel). Following quantification using a Nanodrop™ 1000 spectrophotometer (Thermo Fisher Scientific, USA), RNA concentrations were equalised and reverse transcribed into cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR was performed using an ABI StepOne 7500 instrument. Gene Expression Assays containing primers and a Taqman probe were used to quantify each gene of interest. PCR was performed in PCR plates using a 10 μ l reaction volume (4 μ l of diluted cDNA, 1 μ l of Taqman

gene expression assay, and 5 μ l of Fast Taqman® Universal PCR master mix (Applied Biosystems). Fold change in gene expression from the control group was calculated using the $\Delta\Delta$ Ct method, and β -actin served as the endogenous control to normalise gene expression data.

Primary cortical mixed glia (DIV 14) were treated with IFN γ (10 ng/ml) for 6 hours. Cells were harvested for RNA extraction followed by quantitative PCR to measure mRNA expression of kynurenine pathway enzymes. Assay ID's for the genes examined are as follows: β -actin (4352341E), IDO1 (Mm00492586_m1), TDO2 (Rn00574499_m1) KMO (Mm00505511_m1), KYNU (Mm00551012_m1), and KAT II (Rn00567882_m1).

Collection of Conditioned Media

Primary cortical mixed glia (DIV 14) were treated simultaneously with a combination of control DMEM, IFN γ (10 ng/ml) and with the IDO inhibitor, 1-methyl tryptophan [1-MT (L)] (0.5 mM) for 24 hours. 1-MT is a competitive inhibitor of IDO and exists as either a dextro (D) or levo (L) enantiomer. In vitro enzyme and cellular assays have demonstrated that the L-enantiomer is a more potent inhibitor of IDO (Qian et al. 2009, Lob et al. 2008).

The resulting conditioned media was collected, filter sterilised and supplemented with 1% B27. Conditioned media was transferred onto immature (DIV 3) and mature (DIV 21) primary cortical neuronal cultures for 24 hours.

Analysis of Tryptophan and Kynurenine Pathway Metabolites in Conditioned Media by Liquid Chromatography-tandem Mass Spectrometry (LC/MS)

Conditioned media (200 μ l) was harvested for LC-MS/MS analysis at BEVITAL AS (Bergen, Norway; www.bevital.no). LC-MS/MS was used to quantify concentrations of TRP, KYN, KYNA and QUIN as previously described (Midttun et al. 2009; Midttun et al. 2013). Samples were deproteinized and the supernatant injected onto a stable-bond C8 column. The detector was a triple quadrupole mass spectrometer fitted with electrospray probe and operated in positive multiple reaction monitoring mode. Coefficients of variation (% CV) for the method are as follows: TRP = 6, KYN = 6–9, KYNA = 9–11, QUIN = 7–10. The lower limits of quantification of metabolites in plasma were as follows: TRP = 4 μ mol/L, KYN = 0.007 μ mol/L, KYNA = 0.4 nmol/L, QUIN = 1.6 nmol/L.

Staining of Immature Neurons for β -III Tubulin and Sholl Analysis

Following treatment, media was removed from each well and cells were washed once in PBS. Cells were fixed by adding 300 μ l ice cold methanol to each well and leaving the plates at

-20 °C for 20 minutes. Methanol was then removed and the coverslips were washed 3 times with PBS. After 3 × 5 minute washes, non-specific interactions were blocked by adding 300 µL blocking buffer (4% NGS in PBS) to each well and the plate was left on a rocker at room temperature for 2 hours. The blocking buffer was discarded and cells were washed 3 times in PBS. Primary antibody (200 µL) containing anti- β -III tubulin in PBS (1:1000) was added to each well and left overnight at 4 °C. The primary antibody was removed and the cells were washed 3 times in PBS. Secondary antibody containing Alexa Fluor 488 in PBS (1:2000) was added and left for 2 hours on a rocker at room temperature in a light-protected environment. Secondary antibody was removed and cells were washed 3 times (3 × 5 minute washes) with PBS. Glass coverslips were then removed and mounted onto glass slides. Coverslips were visualised at 20X magnification using an AxioImager Z1 epifluorescent microscope with a Zeiss AxioCam HR camera and AxioVision 4.8.2 software. Following image acquisition, the number of neuritic branches, relative neuritic length, number of primary neurites and Sholl profile were measured for each neuron by Sholl analysis (Sholl 1953) using a protocol adapted from Gutierrez and Davies (Gutierrez and Davies 2007).

Staining of Mature Neurons for MAP2 and Sholl Analysis

Fixation and MAP2 immunocytochemistry were performed to determine the effect of the conditioned media on complexity of mature neurons by Sholl analysis. Following treatment, media was removed from each well and the cells were fixed in 4% paraformaldehyde (RT; 20 minutes). Paraformaldehyde was removed and coverslips were washed 3 times with PBS. Non-specific interactions were blocked by adding 300 µL blocking buffer containing 2% NGS, and 2% BSA in PBS-T to each well (RT; 2 hours). The blocking buffer was discarded and primary antibody containing MAP2 in blocking buffer (1:1000) was added to each well and left overnight at 4 °C. The primary antibody was removed and cells were washed 3 times in PBS (3 × 5 minute washes). Secondary antibody containing Alexa Fluor 546 in PBS-T (1:500) was added (RT; 2 hours). Secondary antibody was removed and cells were washed 3 times with PBS (3 × 5 minute washes). Glass coverslips were then removed and mounted onto glass slides for imaging and Sholl analysis as described above.

Analysis of the Co-localisation of Synaptic Markers in Mature Neurons

Mature neurons were treated for 24 hours and fixed in ice-cold methanol as described above. Cells were permeabilised and blocked using a blocking buffer consisting of 50% NGS + 0.2% triton-X in PBS (RT; 30 minutes). The blocking buffer

was removed and cells were washed with PBS. Primary antibody containing the pre-synaptic marker anti-synaptophysin and the post-synaptic marker anti-PSD-95 diluted in PBS (1:500), was added to each well and left overnight at 4 °C. Secondary antibody containing Alexa Fluor 546 and Alexa Fluor 488 diluted in PBS (1:1000) was added to the wells (RT; 2 hours). Secondary antibody was removed and cells were washed 3 times with PBS (3 × 5 minutes washes). Glass coverslips were then removed and mounted onto glass slides as described above. Coverslips were visualised at 40X magnification using an AxioImager Z1 epifluorescent microscope with a Zeiss AxioCam HR camera and AxioVision 4.8.2 software. The optimum exposure intensity for both the synaptophysin and PSD-95 channels was determined using a control coverslip and was maintained throughout (O'Neill et al. 2016). Image analysis was adapted from Ippolito and Eroglu, using Image J (Eroglu and Barres 2010). The number of co-localised puncta were quantified using the Image J 'Puncta Analyser' plugin. A region of interest was selected which was one cell diameter radially around the soma of interest. The Puncta Analyser was used to adjust the thresholds of the red channel (synaptophysin) and green channel (PSD-95) so that the intensity corresponded to discrete individual puncta. Minimum puncta size was set at a value of 4 for each experiment.

Statistical Analysis

Data is expressed as group means with SEM and were analysed using unpaired Student's *t*-tests or analysis of variance (ANOVA). If any statistically significant change was found following one or two factor ANOVA, *post-hoc* comparisons were performed using a Bonferroni or Newman-Keuls test. Results are deemed significant when $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)

Results

mRNA Expression of KP Enzymes Following IFN γ Stimulation of Primary Cortical Mixed Glia

Stimulation of mixed glia with IFN γ lead to an increase in the mRNA expression of IDO ($T_{(10)} = 8.034$, $P < 0.001$) (Fig. 1a) and a reduction in the expression of KAT-II ($T_{(10)} = 3.131$, $P < 0.05$) (Fig. 1b). TDO, KMO, KYNU were unaffected.

Conditioned Media From IFN γ Treated Mixed Glial Reduces the Complexity of Immature Primary Cortical Neurons (DIV 3)

Conditioned media from IFN γ -stimulated primary mixed glia had no effect on neuronal viability. Direct treatment with IFN γ had no effect on neuronal viability (data not shown).

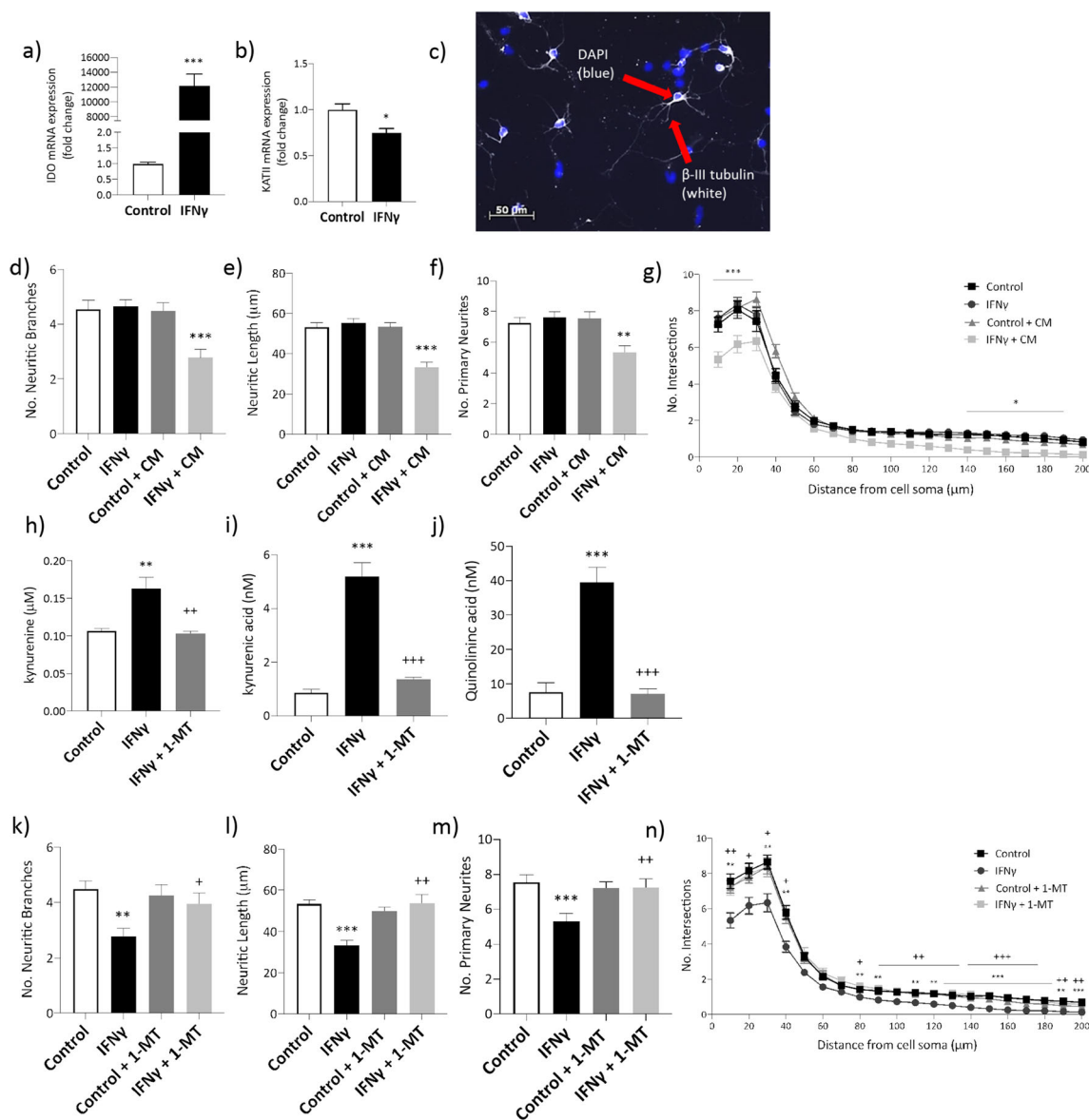


Fig. 1 Effect of IFN γ on KP induction in primary mixed glia and the effect of conditioned media on neurite outgrowth and complexity. Primary cortical mixed glia (DIV 14) were treated with IFN γ (10 ng/ml) for 6 hours. Cells were harvested for mRNA extraction followed by quantitative PCR analysis of the expression of IDO (a) and KAT II (b). Data expressed as mean \pm SEM (n = 5–6). * P < 0.05, *** P < 0.001 vs. control (Student’s t -test a, b). Primary cortical mixed glia (DIV 14) were treated with IFN γ (10 ng/mL) and/or 1-MT (L) (0.5 mM) for 24 hours or IFN γ (10 ng/ml) was placed on immature primary cortical neurons (DIV 3) for 24 hours. Conditioned media was harvested for analysis of the concentrations of kynurenine (h), KYNA (i) and QUIN (j). Data expressed as mean \pm SEM (n = 3). Conditioned media was collected and applied to immature primary cortical neurons (DIV 3)

for 24 hours before fixation and β III-tubulin immunocytochemistry (c-representative image 200x magnification; d–g). Sholl analysis was performed to determine the number of neuritic branches (d, k), the neuritic length (e, l), the number of primary neurites (f,m), and the Sholl profile (g, n). Data are expressed as mean \pm SEM (n = 17–24 coverslips from 3 independent experiments). * P < 0.05, ** P < 0.01, *** P < 0.001 vs. control (Student’s t -test) (a, b, d–f); ** P < 0.01, *** P < 0.001 vs. control CM. + P < 0.05, ++ P < 0.01, +++ P < 0.001 vs. IFN γ CM (Student Newman Keuls test) (h–j, k–m); ** P < 0.01, *** P < 0.001 vs. control CM. + P < 0.05, ++ P < 0.01, +++ P < 0.001 vs. IFN γ CM; two-way repeated measures ANOVA followed Newman Keuls post hoc test (g,n). CM: Conditioned media

Conditioned media from IFN γ -treated mixed glia reduced the number of neuritic branches ($T_{(33)} = 4.187$, $P = 0.0002$), neuritic length [$T_{(33)} = 6.423$, $P < 0.001$] and the number of primary neurites [$T_{(33)} = 3.734$, $P = 0.0007$] (Fig. 1d, e, f respectively). ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma showed an

effect of distance [$F_{(25, 825)} = 335.4$, $P < 0.0001$], IFN γ [$F_{(1, 825)} = 22.15$, $P < 0.001$], and an interaction between distance and IFN γ [$F_{(25, 825)} = 2.189$, $P = 0.0007$]. Post hoc analysis revealed reductions in the number of branches at 10, 20, 30, 40 μ m ($P < 0.001$) and 50 μ m ($P < 0.05$) from the cell soma compared to control (Fig. 1g).

Direct application of IFN γ to primary cortical neurons had no effect on the number of neuritic branches [$T_{(45)} = 0.274$, $P = 0.786$], neurite length [$T_{(45)} = 0.631$, $P = 0.531$] or the number of primary neurites [$T_{(45)} = 0.701$, $P = 0.487$] (Fig. 1d–f, respectively). ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma showed an effect of distance [$F_{(25, 1025)} = 351.5$, $P < 0.001$], no effect of IFN γ [$F_{(1, 1025)} = 0.201$, $P = 0.656$], or an interaction [$F_{(25, 1025)} = 0.384$, $P = 0.998$] (Fig. 1g).

1-MT (L) Attenuates Increased Kynurenine, KYNA and QUIN in Conditioned Media Following IFN γ Stimulation of Primary Cortical Mixed Glia

ANOVA of kynurenine [$F_{(2, 6)} = 14.62$, $P = 0.005$], KYNA [$F_{(2, 6)} = 62.71$, $P < 0.001$], and QUIN [$F_{(2, 6)} = 36.77$, $P < 0.001$] concentrations showed an effect of IFN γ +/- 1-MT. Stimulation of mixed glia with IFN γ led to an increase in kynurenine ($P < 0.05$), KYNA ($P < 0.001$) and QUIN ($P < 0.001$) concentrations when compared to control, which were attenuated by co-treatment with 1-MT (Fig. 1h, i, j, respectively). Tryptophan concentrations were unaffected.

Stimulation of mixed glial cultures with IFN γ provokes a shift in the balance/ratio of QUIN:KYNA from 8.9:1 to 7.5:1. Treatment with 1-MT produced a reduction in the QUIN:KYNA ratio from 7.5:1 to 5.2:1 indicating a shift in balance from QUIN to KYNA.

1-MT Attenuates IFN γ Treated Mixed Glial Conditioned Media-induced Reduction in the Complexity of Primary Cortical Neurons (DIV 3)

ANOVA of the number of neuritic branches showed an effect of conditioned media from IFN γ treated mixed glia [$F_{(1, 66)} = 8.644$, $P = 0.005$], no effect of 1-MT [$F_{(1, 66)} = 1.82$, $P = 0.182$], and an interaction between IFN γ and 1-MT [$F_{(1, 66)} = 4.22$, $P = 0.044$]. Post hoc comparisons revealed reductions in the number of neuritic branches following treatment with conditioned media from IFN γ treated mixed glia ($P < 0.01$) compared to control. Co-treatment with 1-MT attenuated reductions in numbers of branches induced by conditioned media from IFN γ treated mixed glia ($P < 0.05$) (Fig. 1k).

ANOVA of neurite length showed an effect of conditioned media from IFN γ treated mixed glia [$F_{(1, 66)} = 9.12$, $P = 0.003$], 1-MT [$F_{(1, 66)} = 9.909$, $P = 0.003$], and an interaction between IFN γ and 1-MT [$F_{(1, 66)} = 19.661$, $P = 0.004$]. Post hoc comparisons revealed reductions in neurite length following treatment with conditioned media from IFN γ treated mixed glia ($P < 0.001$) compared to control. Co-treatment with 1-MT attenuated reductions in neurite length induced by conditioned media from IFN γ treated mixed glia ($P < 0.001$) (Fig. 1l).

ANOVA of the number of primary neurites showed an effect of conditioned media from IFN γ treated mixed glia [$F_{(1, 66)} = 6.929$, $P = 0.011$], no effect of 1-MT [$F_{(1, 66)} = 3.629$, $P = 0.061$], and an interaction between IFN γ and 1-MT [$F_{(1, 66)} = 7.273$, $P = 0.009$]. Post hoc comparisons revealed reductions in the number of primary neurites following treatment with conditioned media from IFN γ treated mixed glia ($P < 0.01$) compared to control. Co-treatment with 1-MT attenuated reductions in the numbers of primary neurites induced by conditioned media from IFN γ treated mixed glia ($P < 0.01$) (Fig. 1m).

ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma showed an effect of distance [$F_{(25, 1625)} = 750.83$, $P < 0.001$], IFN γ [$F_{(1, 65)} = 15.74$, $P < 0.001$], IFN γ x distance [$F_{(25, 1625)} = 2.672$, $P < 0.001$] and a 1-MT x IFN γ interaction [$F_{(1, 65)} = 8.386$, $P = 0.005$]. Post hoc comparisons revealed reductions in the number of branches following treatment with conditioned media from IFN γ treated mixed glia at 10, 20, 30, 40, ($P < 0.001$) and 50 μm ($P < 0.05$) from the cell soma compared to control. Co-treatment with 1-MT attenuated reductions in the number of branches induced by conditioned media from IFN γ treated mixed glia at 10, 20, 30, 40 ($P < 0.001$) and 50 μm ($P < 0.01$) (Fig. 1n).

1-MT Attenuates IFN γ Treated Mixed Glial Conditioned Media-induced Reduction in the Complexity of Primary Cortical Neurons (DIV 21)

Conditioned media from IFN γ treated mixed glia had no effect on the viability of neurons (DIV 21). Direct treatment with IFN γ had no effect on the viability of neurons (DIV 21) (data not shown).

ANOVA of the number of neuritic branches showed an effect of conditioned media from IFN γ treated mixed glia [$F_{(1, 299)} = 16.02$, $P < 0.001$] and an interaction between IFN γ and 1-MT [$F_{(1, 299)} = 7.091$, $P = 0.0082$]. Post hoc comparisons revealed reductions in the number of neuritic branches following treatment with conditioned media from IFN γ treated mixed glia when compared to control ($P < 0.001$). 1-MT provided some protection against the IFN γ induced reduction in numbers of neuritic branches when compared to the IFN γ alone treated group (Fig. 2a).

ANOVA of neurite length showed an effect of conditioned media from IFN γ treated mixed glia [$F_{(1, 304)} = 11.28$, $P < 0.001$] and an interaction between IFN γ and 1-MT [$F_{(1, 304)} = 11.91$, $P < 0.001$]. Post hoc comparisons revealed reductions in neurite length following treatment with conditioned media from IFN γ treated mixed glia ($P < 0.001$) compared to control. Co-treatment with 1-MT attenuated IFN γ induced reduction in neuritic length when compared to IFN γ treatment alone ($P < 0.001$) (Fig. 2b). Three were no effects of

conditioned media from IFN γ treated mixed glia alone or in combination with 1-MT on the number of primary neurites (Fig. 2c).

ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma showed an effect of distance [$F_{(19, 5814)} = 498.1, P < 0.001$], an effect of treatment [$F_{(3, 306)} = 6.670, P < 0.001$] and an interaction between distance and treatment [$F_{(57, 5814)} = 2.870, P < 0.001$]. Post hoc comparisons revealed that conditioned media from IFN γ treated mixed glia induced a reduction in neuritic branching at 10–90 μm from the cell soma compared to conditioned media from untreated mixed glia. Co-treatment with 1-MT attenuated these reductions compared to IFN γ alone (Fig. 2d).

Conditioned Media From IFN γ Treated Mixed Glia Reduces Co-localisation of Synaptic Markers in Primary Cortical Neurons (DIV 21)

ANOVA of co-localised synaptic markers (% control) showed an effect of conditioned media from IFN γ treated mixed glia [$F_{(3, 179)} = 11.40, P < 0.001$]. Post hoc comparisons revealed reductions in co-localised synaptic puncta following treatment with conditioned media from IFN γ treated mixed glia ($P < 0.001$) compared to control. Co-treatment with 1-MT attenuated these reductions when compared to treatment with conditioned media from IFN γ treated mixed glia alone ($P < 0.001$) (Fig. 2g).

KYNA Increases the Complexity of Primary Cortical Neurons and Co-localisation of Synaptic Markers

ANOVA of the number of neuritic branches showed an effect of KYNA approaching significance [$F_{(3, 402)} = 2.480, P = 0.06$]. ANOVA of neurite length showed an effect of KYNA [$F_{(3, 403)} = 13.17, P < 0.001$]. Post hoc analysis revealed an increase in neurite length following treatment with KYNA (0.1 and 0.3 μM) compared to control ($P < 0.001$) (Fig. 3b). There was no effect of KYNA on the number of primary neurites. ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma showed an effect of distance [$F_{(19, 7695)} = 698.1, P < 0.001$], KYNA [$F_{(3, 405)} = 11.56, P < 0.001$] and a distance \times KYNA interaction [$F_{(57, 7695)} = 4.063, P < 0.001$]. Post-hoc comparisons revealed that KYNA increased neuritic branching at all distances between 40 and 140 μm from the cell soma compared to control (Fig. 3d).

ANOVA of co-localised synaptic markers (% control) showed an effect of KYNA [$F_{(3, 557)} = 7.531, P < 0.001$]. Post hoc comparisons revealed an increase in co-localised puncta following treatment with KYNA (0.03 and 0.1 μM) compared to control ($P < 0.001$) and following treatment with KYNA (0.3 μM) compared to control ($P < 0.05$) (Fig. 3e).

KYNA Attenuates QUIN-induced Reductions in the Complexity of Primary Cortical Neurons and Co-localisation of Synaptic Markers

QUIN reduced the number of neuritic branches [$T_{(132)} = 4.896, P < 0.001$] compared to control ($P < 0.001$; Student's *t* test). ANOVA showed an effect of KYNA on QUIN-induced reductions [$F_{(3, 236)} = 5.584, P = 0.001$]. Post hoc comparisons revealed that KYNA (0.3 μM) dose dependently attenuated QUIN related reduction in neuritic branches compared to treatment with QUIN alone ($P < 0.01$) (Fig. 4a). QUIN reduced neuritic length [$T_{(131)} = 6.885, P < 0.001$] compared to control ($P < 0.001$; Student's *t* test). ANOVA showed an effect of KYNA on QUIN-induced reductions [$F_{(3, 234)} = 14.78, P < 0.001$]. Post hoc comparisons revealed that KYNA (0.1 μM and 0.3 μM) dose dependently attenuated QUIN related reduction in neuritic length when compared to treatment with QUIN alone ($P < 0.01$; $P < 0.001$ respectively) (Fig. 4b). QUIN reduced the number of primary neurites [$T_{(133)} = 2.057, P = 0.04$] compared to control ($P < 0.001$; Student's *t* test). KYNA has no effect on QUIN induced reductions in the number of primary neurites (Fig. 4c).

ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma showed an effect of distance [$F_{(19, 5548)} = 515.3, P < 0.001$], an effect of treatment [$F_{(4, 292)} = 12.21, P < 0.001$] and an interaction between distance and treatment [$F_{(76, 5548)} = 1.670, P < 0.001$]. *Post hoc* analysis revealed a decrease in the Sholl profile at all distances between 40 and 150 μm following treatment with QUIN compared to control. *Post hoc* analysis also revealed KYNA (0.3 μM) attenuated the QUIN related decrease in the Sholl profile at all distances between 50 and 120 μm compared to treatment with QUIN alone (Fig. 4d).

QUIN reduced co-localised synaptic puncta ($T_{(155)} = 4.228, P < 0.001$) compared to control (Student's *t* test, $P < 0.01$). ANOVA of co-localised synaptic markers (% control) showed an effect of KYNA [$F_{(3, 365)} = 15.9, P < 0.001$]. Post hoc comparisons revealed that co-treatment with KYNA (0.3 μM) increased the number of co-localised puncta compared to treatment with QUIN alone ($P < 0.001$) (Fig. 4e).

Effect of KYNA on Reductions in the Complexity of Mature Primary Cortical Neurons Induced by Conditioned Media From IFN γ Treated Mixed Glia

Conditioned media from IFN γ treated mixed glia reduced the number of neuritic branches [$T_{(158)} = 3.907, P < 0.001$] compared to control (Student's *T* test). ANOVA of the numbers of neuritic branches showed an effect of KYNA on these IFN γ treated mixed glia conditioned media related reductions [$F_{(3, 284)} = 4.253, P = 0.006$]. Post hoc comparisons revealed an increase in the number of neuritic branches following co-

treatment with KYNA 0.3 μM compared to control ($P < 0.05$) (Fig. 5a).

Conditioned media from IFN γ treated mixed glia reduced neurite length [$T_{(156)} = 5.781$, $P < 0.001$] compared to control (Student's T test). ANOVA of neuritic length showed an effect of KYNA on these IFN γ treated mixed glia conditioned media related reductions [$F_{(3, 280)} = 7.920$, $P < 0.001$]. Post hoc comparisons revealed an increase in neuritic length following co-treatment with KYNA 0.3 μM compared to control ($P < 0.001$) (Fig. 5b). There was no effect of IFN γ treated mixed glia conditioned media or KYNA on the number of primary neurites (Fig. 5c).

ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma showed an effect of distance [$F_{(19, 6878)} = 482.4$, $P < 0.001$], an effect of treatment [$F_{(4, 362)} = 10.17$, $P < 0.001$] and an interaction between distance and treatment [$F_{(76, 6878)} = 3.340$, $P < 0.001$]. Post hoc comparisons revealed a decrease in the Sholl profile at 60–110 μm following treatment with conditioned media from IFN γ treated mixed glia compared to control ($P < 0.001$). Post hoc analysis also revealed that co-treatment with KYNA 0.3 μM increased the Sholl profile at 60–90 μm and at 100–120 μm ($P < 0.001$) compared to conditioned media from IFN γ treated mixed glia alone (Fig. 5d).

Effect of KYNA on Reductions in the Co-localisation of Synaptic Proteins in Mature Primary Cortical Neurons Induced by Conditioned Media From IFN γ Treated Mixed Glia

The application of conditioned media from mixed glia has a trophic action and provokes an increase in co-localisation of synaptic proteins. When comparing experiments where QUIN was applied directly to neurons with conditioned media from mixed glia, there is a notable difference under control conditions (approximately 10-fold) in numbers of co-localised puncta.

Conditioned media from IFN γ treated mixed glia reduced co-localisation of synaptic proteins ($T_{(252)} = 8.572$, $P < 0.001$) compared to control (Student's t test, $P < 0.001$). ANOVA of co-localised synaptic markers (% control) showed an effect of KYNA [$F_{(3, 504)} = 19.91$, $P < 0.001$]. Post hoc analysis revealed that co-treatment with KYNA (0.1 μM and 0.3 μM) increased the number of co-localised puncta compared to treatment with conditioned media from IFN γ treated mixed glia alone ($P < 0.001$) (Fig. 5e).

Discussion

Stimulation of primary mixed glia with IFN γ activates the KP via induction of the rate limiting enzyme, IDO (103-fold

increase) in these cells. Other KP enzymes were not affected aside from a modest reduction in the expression of KAT-II, the enzyme responsible for the catalytic conversion of kynurenine to KYNA. In tandem, raised concentrations of kynurenine, KYNA and QUIN were evident in conditioned media. QUIN is present at concentrations approximately 8–9 fold higher than KYNA with the balance/ratio of QUIN:KYNA falling from 8.9 to 7.5 upon induction of the KP. Thus, although a robust induction of the KP was evident in response to stimulation of mixed glia with IFN γ , a shift in balance of KYNA to QUIN concentrations was not apparent.

Conditioned media from IFN γ -stimulated mixed glia suppressed all measures of neurite outgrowth and complexity of primary cortical neurons (DIV 3). Pre-treatment of these cells with the IDO inhibitor, 1-MT (L), reversed the IFN γ -induced changes in metabolite concentrations in the conditioned media and protected against the IFN γ stimulated mixed glial conditioned media-induced reductions in neurite outgrowth and complexity. Mature neurons (from DIV 14) are better developed in terms of both enzyme and protein expression, structural morphology, function and sensitivity to external stimuli. Similarly, 1-MT (L), reversed the IFN γ -stimulated mixed glial conditioned media-induced reductions in neurite branching and length of primary cortical neurons (DIV 21) and attenuated the IFN γ -induced reductions in co-localisation of synaptic markers in primary cortical neurons (DIV 21). Direct application of IFN γ to primary cortical neurons (DIV 3 and 21) had no effect on outgrowth or complexity. Considering that the conditioned media from IFN γ -stimulated glia was found to reduce measures of neuronal complexity and synapse formation, such effects are likely due to the production and release of factors from the glial cells into the media in which they are cultured. Overall, these data support a role for KP

Fig. 2 Effect of 1-MT (L) on IFN γ -stimulated mixed glia conditioned media induced reductions in the complexity and co-localised expression of synaptic markers in mature primary cortical neurons. Primary cortical mixed glia (DIV 14) were treated with IFN γ (10 ng/mL) and 1-MT (L) (0.5 mM) for 24 hours. The resulting conditioned media was collected and applied to mature primary cortical neurons (DIV 21) for 24 hours before fixation and MAP2, synaptophysin and PSD-95 [representative images e- MAP2; f- synaptophysin (red); PSD-95 (green); and merge image showing anti- synaptophysin/PSD-95 co-staining (yellow) and the nuclear marker, DAPI (blue); Representative individual co-localised puncta of synaptic markers are displayed as black dots in the inset image. MAP2 20X magnification, synaptogenesis 40X magnification]. Sholl analysis was performed to analyse the number of neuritic branches (a), the neuritic length (b), the number of primary neurites (c), and the Sholl profile (d). Data are expressed as mean \pm SEM, $n = 6$ coverslips per treatment group from 4 independent experiments. Co-localised synaptic puncta (g) are presented as % control (Control = 26.02 \pm 3.30). Data are expressed as mean \pm SEM ($n = 37$ –53 neurons from 3 independent experiments). * $P < 0.05$, *** $P < 0.001$, vs. control, +++ $P < 0.001$, + $P < 0.05$ vs. IFN γ [two-way ANOVA followed by Newman Keuls post hoc test (a–c, g)]. Two-way repeated measures ANOVA Bonferroni post hoc test (d). 1-MT: 1-methyl tryptophan

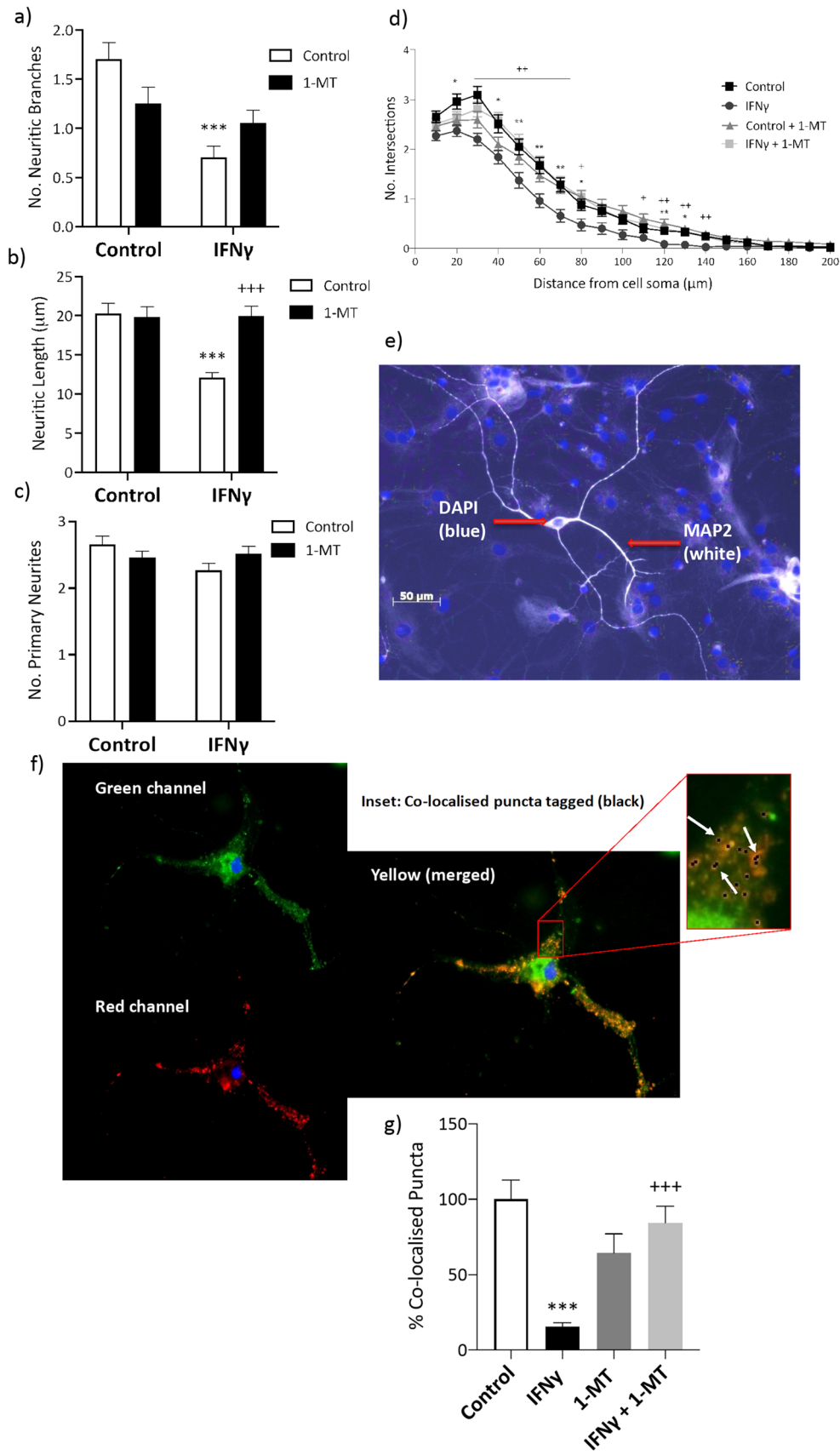
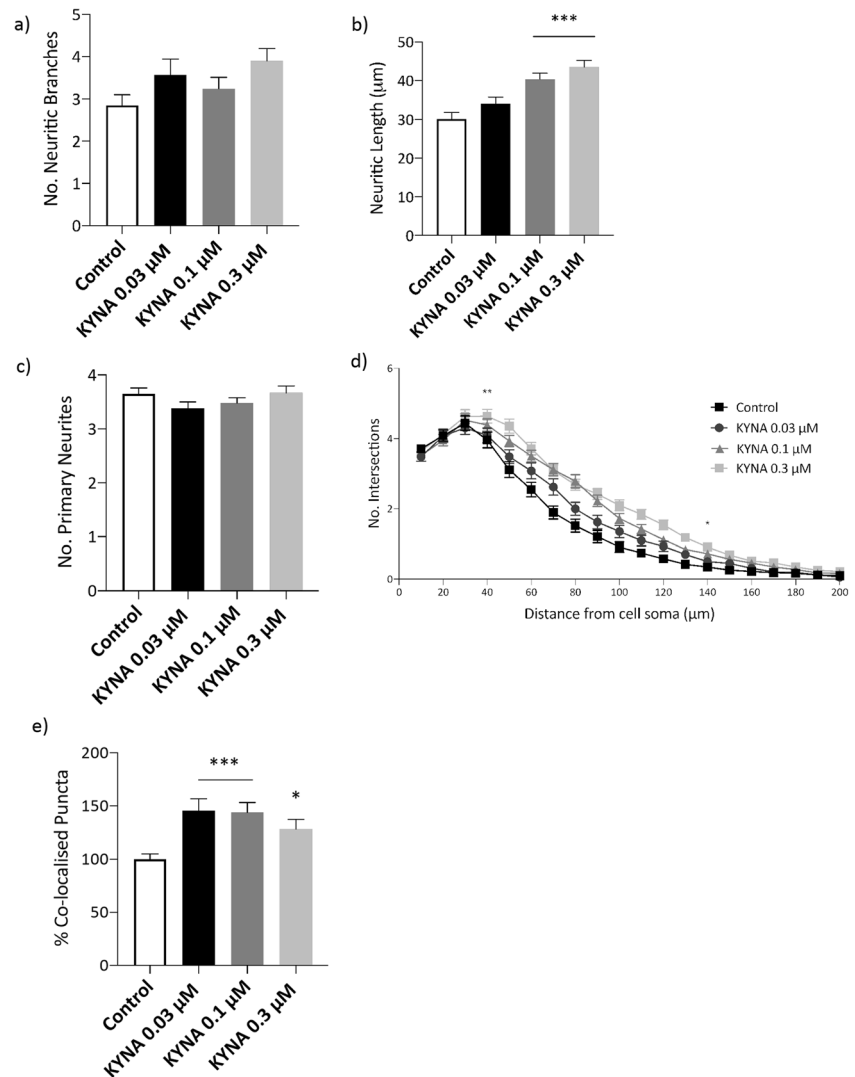


Fig. 3 Effect of KYNA on the complexity and co-localised expression of synaptic markers in mature primary cortical neurons. Primary cortical mature neurons (DIV 21) were treated with KYNA (0.03, 0.1 and 0.3 μ M) for 24 hours before fixation and MAP2 (**a-d**) or synaptophysin and PSD-95 immunocytochemistry (**e**). Sholl analysis was performed to analyse the number of neuritic branches (**a**), the neuritic length (**b**), the number of primary neurites (**c**), and the Sholl profile (**d**). Co-localised synaptic puncta (**e**) are presented as % control (20.21 ± 1.02). Data are expressed as mean \pm SEM, $n = 4-6$ coverslips per treatment group from 3–4 independent experiments. *** $P < 0.001$, ** $P < 0.01$ vs. control [Newman-Keuls post hoc test or Two way repeated measures ANOVA followed by Bonferroni post hoc test (**d**)]



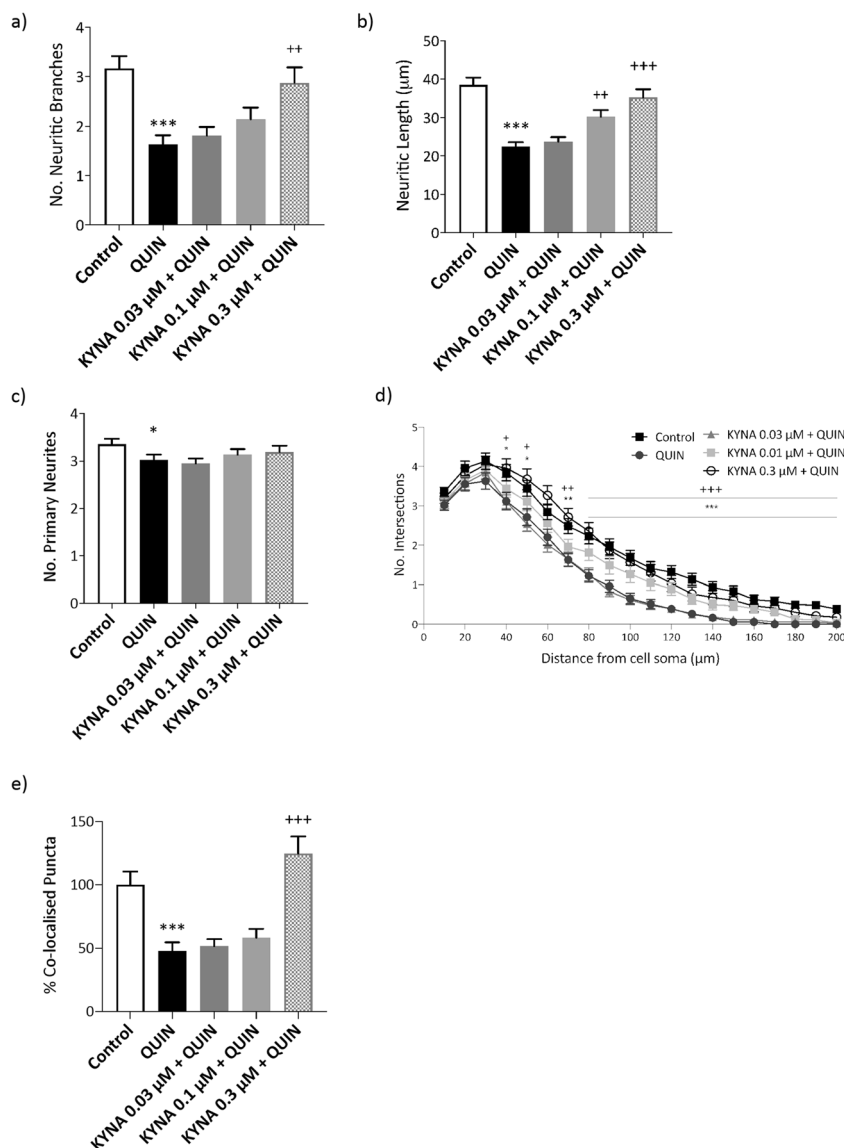
activation in mediating reactive glial associated loss of neuronal complexity and synapse formation. The results are consistent with those previously reported in BV microglial cells (O'Farrell et al. 2017) and further show a role for the KP in reactive primary glial-associated reductions in the complexity of mature primary cortical neurons.

In support of a role for KP activation and associated metabolites, the direct application of metabolites to primary neuronal cultures (DIV 21) were assessed. Previously 3-HK, 3-HAA and QUIN in combination were reported to suppress neurite outgrowth and complexity of primary cortical neurons (DIV 3). These effects were not apparent when the metabolites were applied in isolation indicating a synergistic action of the combination (O'Farrell et al. 2017). Moreover, changes in neuronal complexity were attenuated by pre-treatment with the selective NMDA-R antagonist MK-801 indicating an important role for the NMDA receptor in mediating these actions. Following on from these findings it was of interest to

assess if KYNA, with NMDA receptor antagonist properties, might attenuate the actions of 3-HK, 3-HAA and QUIN. Furthermore, the effects of these metabolites on mature neurons from DIV 14 are unknown.

Direct application of KYNA to primary cortical neurons (DIV 21) had no effect on the number of primary neurites but provoked an increase in neurite length and complexity (Sholl profile). Furthermore, KYNA increased numbers co-localisation of synaptic markers in primary cortical neurons (DIV 21). By contrast direct application of QUIN to primary cortical neurons (DIV 21) provoked a reduction in all measures of complexity (primary neurites, neuritic branches and length). In addition, the application of QUIN to primary cortical neurons (DIV 21) decreased co-localisation of synaptic markers. In a concentration dependent fashion, co-treatment with KYNA blocked all QUIN-induced changes consistent with a counter-interaction between glial derived metabolites KYNA and QUIN in mediating reactive glial associated

Fig. 4 Effect of KYNA on QUIN induced reductions in the complexity and co-localised expression of synaptic markers in mature primary cortical neurons. Primary cortical mature neurons (DIV 21) were co-treated with KYNA (0.03, 0.1 and 0.3 μ M) and QUIN (0.1 μ M) for 24 hours before fixation and MAP2 (**a-d**) or synaptophysin and PSD-95 immunocytochemistry (**e**) Sholl analysis was performed to analyse the number of neuritic branches (**a**), the neuritic length (**b**), the number of primary neurites (**c**), and the Sholl profile (**d**). Co-localised synaptic puncta (**e**) are presented as % control (11.00 ± 1.15). Data are expressed as mean \pm SEM, $n = 4-6$ coverslips per treatment group from 3–4 independent experiments. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ vs. control; +++ $P < 0.001$, ++ $P < 0.01$, vs. QUIN [Student’s *t*-test or Newman-Keuls post hoc test or Two way repeated measures ANOVA followed by Bonferroni post hoc test (**d**)]



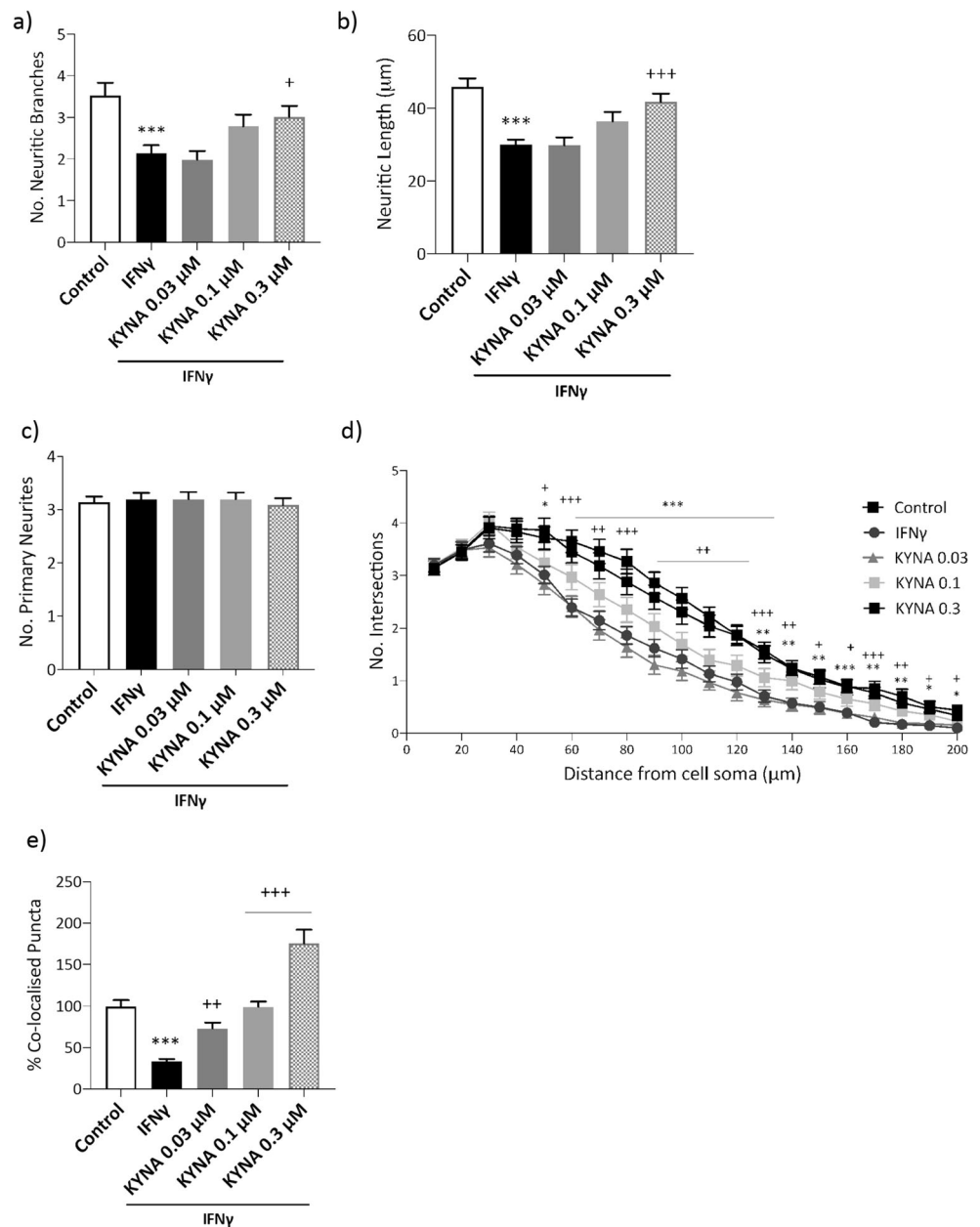
neuronal atrophy. Moreover KYNA rescued the effects of QUIN on the effects of conditioned media derived from IFN-g stimulated glia on co-localisation of synaptic markers.

Co-treatment with KYNA did not however rescue reduced complexity of primary cortical neurons (DIV 21) or the co-localisation of synaptic markers in primary cortical neurons (DIV 21) in response to co-application of QUIN with the pro-oxidative metabolites 3-HK and 3-HAA (data not shown).

Within the brain the KP is compartmentalized as a consequence of different cells showing preferential expression of the various enzymes responsible for tryptophan metabolism. Differential expression of KAT II and KMO occurs with the former being expressed in both microglia and astrocytes while the latter is preferentially expressed in microglia and neurons. Consequently, it is proposed that astrocytes are responsible for the generation of KYNA, while microglia are accountable for the generation of 3-HK, 3-HAA and QUIN. Considering that

both glial cell types express IDO, stimulation with IFN γ has the potential to activate each arm. QUIN is a neurotoxic metabolite with pro-oxidative functions and excitotoxic effects via activation of the NMDA receptor (Lugo-Huitron et al. 2013) which disrupt the cytoskeleton (Pérez-De La Cruz et al. 2012; Rahman et al. 2009; Stroissnigg et al. 2007; Wilson and González-Billault 2015), dendritic architecture, the stability of microtubules and axonal trafficking of vesicles, which manifest as reduced neuronal outgrowth and complexity (Pérez-De La Cruz et al. 2012; Wilson and González-Billault 2015). Moreover QUIN has been linked to a reduction in the expression of synaptic proteins including PSD-95 (Rahman et al. 2018). By contrast KYNA is an endogenous, competitive antagonist of the NMDA receptor (Schwarz et al. 2012; Vecsei et al. 2013) and cholinergic $\alpha 7$ nicotinic receptors at physiological concentrations (Majláth et al. 2016; Moroni et al. 2012; Prescott et al. 2006; Szalardy et al. 2012)

Fig. 5 Effect of KYNA on IFN γ -stimulated mixed glia conditioned media induced reductions in the complexity and co-localised expression of synaptic markers in mature primary cortical neurons. Primary cortical mature neurons (DIV 21) were co-treated with KYNA (0.03, 0.1 and 0.3 μ M) and conditioned media from IFN γ (10 ng/mL) treated mixed glia, for 24 hours before fixation and MAP2 (**a–d**) or synaptophysin and PSD-95 immunocytochemistry (**e**). Sholl analysis was performed to analyse the number of neuritic branches (**a**), the neuritic length (**b**), the number of primary neurites (**c**), and the Sholl profile (**d**). Co-localised synaptic puncta (**e**) are presented as % control (107.9 ± 8.26). Data are expressed as mean \pm SEM, $n = 4–6$ coverslips per treatment group from 3–4 independent experiments. *** $P < 0.001$, ** $P < 0.01$ vs. control; +++ $P < 0.001$, ++ $P < 0.01$ conditioned media from IFN γ treated mixed glia (Student's *t*-test or Newman-Keuls post hoc test) or Two way repeated measures ANOVA followed by Bonferroni post hoc test (**d**.)



and is associated with protection against oxidative stress, excitotoxic insult (García-Lara et al. 2015), and QUIN induced neurotoxicity (Ferreira et al. 2018).

The ability of KYNA to attenuate QUIN induced reductions in neuronal complexity and markers of synapse formation support a bipartite role of the KP and the potential for manipulating either the neurotoxic or neuroprotective arms of the pathway as a strategy to ameliorate reactive glial associated neuronal atrophy and/or synapse loss. Moreover while KYNA has the capacity to block all ionotropic glutamate receptors, with highest affinity for the NMDA receptor, KYNA acts at other targets including the G protein-coupled receptor 35 (GPR35) and the Aryl hydrocarbon Receptor (AhR) a transcription factor which regulates the transcription of

inflammatory factors including cytokines (DiNatale et al. 2010; Nguyen et al. 2010). These mechanisms, which were not investigated in these experiments, may also contribute to KYNA related protective effects on primary cortical neurons.

The understanding whereby the neurotoxic arm of the kynurenine pathway is confined to microglia while the neuroprotective arm is restricted to astrocytes may be called into question and further assessment of the cellular localisation and the functional relationship between both arms of the pathway in the brain is required. In our experiments to date, mixed glial cultures have been necessary as conditioned media drawn from IFN γ enriched microglial or astroglial cultures fail to induce changes in the complexity of primary cortical neurons (DIV 3). This may be related to the fact that either glial cell do

not function well in enriched or isolated conditions and therefore mixed cultures represent a more naturalistic setting. Future work to examine the extent to which brain cell types contribute to concentrations of these metabolites is of importance to allow for discrete pathway manipulation. Preferentially targeting one arm may be potentially beneficial by way of counterbalancing shifts in pathway activation that occurs in neurological disorders (O'Farrell and Harkin 2017).

Unbridled production of pro-inflammatory cytokines, chemokines and infiltration of circulating immune cells are characteristics of neuroinflammation that have been linked with KP enzyme induction, neuronal atrophy, and loss of synaptic connections in the brain. Effects on neuronal viability, complexity, and synapse formation may be extrapolated to give insight into the effects of inflammatory-driven KP activation on neuronal complexity, function and connectivity. Modulation of the KP may represent an attractive and effective approach to ameliorate inflammatory related neurodegenerative diseases (O'Farrell and Harkin 2017).

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Data Availability Data will be made available upon request.

Compliance with Ethical Standards

Conflicts of Interest The authors have no conflicts of interest to declare.

Ethics Approval The experimental protocols were in compliance with the European directive 2010/63/EU on the protection of animals used for scientific purposes, approved by the Animal Research Ethics Committee in Trinity College Dublin and performed under license granted by the Health Products Regulatory Authority.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Code Availability Not applicable.

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