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RESEARCH PAPER

Effects of levetiracetam on astroglial release of kynurenine-pathway metabolites

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BACKGROUND AND PURPOSE

Several preclinical studies have demonstrated the unique profiles of levetiracetam (LEV), inhibits spontaneous absence epilepsy models but does not affect traditional convulsion models; however, the detailed pharmacological mechanisms of action of LEV remain to be clarified.

EXPERIMENTAL APPROACH

We determined the interaction between LEV and IFNγ regarding astroglial release of anti-convulsive (kynurenic acid and xanthurenic acid), pro-convulsive (quinolinic acid) and anti-convulsive but pro-absence (cinnabarinic acid) kynurenine-pathway metabolites from rat cortical primary cultured astrocytes using ultra-HPLC equipped with MS.

KEY RESULTS

IFNγ increased basal astroglial release of cinnabarinic acid and quinolinic acid but decreased that of kynurenic acid and xanthurenic acid. IFNγ enhanced inositol 1,4,5-trisphosphate (IP_3) receptor agonist (adenophostin A, AdA)-induced astroglial release of kynurenine-pathway metabolites, without affecting AMPA-induced release. LEV increased basal astroglial release of kynurenic acid and xanthurenic acid without affecting cinnabarinic acid or quinolinic acid. Chronic and acute LEV administration inhibited AMPA- and AdA-induced kynurenine-pathway metabolite release. Upon chronic administration, LEV enhanced stimulatory effects of IFNγ on kynurenic acid and xanthurenic acid, and reduced its stimulatory effects on cinnabarinic acid and quinolinic acid. Furthermore, LEV inhibited stimulatory effects of chronic IFNγ on AdA-induced release of kynurenine-pathway metabolites.

CONCLUSIONS AND IMPLICATIONS

This study demonstrated several mechanisms of LEV: (i) inhibition of AMPA- and AdA-induced astroglial release, (ii) inhibition of IFNγ-induced IP_3 receptor activation and (iii) inhibition of release of cinnabarinic acid and quinolinic acid with activation of that of kynurenic acid induced by IFNγ. These combined actions of LEV may contribute to its unique profile.

Abbreviations

3OH-kynurenine, 3-hydroxykynurenine; ACSF, artificial CSF; AdA, adenophostin A; fDMEM, DMEM containing 10% FCS; IP_3, inositol 1,4,5-trisphosphate; KAT, kynurenine aminotransferase; LEV, levetiracetam; UHPLC/MS, ultra-HPLC equipped with MS; WAG/Rij, Wistar albino Glaxo rats of Rijswijk

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Introduction

The anti-convulsant drug (S)-α-ethyl-2-oxo-pyrrolidine (**levetiracetam**, LEV) has neuroprotective effects and a wide clinical anti-epileptic spectrum (Berkovic *et al.*, 2007; De Smedt *et al.*, 2007a; Belcastro *et al.*, 2011). LEV also exhibits unique effects on animal models of convulsion and epilepsy. It is ineffective in traditional convulsion screening models, namely, maximal-electroshock and pentylenetetrazol-induced convulsion models (De Smedt *et al.*, 2007b), whereas LEV provides protection in several spontaneous absence epilepsy animal models, such as Strasbourg and Wistar albino Glaxo rats of Rijswijk (WAG/Rij) (Privitter and Cavitt, 2007). These preclinical findings in traditional convulsion and spontaneous epilepsy models suggest that LEV is an anti-absence drug but lacks anti-convulsant activity.

The major mechanism of action of LEV is considered to involve binding to **synaptic vesicle glycoprotein 2A** (Lynch *et al.*, 2004), although other pharmacological actions have also been demonstrated, such as inhibition of N-type **voltage-sensitive Ca^{2+}** and **K^+ channels**, **AMPA receptors** and Ca^{2+} release from intracellular Ca^{2+} stores mediated by **inositol 1,4,5-trisphosphate (IP_3) receptors** (Carunchio *et al.*, 2007; De Smedt *et al.*, 2007b; Fukuyama *et al.*, 2012). In particular, activation of the AMPA receptor plays important roles in the initiation of epileptic seizures and propagation of epileptic discharge (Rogawski, 2002), whereas hyper-activation of the IP_3 receptor contributes to the neuronal damage induced by epileptic seizure, seizure maintenance and dysfunction of GABA exocytosis during epileptic discharge (Pal *et al.*, 2001; Fukuyama *et al.*, 2012).

Reactive astrocytes accompany chronic neurological disease. Both clinical and preclinical studies have strongly indicated that several immunological reactions play important roles in the ictogenesis and development of epileptogenesis (Getts *et al.*, 2007; Somera-Molina *et al.*, 2007). In particular, several clinical studies have suggested that pro-inflammatory reactions contribute to the development of epileptogenesis (Sinha *et al.*, 2008; Li *et al.*, 2017). Despite these clinical and preclinical efforts, the mechanisms of development of epileptogenesis associated with pro-inflammatory and anti-inflammatory reactions have remained to be clarified. Astrocytes and microglia, the immune cells within the CNS, are extremely heterogeneous (Liddelow and Barres, 2017). Recently, it has been proposed that reactive astrocytes are composed of at least two phases, toxic 'A1' and trophic 'A2' reactive astrocytes, which parallel the 'M1' and 'M2' microglia respectively (Liddelow and Barres, 2017). Basically, reactive astrocytes might well have more than two states of polarization, which is an important feature for immunological research (Liddelow and Barres, 2017). Furthermore, trophic A2 reactive astrocytes up-regulate some genes responsible for the induction of synapse formation; however, these changes might also result in unwanted synapses that lead toepilepsy (Eftekhari *et al.*, 2014). Therefore, we should assume that the mechanisms of epileptogenesis are extremely complex. To understand the detailed mechanisms of epileptogenesis and ictogenesis, each specific chemical mediator reaction needs to be identified as well as the more complex interactions amongst immunological mediators on tripartite synaptic transmission that provide the numerous mechanisms behind the pathophysiology of epilepsy.

IFNγ is involved in seizure generation and the process of epileptogenesis (Getts *et al.*, 2007; Somera-Molina *et al.*, 2007). It is also reported to increase the expression of IP_3 receptors and enhance AMPA receptor-induced neurotoxicity (Sahu *et al.*, 2007; Park *et al.*, 2009). Furthermore, activation of IFNγ is reported to enhance the activity of the kynurenine pathway in glia (Myint, 2012). Several preclinical studies have demonstrated that LEV reduces and promotes the expression of pro-inflammatory and anti-inflammatory cytokines respectively (Haghikia *et al.*, 2008; Christensen *et al.*, 2010; Kim *et al.*, 2010; Stienen *et al.*, 2011). However, chronic LEV intake does not affect cytokine levels in patients with epilepsy (Guenther *et al.*, 2014).

The kynurenine pathway synthesizes various endogenous neuroactive metabolites from tryptophan (Figure 1) (Myint, 2012). **Kynurenic acid**, which is a broad-spectrum endogenous neuroprotective antagonist of AMPA and **NMDA receptors** (Hilmas *et al.*, 2001), inhibits absence seizures in WAG/Rij (Peeters *et al.*, 1994; Kaminski *et al.*, 2003). In contrast to kynurenic acid, quinolinic acid is a pro-convulsive metabolite, since it produces convulsions and neuronal damage through activation of the NMDA receptor (Lapin, 1978; Stone, 2001). Recently, xanthurenic acid and cinnabarinic acid have been identified as endogenous agonists of group II (II-mGlu) and group III (III-mGlu) **metabotropic glutamate (mGlu) receptors** respectively (Fazio *et al.*, 2012; Copeland *et al.*, 2013; Fazio *et al.*, 2017). Agonists of II-mGlu and III-mGlu receptors attenuate chemical-induced convulsions, whereas antagonists of them abolish the anti-convulsive effects of their agonists (Folbergrova *et al.*, 2001; Folbergrova *et al.*, 2003). Contrary to the findings in convulsion models, the activation of II-mGlu and III-mGlu receptors suppressed and enhanced absence seizures in spontaneous epileptic models respectively (Moldrich *et al.*, 2001; Ngomba *et al.*, 2008). These previous finidings suggest that kynurenic acid and xanthurenic acid are anti-convulsive metabolites, while quinolinic acid is a pro-convulsive one. Interestingly, the candidate endogenous III-mGlu agonist cinnabarinic acid is probably an anti-convulsive but pro-absence metabolite.

Based on previous demonstrations, the mechanisms behind the anti-absence effect but lack of anti-convulsive action of LEV in animal models are considered to involve the regulation of IFNγ and astroglial release of kynurenine-pathway metabolites (especially the inhibition of cinnabarinic acid); however, the effects of LEV on either astroglial release of kynurenine-pathway metabolites or IFNγ-induced astroglial release have not been clarified. Therefore, to clarify the mechanisms of the anti-absence effect with a lack of anti-convulsive action of LEV, the present study determined the effects of LEV on the astroglial release of kynurenine-pathway metabolites (kynurenine, kynurenic acid, quinolinic acid, xanthurenic acid and cinnabarinic acid) and IFNγ-induced astroglial release of kynurenine-pathway metabolites from cortical primary cultured astrocytes using ultra-HPLC equipped with MS (UHPLC/MS). Furthermore, to explore the mechanism of the neuroprotective action of LEV, we determined its effects on the astroglial release of kynurenine-pathway metabolites induced by AMPA- and IP_3-receptors, since the activation of AMPA- and

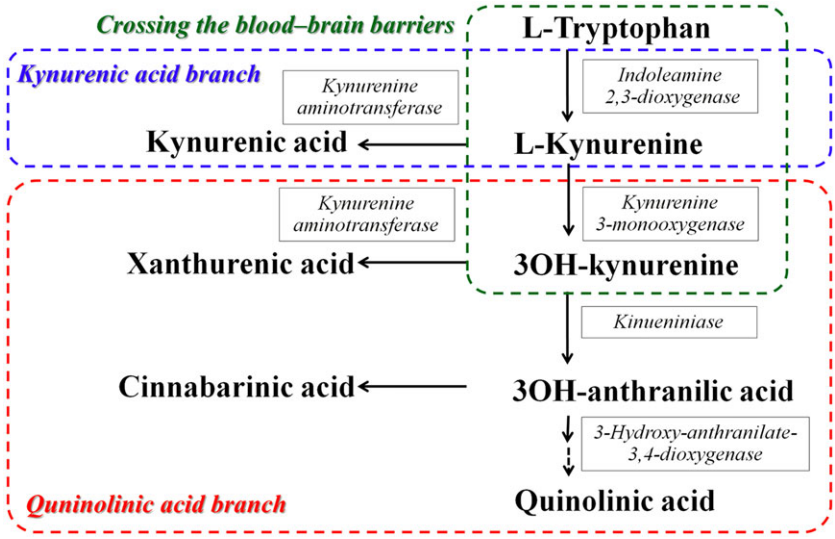

Figure 1

Kynurenine-pathway of tryptophan metabolism. L-kynurenine is synthesized from **L-tryptophan** by indoleamine 2,3-dioxygenase. L-kynurenine and 3OH-kynurenine are transaminated by KAT into kynurenic acid and xanthurenic acid respectively. Kynurenine 3-monooxygenase transforms L-kynurenine into 3OH-kynurenine. Kynureninase transforms 3OH-kynurenine into 3-hydroxyanthranilic acid. Quinolinic acid is formed from 3-hydroxyanthranilic acid by 3-hydroxyanthranilic acid oxygenase. Cinnabarinic acid is an endogenous oxidation product of 3OH-anthranilic acid. Blue square indicates 'kynurenic acid branch' which is the major kynurenine-pathway of astrocytes. In physiological conditions, astrocytes can synthesize mainly kynurenic acid but not 3OH-kynurenine, since astrocytes contain KAT but not kynurenine 3-monooxygenase (Guillemin *et al.*, 2001). Red square indicates 'quinolinic acid branch', which is dormant during the physiological stage (Guillemin *et al.*, 2001). Green square indicates the kynurenine-pathway metabolites, which can be transported across the blood–brain barrier (Fukui *et al.*, 1991).

IP_3-receptors is reported to enhance the release of several gliotransmitters (Hamilton and Attwell, 2010; Tanahashi *et al.*, 2012; Yamamura *et al.*, 2013).

Methods

All animal care and experimental procedures described in this report complied with the Ethical Guidelines established by the Institutional Animal Care and Use Committee at Mie University (No. 24–35). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010).

Primary astrocyte culture

Astrocytes were prepared using a protocol adapted from previously described methods (Tanahashi *et al.*, 2012; Yamamura *et al.*, 2013; Fukuyama *et al.*, 2014). Pregnant Sprague–Dawley rats (SLC, Shizuoka, Japan) were used, which were housed individually in cages in air-conditioned rooms (temperature, 22 ± 2°C) under a 12 h light/dark cycle, and subsequently had *ad libitum* access to food and water. For the preparation of cultured astrocytes, cortical astrocyte cultures were prepared from neonatal Sprague–Dawley rats (n = 60) killed by decapitation at 0–24 h of age and subjected to the removal of cerebral hemispheres under a dissecting microscope. Tissue was chopped into fine pieces using scissors and then triturated briefly with a micropipette. The suspension was filtered using 70 μm nylon mesh (BD, Franklin Lakes, NJ) and centrifuged. Pellets were re-suspended in 10 mL of DMEM containing 10% FCS (fDMEM) (repeated three times). After a 14 day culture (DIV14), contaminating cells were removed by shaking in a standard incubator for 16 h at 200 rpm. On DIV21, astrocytes were removed from flasks by trypsinization and seeded onto a translucent PET membrane (1.0 μm) with 12- or 24-well plates (BD) directly at a density of 10^5 cells cm^{-2} for use in experiments (Tanahashi *et al.*, 2012; Yamamura *et al.*, 2013; Fukuyama *et al.*, 2014).

During DIV21–DIV28, astrocytes in each well were incubated in culture medium containing various target agents [LEV, IFNγ, **kynurenine** or 3-hydroxykynurenine (3OH-kynurenine)], which was changed twice a week for chronic administration (for 7 days) (detailed methods are described under 'Treatment of astrocytes and study design' section). The target agent in the culture medium was randomly set so as not to be exposed to the same drug within the same individual. There were 12 rats included in each of Studies 1–6. On DIV28, cultured astrocytes were washed out using artificial CSF (repeated three times) (ACSF: 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5.5 mM glucose, and buffered with 20 mM HEPES buffer to pH 7.3) (washout). The remaining adherent cells included 95% glial fibrillary acidic protein (GFAP) positive and A2B5-negative cells, as detected using immunohistochemical staining (Tateishi *et al.*, 2006).

After the washout, astrocytes were incubated in ACSF (100 μL per translucent PET membrane) at 35°C for 60 min in a CO_2 incubator (pretreatment incubation). After this pretreatment incubation, astrocytes were incubated in ACSF containing agents (60 min) for acute administration, and ACSF was collected for analysis. Each 100 μL ACSF sample was filtered by Vivaspin 500-3 K (Sartorius, Goettingen, Germany). Filtered samples were freeze-dried and stored at

−80°C until analyses. The freeze-dried samples were treated with 50 μL of acetonitrile containing 5% ammonium ($v \cdot v^{-1}$) for UHPLC/MS analysis.

Treatment of astrocytes and study design

Each study was designed to ensure administration, including acute and chronic administrations, of different agents to astrocyte cultures prepared from the same rat.

Study 1: Concentration-dependent effects of chronic IFNγ administration on astroglial release of kynurenine-pathway metabolites

Astrocyte cultures were incubated in fDMEM containing IFNγ (0, 10, 30 and 100 $U \cdot mL^{-1}$) for 7 days (from DIV21 to DIV28). On DIV28, after washout and pretreatment incubation, astrocytes were incubated in ACSF for 60 min and ACSF was collected for later analysis.

Study 2: Effects of chronic IFNγ administration on kynurenine aminotransferase activity

To study the effects of chronic IFNγ administration on kynurenine aminotransferase (KAT) index, the KAT index was calculated as the ratio of kynurenic acid to kynurenine (Kocki *et al.*, 2006; Myint *et al.*, 2007) using data from Study 1.

To study the effects of the chronic administration of IFNγ on KAT activity, astrocyte cultures were incubated in fDMEM containing IFNγ (0, 10, 30 or 100 $U \cdot mL^{-1}$) plus kynurenine (1 μM) (Kocki *et al.*, 2006) on a translucent PET membrane from DIV21 to DIV28. On DIV28, after washout and pretreatment incubation, astrocytes were incubated in ACSF for 60 min and ACSF was collected for analysis.

Study 3: Effects of chronic IFNγ administration on astroglial release of kynurenine-pathway metabolites under the chronic application of 3OH-kynurenine

Astrocyte cultures were incubated in fDMEM containing IFNγ (0, 10, 30 and 100 $U \cdot mL^{-1}$) plus 3OH-kynurenine (1 μM) from DIV21 to DIV28. On DIV28, after washout and pretreatment incubation, astrocytes were incubated in ACSF for 60 min and ACSF was collected for analysis.

Study 4: Interaction between chronic administrations of IFNγ and LEV on astroglial release of kynurenine-pathway metabolites

Astrocyte cultures were incubated in fDMEM containing LEV (0, 10, 30 and 100 μM), IFNγ (0 and 100 $U \cdot mL^{-1}$) without (for kynurenic acid) or with (for xanthurenic acid, cinnabarinic acid and quinolinic acid) 1 μM 3OH-kynurenine from DIV21 to DIV28. On DIV28, after washout and pretreatment incubation, astrocytes were incubated in ACSF for 60 min and ACSF was collected for analysis.

Study 5: Time-dependent effects of acute LEV administration on AMPA- and AdA-induced astroglial release of kynurenine-pathway metabolites

After incubation in fDMEM without (for kynurenic acid) or with (for xanthurenic acid, cinnabarinic acid and quinolinic acid) 1 μM 3OH-kynurenine, on DIV28, astrocyte cultures were washed out by ACSF (repeated three times). To obtain control data, after the pretreatment incubation for 60 min (repeated twice), astrocytes were incubated in ACSF containing **AMPA** (100 μM) or **adenophostin A** (AdA; 1 μM) for 60 min. To study the effects of acute LEV (100 μM) administration (for 1 h) on AMPA- and AdA-induced release of kynurenine-pathway metabolites, after pretreatment incubation for 60 min, astrocytes were incubated in ACSF containing LEV for 60 min. After pre-incubation of LEV (1 h), astrocytes were incubated in ACSF containing LEV plus AMPA or AdA for 60 min. To study the effects of acute LEV (100 μM) administration (for 2 h) on AMPA- and AdA-induced release of kynurenine-pathway metabolites, after washout, astrocytes were incubated in ACSF containing LEV for 1 h (repeated twice). After pre-incubation of LEV (2 h), astrocytes were incubated in ACSF containing LEV plus AMPA or AdA for 60 min.

Study 6: Interaction between chronic administrations of LEV and IFNγ on AMPA- and AdA-induced astroglial release of kynurenine-pathway metabolites

To study the interaction between chronic administrations of LEV and IFNγ on AMPA- and AdA-induced astroglial release of kynurenic acid, astrocyte cultures were incubated in fDMEM without (control) or with IFNγ (100 $U \cdot mL^{-1}$), LEV (100 μM) or IFNγ (100 $U \cdot mL^{-1}$) plus LEV (100 μM) from DIV21 to DIV28. To study the interaction between chronic administrations of LEV and IFNγ on AMPA- and AdA-induced astroglial release of xanthurenic acid, cinnabarinic acid and quinolinic acid, astrocytes were incubated in fDMEM containing 1 μM 3OH-kynurenine without (control) or with IFNγ (100 $U \cdot mL^{-1}$), LEV (100 μM) or IFNγ (100 $U \cdot mL^{-1}$) plus LEV (100 μM) from DIV21 to DIV28. On DIV28, after washout and pretreatment incubation, astrocytes were incubated in ACSF containing AMPA (100 μM) or AdA (1 μM) for 60 min.

Determination of levels of kynurenine-pathway metabolites

Levels of kynurenine, kynurenic acid, xanthurenic acid, cinnabarinic acid and quinolinic acid were determined by UHPLC (PU-4185; Jasco, Tokyo, Japan) with MS (Acquity SQ detector; Waters, Milford, MA). Twenty microlitres of filtrated samples were injected using an auto-sampler (AS-4150; Jasco). The concentrations of kynurenine-pathway metabolites were determined by UHPLC equipped with a Hypercarb column (particle 3 μm, 150 × 2.1 mm; Thermo, Waltham, MA) at 35°C, and the mobile phase was set at 500 $\mu L \cdot min^{-1}$. A linear gradient elution programme was performed over 10 min with mobile phases A (5 mM ammonium acetate buffer, pH 11) and B (acetonitrile). Nitrogen flows of desolvation and cone were set at 800 and 5 $L \cdot h^{-1}$, respectively, and desolvation temperature was set at 450°C. Cone voltages for the determination of kynurenine (m/z = 209.1), kynurenic acid (m/z = 190.2), xanthurenic acid (m/z = 207.1), cinnabarinic acid (m/z = 301.3) and quinolinic acid (m/z = 167.2) were 20, 25, 25, 40 and −10 V respectively. Where possible, we sought to randomize and blind sample data. In particular, for determination of the extracellular levels of kynurenine-

pathway metabolites, each sample was set on the auto-sampler according to a table of random numbers.

Data analysis

All experiments were designed with equal sizes (n = 12) per group based on our previous studies (Tanahashi *et al.*, 2012; Yamamura *et al.*, 2013; Fukuyama *et al.*, 2014). Values were expressed as mean ± SD. A P value less than 0.05 ($P < 0.05$) was considered statistically significant. Data were confirmed to be normally distributed using the Kolmogorov–Smirnov test (BellCurve for Excel; Social Survey Research Information Co., Ltd., Tokyo, Japan). There was no significant variance inhomogeneity as determined by Bartlett's test and Levene's test; the data were statistically analysed using one-way or two-way ANOVA respectively. When the F-value was significant ($P < 0.05$), data were subsequently analysed by Tukey's *post hoc* test (BellCurve for Excel). The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2018).

Materials

LEV and AMPA (AMPA-receptor agonist) were obtained from Wako Chemicals (Osaka, Japan). 3-Hydroxykynurenine (3OH-kynurenine) and adenophostin A (AdA) (IP_3 receptor agonist) were purchased from Sigma-Aldrich (St. Louis, MO). Rat recombinant IFNγ was purchased from Biolegend (San Diego, CA).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b,c,d,e).

Results

Concentration-dependent effects of chronic IFNγ administration on astroglial release of kynurenine-pathway metabolites (Study 1)

The levels of tryptophan and kynurenine in culture medium (fDMEM) were 73.9 ± 13.8 μM and 7.3 ± 0.8 nM respectively; however, the levels of other kynurenine-pathway metabolites, kynurenic acid, 3OH-kynurenine, xanthurenic acid, cinnabarinic acid and quinolinic acid, in fDMEM were not detectable.

After incubation in fDMEM with no agents (non-treatment conditions), the astroglial release of kynurenine and kynurenic acid in ACSF was 60.7 ± 11.7 nM (n = 24) and 5.3 ± 1.0 nM, respectively, but that of xanthurenic acid, cinnabarinic acid and quinolinic acid was not detectable (Fukuyama *et al.*, 2014). After incubation in fDMEM containing IFNγ (0, 10, 30 and 100 U·mL^{-1}), the astroglial release of xanthurenic acid, cinnabarinic acid and quinolinic acid remained undetectable; however, the astroglial release of kynurenine and kynurenic acid was increased in an IFNγ concentration-dependent manner (Figure 2).

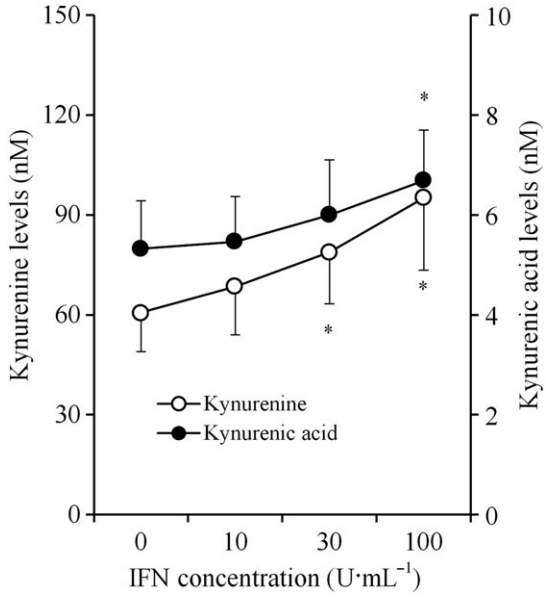

Figure 2

Concentration-dependent effects of chronic IFNγ administration on astroglial release of kynurenine-pathway metabolites. Concentration-dependent effects of chronic IFNγ administration on astroglial release of kynurenine and kynurenic acid, after incubation in culture medium containing IFNγ (for 7 days), are indicated in the figure. Concentration-dependent effects of chronic IFNγ administration on astroglial releases of kynurenine and kynurenic acid and KAT activity were analysed using one-way ANOVA followed by Tukey's *post hoc* test. *$P < 0.05$ versus control (free IFNγ) (n = 12). In this study (Study 1), astrocyte cultures prepared from the same rat were administered different agents.

Effects of chronic IFNγ administration on kynurenine aminotransferase activity (Study 2)

The KAT activity index (ratio of kynurenic acid to kynurenine) (Kocki *et al.*, 2006; Myint *et al.*, 2007) was concentration-dependently reduced by chronic IFNγ administration (Figure 3A). Similar to the KAT activity index, upon the chronic administration of IFNγ after incubation in fDMEM containing IFNγ with 1 μM kynurenine for 7 days (from DIV21 to DIV28), IFNγ concentration-dependently reduced the astroglial release of kynurenic acid (Figure 3B).

Effects of chronic IFNγ administration on astroglial release of kynurenine-pathway metabolites under the chronic application of 3OH-kynurenine (Study 3)

After incubation in fDMEM containing IFNγ (0, 10, 30 and 100 U·mL^{-1}) without 3OH-kynurenine for 7 days, the astroglial release of xanthurenic acid, cinnabarinic acid and quinolinic acid was not detectable. After incubation in fDMEM containing 1 μM 3OH-kynurenine for 7 days, the astroglial release of xanthurenic acid, cinnabarinic acid and quinolinic acid became detectable (Figure 4) without affecting that of kynurenine or kynurenic acid (data not shown) (Fukuyama *et al.*, 2014). Chronic IFNγ administration

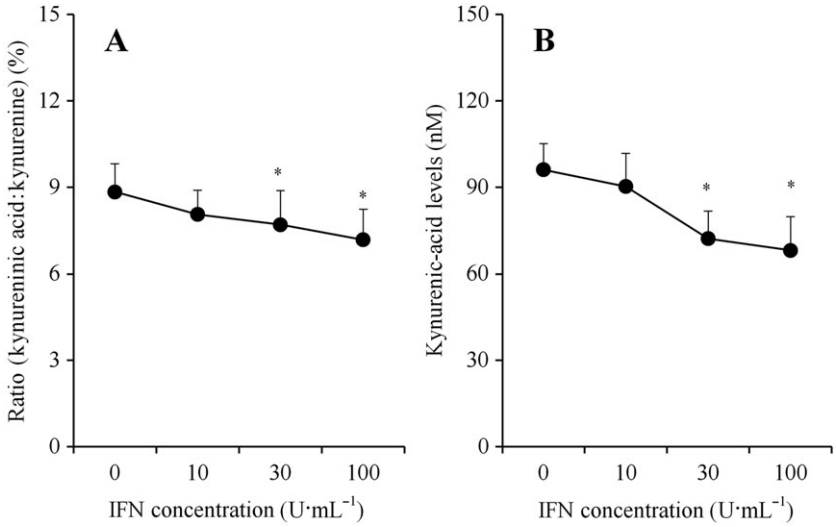

Figure 3

Effects of chronic IFNγ administration on KAT activity. Concentration-dependent effects of chronic IFNγ administration (for 7 days) on KAT activity index is indicated in (A). KAT activity index was calculated as the ratio of kynurenic acid:kynurenine from results in Study 1. After incubation in culture medium containing 1 μM kynurenine plus IFNγ for 7 days, concentration-dependent effect of chronic IFNγ administration (for 7 days) on astroglial kynurenic acid release is indicated in (B). Concentration-dependent effects of chronic IFNγ administration on KAT activity index and astroglial kynurenic acid release were analysed using one-way ANOVA followed by Tukey's *post hoc* test. *$P < 0.05$ versus control (free IFNγ) ($N = 12$). In this study (Study 2), astrocyte cultures prepared from the same rat were administered different agents.

increased the astroglial release of cinnabarinic acid and quinolinic acid but decreased that of xanthurenic acid (Figure 4).

Interaction between chronic administrations of IFNγ and LEVon astroglial release of kynurenine-pathway metabolites (Study 4)

LEV (0, 10, 30 and 100 μM) concentration-dependently increased the astroglial release of kynurenic acid (Figure 5B) without affecting that of kynurenine (Figure 5A). IFNγ (100 U·mL^{-1}) increased the astroglial release of both kynurenine and kynurenic acid (Figure 5A, B). Interestingly, IFNγ enhanced the concentration-dependent stimulatory effects of LEV on kynurenic acid release (Figure 5B).

After incubation in fDMEM containing 1 μM 3OH-kynurenine, LEV (0, 10, 30 and 100 μM) and IFNγ (100 U·mL^{-1}) for 7 days, interactions between LEV and IFNγ regarding the astroglial release of xanthurenic acid, cinnabarinic acid and quinolinic acid were detected (Figure 6A–C). LEV concentration-dependently increased the astroglial release of xanthurenic acid release (Figure 6A) but did not affect that of cinnabarinic acid or quinolinic acid (Figure 6B, C). In contrast to LEV, IFNγ (100 U·mL^{-1}) decreased the astroglial release of xanthurenic acid (Figure 6A) but increased that of cinnabarinic acid and quinolinic acid (Figure 6B, C). Interestingly, under the IFNγ-free conditions, 100 μM LEV did not affect the astroglial release of cinnabarinic acid or quinolinic acid; however, upon incubation in IFNγ with LEV, LEV concentration-dependently reduced the astroglial release of cinnabarinic acid and quinolinic acid (Figure 6B, C). The concentration-dependent stimulatory effect of LEV on xanthurenic acid release was enhanced by IFNγ (Figure 6A).

Time-dependent effects of acute LEV administration on AMPA- and AdA-induced astroglial release of kynurenine-pathway metabolites (Study 5)

The acute administration of both 100 μM AMPA (AMPA receptor agonist) and 1 μM AdA (IP_3 receptor agonist) for 60 min increased the astroglial release of kynurenic acid, xanthurenic acid, cinnabarinic acid and quinolinic acid (control) (Figure 7A–D). AMPA- and AdA-induced release was calculated by subtraction of the basal release level (open column: astroglial release before administration of 100 μM AMPA or 1 μM AdA) from the increased astroglial release following the administration of 100 μM AMPA and 1 μM AdA respectively.

Acute LEV (100 μM) did not affect the astroglial release of kynurenine-pathway metabolites (Figure 7A–D). Acute LEV administration time-dependently inhibited the AMPA-induced release of kynurenic acid (Figure 7A), xanthurenic acid (Figure 7B), cinnabarinic acid (Figure 7C) and quinolinic acid (Figure 7D). Acute LEV administration also time-dependently inhibited the AdA-induced release of kynurenic acid (Figure 7A), xanthurenic acid (Figure 7B), cinnabarinic acid (Figure 7C) and quinolinic acid (Figure 7D). In particular, 1 h of LEV administration did not affect AMPA- or AdA-induced release of kynurenine-pathway metabolites; however, 2 h of LEV administration inhibited both AMPA- and AdA-induced release of kynurenine-pathway metabolites (Figure 7A–D).

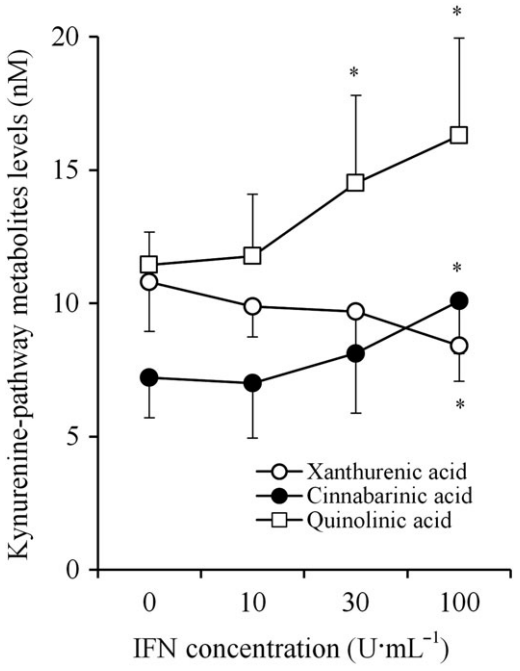

Figure 4

Concentration-dependent effects of chronic IFNγ administration on astroglial release of kynurenine-pathway metabolites in quinolinic acid branch under the chronic application of 3OH-kynurenine. Concentration-dependent effects of chronic IFNγ administration (for 7 days) on astroglial release of xanthurenic acid, cinnabarinic acid and quinolinic acid, after incubation in culture medium containing 1 μM 3OH-kynurenine plus IFNγ for 7 days, are indicated. Concentration-dependent effects of chronic IFNγ administration on astroglial releases of kynurenine-pathway metabolites were analysed using one-way ANOVA followed by Tukey's *post hoc* test. *$P < 0.05$ versus control (free IFNγ) ($n = 12$). In this study (Study 3), astrocyte cultures prepared from the same rat were administered different agents.

Interaction between chronic administrations of LEV and IFNγ on AMPA- and AdA-induced astroglial release of kynurenic acid (Study 6)

After incubation in fDMEM containing IFNγ (100 U·mL^{-1}), LEV (100 μM) or IFNγ (100 U·mL^{-1}) plus LEV (100 μM) for 7 days, significant interactions between LEV and IFNγ on the AMPA- and AdA-induced astroglial release of kynurenic acid, xanthurenic acid, cinnabarinic acid and quinolinic acid were detected (Figure 8A–D).

IFNγ enhanced the AdA-induced release of all kynurenine-pathway metabolites, namely, kynurenic acid, xanthurenic acid, cinnabarinic acid and quinolinic acid, but did not affect AMPA-induced release (Figure 8A–D). LEV inhibited both AMPA- and AdA-induced release of kynurenic acid, xanthurenic acid, cinnabarinic acid and quinolinic acid (Figure 8A–D). LEV also inhibited the stimulatory effects of IFNγ on AdA-induced release of all kynurenine-pathway metabolites (Figure 8A–D).

Discussion

The occurrence of the kynurenine pathway in microglia of the CNS was confirmed in previous studies (Guillemin *et al.*, 2003), although it is only present in some astrocytes due to the lack of **kynurenine 3-monooxygenase** (Guillemin *et al.*, 2001). Thus, the major product of the kynurenine pathway in astrocytes is considered to be kynurenic acid, but astrocytes cannot synthesize quinolinic acid (Guillemin *et al.*, 2001). The present study also demonstrated that, under non-treatment conditions, astroglial release of kynurenine and kynurenic acid was detectable, whereas that of xanthurenic acid, cinnabarinic acid and quinolinic acid, which are kynurenine-pathway metabolites downstream from kynurenine 3-monooxygenase, could not be detected. However, upon incubation in fDMEM containing 1 μM 3OH-kynurenine, which is synthesized by kynurenine 3-monooxygenase from kynurenine, astroglial release of xanthurenic acid, cinnabarinic acid and quinolinic acid became detectable. These results suggest that the kynurenine pathway in astrocytes comprises two sub-branches, namely, the 'kynurenic acid branch' and 'quinolinic acid branch' (Figure 1). Under physiological conditions, the kynurenic acid branch is the major astroglial metabolic kynurenine pathway, which can mainly synthesize kynurenic acid but not 3OH-kynurenine (Guillemin *et al.*, 2001). The other quinolinic acid branch, which can synthesize the downstream kynurenine-pathway metabolites from 3OH-kynurenine, is dormant under physiological conditions (Guillemin *et al.*, 2001). In other words, if astrocytes are supplied with 3OH-kynurenine from neighbouring microglia or is transported across the blood–brain barrier from the circulation (Fukui *et al.*, 1991), they can synthesize kynurenine-pathway metabolites in the quinolinic acid branch, including xanthurenic acid, cinnabarinic acid and quinolinic acid (Guillemin *et al.*, 2001).

Effects of IFNγ on astroglial release of kynurenine-pathway metabolites

IFNγ enhances kynurenine-pathway turnover in astrocytes through the up-regulated expression of **indoleamine 2,3-dioxygenase** (Guillemin *et al.*, 2007; Asp *et al.*, 2011); however, kynurenine 3-monooxygenase is decreased by IFNγ (Asp *et al.*, 2011). In the present study, the chronic administration of IFNγ (for 7 days) concentration-dependently increased the astroglial release of both kynurenine and kynurenic acid; however, the KAT activity index (Kocki *et al.*, 2006; Myint *et al.*, 2007) and extracellular xanthurenic acid level were reduced by IFNγ in a concentration-dependent manner. Indeed, after incubation in fDMEM containing 1 μM kynurenine (for 7 days), the chronic administration of IFNγ decreased extracellular kynurenic acid levels. These results suggest that IFNγ probably reduces KAT activity (Asp *et al.*, 2011) but at the same time enhances the production of kynurenine (kynurenic acid precursor) resulting in an increased kynurenic acid level.

After incubation in fDMEM containing 1 μM 3OH-kynurenine, chronic IFNγ administration decreased the astroglial release of xanthurenic acid, which is produced by KAT from 3OH-kynurenine. Consistent with the findings for xanthurenic acid, chronic IFNγ administration increased the astroglial release of cinnabarinic acid and quinolinic acid. Furthermore, an increase in 3OH-kynirenine in the culture medium (higher than 1 μM) reduced the astroglial release of

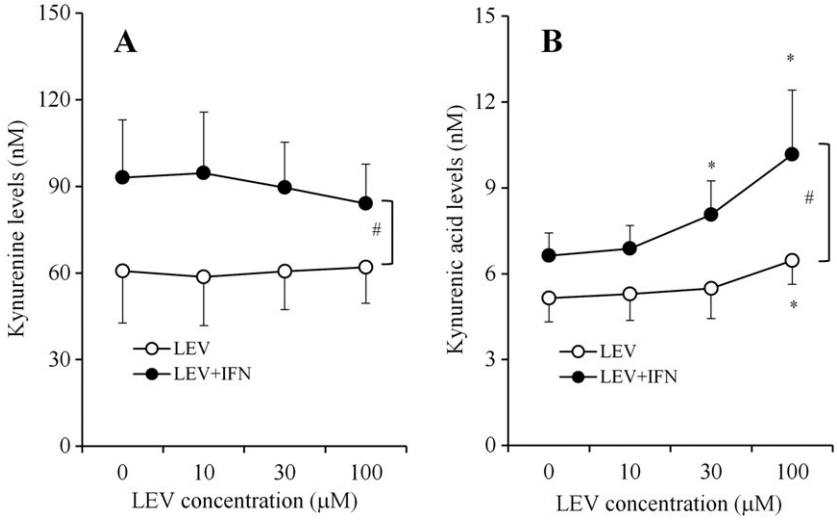

Figure 5

Interaction between chronic administration of IFNγ and LEV on astroglial release of kynurenine-pathway metabolites. After incubation in culture medium containing LEV (10, 30 and 100 μM) with or without 100 U·mL^{-1} IFNγ for 7 days; concentration-dependent effects of LEV (0, 10, 30 and 100 μM) on astroglial release of kynurenine and kynurenic acid are indicated in (A) and (B) respectively. Concentration-dependent effects of LEV on astroglial releases of kynurenine-pathway metabolites were analysed using two-way ANOVA followed by Tukey's *post hoc* test. *$P < 0.05$ versus control (free LEV), #$P < 0.05$ versus LEV + IFNγ ($n = 12$). In this study (Study 4), different agents were administered to astrocyte cultures prepared from the same rat.

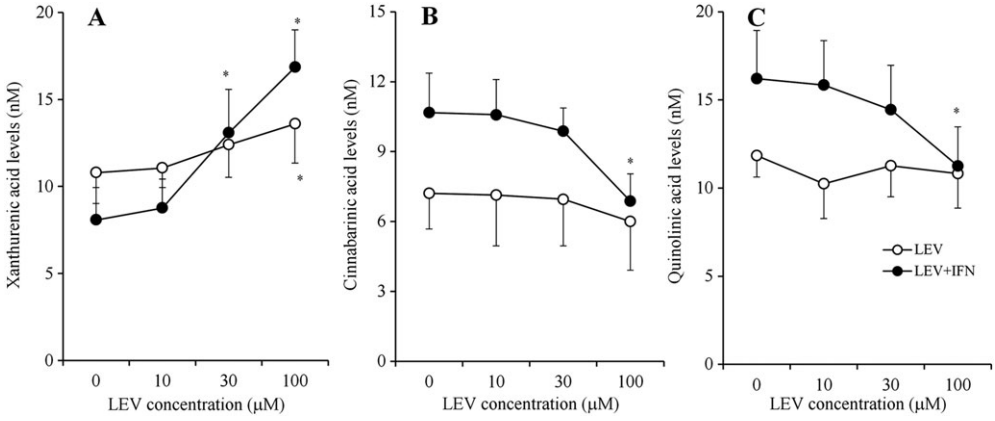

Figure 6

Interaction between chronic administration of IFNγ and LEV on astroglial release of kynurenine-pathway metabolites under the application of 3OH-kynurenine. After incubation in culture medium containing 1 μM 3OH-kynurenine, LEV (10, 30 and 100 μM) with or without 100 U·mL^{-1} IFNγ for 7 days; concentration-dependent effects of chronic administration of LEV (10, 30 and 100 μM) on astroglial release of xanthurenic acid, cinnabarinic acid and quinolinic acid are indicated in (A–C) respectively. Concentration-dependent effects of LEV on astroglial release of kynurenine-pathway metabolites were analysed using two-way ANOVA followed by Tukey's *post hoc* test ($n = 12$). *$P < 0.05$ versus control (free LEV) ($n = 12$). In this study (Study 4), different agents were administered to astrocyte cultures prepared from the same rat.

kynurenine and kynurenic acid (Fukuyama *et al.*, 2014). These results suggest that, if epileptic reactive astrocytes are supplied with 3OH-kynurenine, chronic exposure to IFNγ probably induces a reduction in anti-convulsive metabolites (kynurenic acid and xanthurenic acid) and an enhancement of pro-absence (cinnabarinic acid) and pro-convulsive (quinolinic acid) metabolites.

A clinical study demonstrated that the peripheral 3OH-kynurenine level of patients with uncontrolled epilepsy was higher than that of controlled patients (Dolina *et al.*, 2012). Thus, epileptic reactive astrocytes possibly take 3OH-kynurenine from the peripheral circulation by crossing the blood–brain barrier (Fukui *et al.*, 1991). Additionally, the expression of quinolinic acid in astrocytes in patients with epilepsy has not been studied; however, quinolinic acid was detected in astrocytes in cases of Alzheimer's disease (Guillemin *et al.*, 2005). Reactive microglia contribute to acute seizures, degeneration and aberrant neurogenesis in

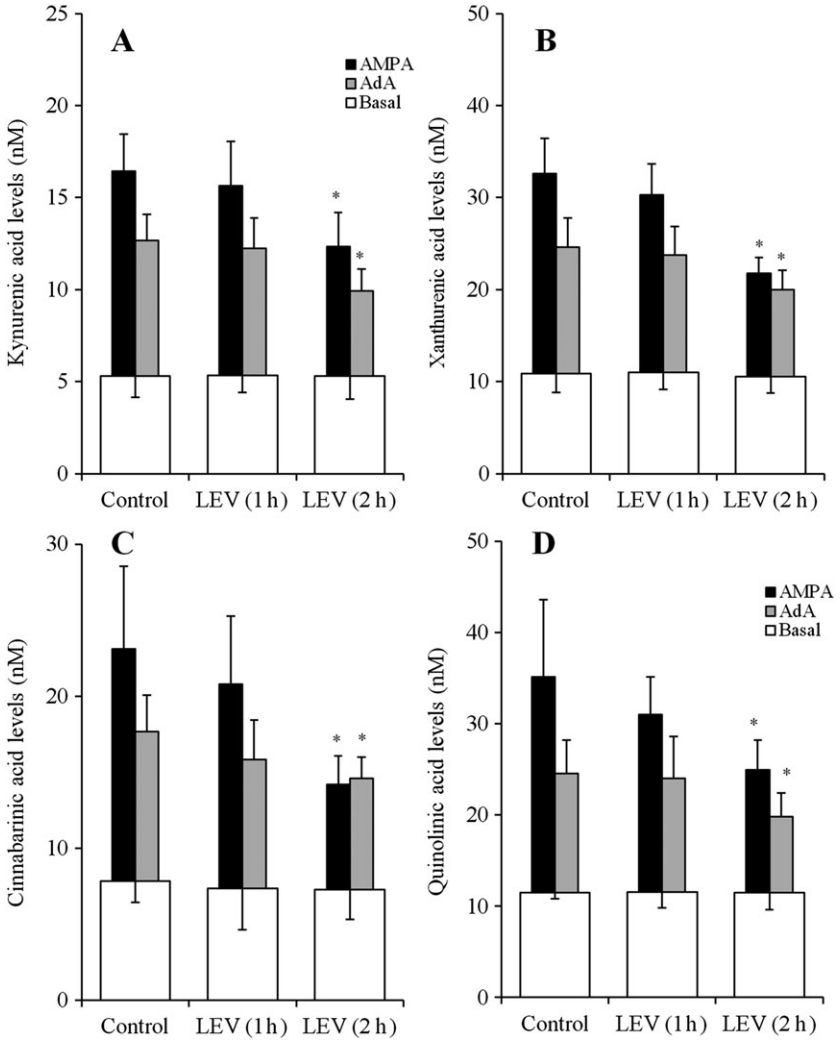

Figure 7

Time-dependent effects of acute LEV administration on AMPA- and AdA-induced astroglial release of kynurenine-pathway metabolites. Time-dependent effects of acute 100 μM LEV administration on 100 μM AMPA- and 1 μM AdA-induced astroglial release of kynurenic acid, xanthurenic acid, cinnabarinic acid and quinolinic acid are indicated in (A–D) respectively. AMPA- and AdA-evoked release of kynurenine-pathway metabolites were calculated by subtraction of basal release from the astroglial release after AMPA- and AdA-evoked stimulation (for 60 min). *$P < 0.05$ versus control by two-way ANOVA followed by Tukey's *post hoc* test ($n = 12$). In this study (Study 5), different agents were administered to astrocyte cultures prepared from the same rat.

epileptic models. Therefore, reactive astrocytes can probably take in 3OH-kynurenine from neighbouring microglia or the peripheral circulation. Upon 3OH-kynurenine exposure, the activation of IFNγ in the CNS can induce a reduction in anti-convulsive metabolites (kynurenic acid and xanthurenic acid) and enhancement of pro-absence (cinnabarinic acid) and pro-convulsive (quinolinic acid) metabolites. To clarify our hypothesis, we will explored the epileptogenetic microglia-induced 3OH-kynurenine production (Eyo *et al.*, 2017) in the CNS of patients with epilepsy.

Chronic exposure to IFNγ increased the expression of IP_3 receptors without affecting that of AMPA receptors (Sahu *et al.*, 2007; Park *et al.*, 2009). The present study also demonstrated that chronic IFNγ administration enhanced AdA-induced astroglial release without affecting AMPA-induced release. Hyperactivation of the IP_3 receptor contributes to seizure maintenance and seizure-related neuronal damage (Pal *et al.*, 2001; Fukuyama *et al.*, 2012). Therefore, the present results suggest that the process of epileptogenesis induced by chronic activation of IFNγ probably includes the combined increase in the release of cinnabarinic acid and quinolinic acid, decrease in the release of kynurenic acid and xanthurenic acid and activation of the IP_3 receptor in astrocytes (Getts *et al.*, 2007; Somera-Molina *et al.*, 2007).

Effects of LEV on astroglial release of kynurenine-pathway metabolites

Several conventional anti-convulsants, such as carbamazepine, phenytoin, phenobarbital, felbamate and lamotrigine, enhance the production of kynurenic acid (Kocki *et al.*, 2004; Kocki *et al.*, 2006). We recently demonstrated that

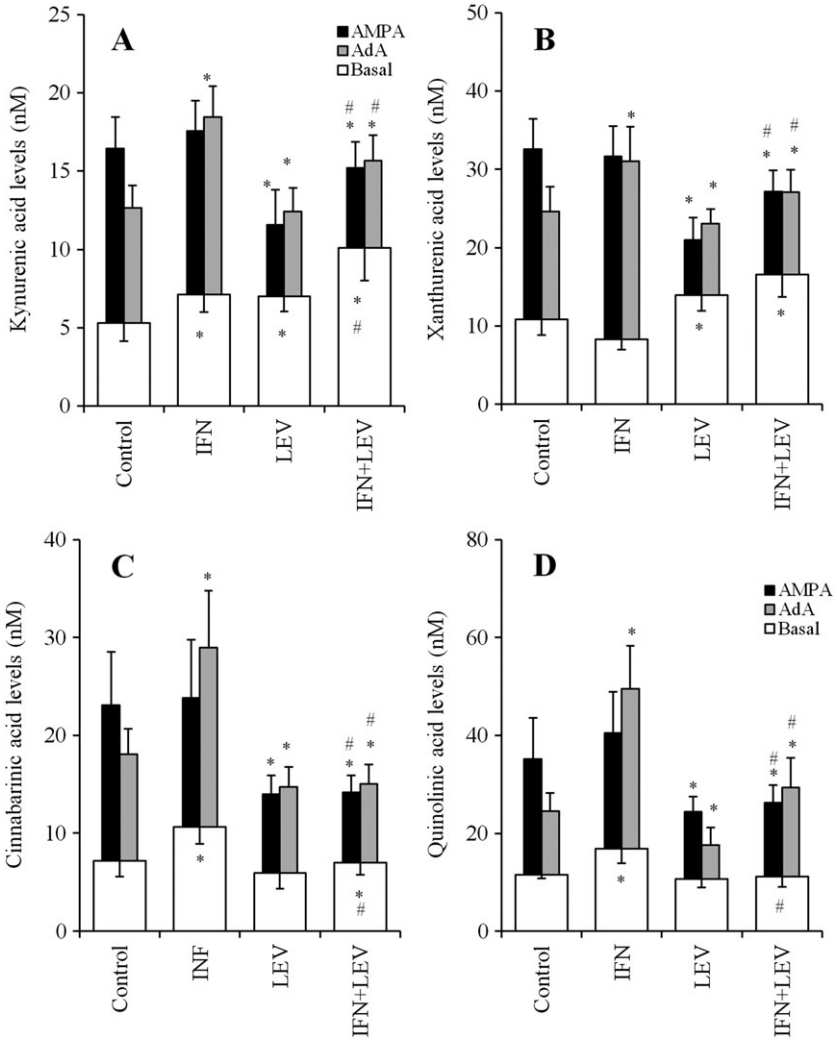

Figure 8

Interaction between chronic administration of LEV and IFNγ on AMPA- and AdA-induced astroglial release of kynurenine-pathway metabolites. Interaction between incubation of 100 U·mL^{-1} IFNγ and 100 μM LEV for 7 days on 100 μM AMPA- and 1 μM AdA-induced astroglial release of kynurenic acid, xanthurenic acid, cinnabarinic acid and quinolinic acid are indicated in (A–D) respectively. AMPA- and AdA-evoked release were calculated by subtraction of basal release (opened columns) from the astroglial release during AMPA- and AdA-evoked stimulation. *$P < 0.05$ versus control, #$P < 0.05$ versus IFNγ by two-way ANOVA followed by Tukey's *post hoc* test ($n = 12$). In this study (Study 6), different agents were administered to the astrocyte cultures prepared from the same rat.

ONO-2506, which inhibits absence seizure of Cacna1a conditional knockdown mice but does not affect traditional convulsion models, also increased astroglial kynurenic acid release (Yamamura *et al.*, 2013). Unfortunately, the effects of conventional anti-convulsants on other kynurenine-pathway metabolites have not been demonstrated. Similar to conventional anti-convulsants, chronic LEV administration increased the astroglial release of kynurenic acid. Furthermore, chronic LEV administration increased the astroglial release of anti-convulsive metabolites, kynurenic acid and xanthurenic acid, without affecting pro-absence cinnabarinic acid and pro-convulsive quinolinic acid. Surprisingly, during chronic IFNγ exposure, chronic LEV administration increased the astroglial release of kynurenic acid and xanthurenic acid but decreased that of cinnabarinic acid and quinolinic acid. Therefore, LEV appears to antagonize the pharmacological pro-convulsive and pro-absence actions of IFNγ. In other words, the inhibitory effects of LEV on the anti-convulsive and pro-absence cinnabarinic acid appear to be well correlated with the anti-absence but lack of anti-convulsive profile of LEV (De Smedt *et al.*, 2007b; Privitter and Cavitt, 2007). Recent preclinical studies suggested that IFNγ-induced negative feedback on anti-inflammatory and repair functional processes associated with M2 microglia or A2 astrocytes probably plays an important role in the development of epileptogenesis (Sinha *et al.*, 2008; Li *et al.*, 2017). Therefore, the suppression of IFNγ-induced astroglial responses by LEV also possibly contributes to the anti-convulsive and anti-absence actions of LEV.

In vitro experiments have also shown that LEV prevents the Ca^{2+} influx accompanying seizure generation, reducing the fluctuations in membrane excitability occurring during

epileptic discharges (Pisani *et al.*, 2004) *via* inhibition of the IP_3 receptor (Nagarkatti *et al.*, 2008). LEV also decreases kainate- and AMPA-induced currents in cultured cortical neurons, as demonstrated in a whole-cell patch clamp study (Carunchio *et al.*, 2007). Our microdialysis study demonstrated that LEV inhibited AdA-evoked releases of frontal monoamine, L-glutamate and GABA (Fukuyama *et al.*, 2012). The present study indicates that both acute and chronic administration of LEV inhibited hyperexcitable astroglial release induced by AMPA and IP_3 receptor activation.

Using hippocampal slices, 30 min exposure to LEV suppressed the later field potential of a burst, whereas such suppression of the relative size of later field potential required more than 60 min of exposure to LEV (Yang *et al.*, 2007; Yang and Rothman, 2009). In the present study, acute LEV administration inhibited both AMPA- and AdA-induced astroglial release of all kynurenine-pathway metabolites, such as kynurenic acid, xanthurenic acid, cinnabarinic acid and quinolinic acid. In particular, acute LEV-induced suppression of AMPA- and AdA-induced astroglial release of kynurenine-pathway metabolites required exposure to LEV for longer than 2 h.

Our results provide a mechanism for the anti-convulsive, anti-absence and neuroprotective actions of LEV but do not explain the mechanisms behind the contradictory actions of LEV in epileptic patients and animal models of convulsion and absence epilepsy. Differences in astroglial functions have been demonstrated between humans and rodents. In the present study, chronic IFNγ exposure reduced KAT activity. IFNγ is also known to reduce KAT expression in fibroblasts (Asp *et al.*, 2011). However, chronic IFNγ administration increased the kynurenic acid level in human astrocytes *via* an increase in the expression of KAT (Guillemin *et al.*, 2007). Additionally, recent studies have demonstrated that the transplantation of human astrocytes in rodent brain enhanced synapse plasticity and learning (Han *et al.*, 2013). Therefore, human astrocytes are an important player in the patho-mechanisms of neuropsychiatric diseases, neurodevelopmental disorders and epilepsy. To clarify the different effects of LEV in patients with epilepsy and animal models of convulsion and epilepsy, we need to study the effects of LEV on the astroglial release of metabolites of the kynurenine pathway using cultured human astrocytes.

Conclusion

The present paper describes the extremely complex mechanisms of action of LEV on the astroglial release of kynurenine-pathway metabolites. (i) Under physiological conditions, LEV increased the basal astroglial release of anti-convulsive and anti-absence metabolites, kynurenic acid and xanthurenic acid, without affecting the release of cinnabarinic acid and quinolinic acid. (ii) In contrast, under chronic exposure to IFNγ, which can modulate seizure susceptibility (Getts *et al.*, 2007), LEV enhanced the IFNγ-induced astroglial release of kynurenic acid and xanthurenic acid but inhibited the anti-convulsive and pro-absence metabolite cinnabarinic acid, and the pro-convulsive metabolite quinolinic acid. (iii) LEV inhibited the enhanced astroglial release of all kynurenine-pathway metabolites induced by hyperactivation of IP_3 and AMPA receptors. (iv) Chronic IFNγ exposure increased the AdA-induced release of all kynurenine-pathway metabolites, whereas chronic LEV administration inhibited the stimulatory effects of IFNγ on IP_3 receptor-associated astroglial release. These four effects of LEV provide insights into the mechanisms behind the anti-epileptic action of LEV. In particular, the inhibitory effects of LEV on the enhanced release of cinnabarinic acid induced by IFNγ at least partly contribute to the anti-absence but lack of anti-convulsive profile of LEV.

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Author contributions

M.O. designed the research. K.F. and M.O. performed experiments. Both authors have given their final approval for the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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