UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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Letícia Barbieri Caus

AVALIAÇÃO DO PERFIL ELETROENCEFALOGRÁFICO APÓS INJEÇÃO INTRAESTRIATAL DE ÁCIDO QUINOLÍNICO EM MODELO ANIMAL DE ACIDEMIA GLUTÁRICA TIPO I

Porto Alegre
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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Bacharel em Biomedicina.

Orientadora: Profª. Drª. Maria Elisa Calcagnotto

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RESUMO

A acidemia glutárica tipo I (AG-I) é uma doença autossômica recessiva do metabolismo dos aminoácidos lisina, hidroxilisina e triptofano, causada pela deficiência da enzima glutaril-CoA-desidrogenase (GCDH). Nesta doença ocorre o acúmulo de ácidos orgânicos em fluidos e tecidos, principalmente no Sistema Nervoso Central (SNC), o que caracteriza a AG-I como uma acidemia com sinais e sintomas principalmente de cunho neurológico e leva a alterações neurológicas que incluem discinesia, déficits neurológicos e epilepsia, geralmente associadas à atrofia frontotemporal e degeneração estriatal. O modelo animal para AG-I, camundongos deficientes na atividade da GCDH (*Gcdh*$^{-/-}$) e que recebem dieta com sobrecarga de lisina (*Gcdh*$^{-/-}$-Lis) replica genótipo e fenótipo de pacientes com a doença. Apesar da intensa investigação, a compreensão das causas da degeneração estriatal e da neuropatologia na AG-I ainda é limitada, e uma das hipóteses postuladas traz o ácido quinolínico (AQ), um intermediário da rota das quinureninas, a principal rota catabólica do triptofano, como um dos responsáveis pelas alterações neurológicas vistas na doença. O AQ é um agonista de receptores NMDA, exercendo excitotoxicidade através do sistema glutamatérgico, podendo exercer uma toxicidade sinérgica com os ácidos orgânicos que se acumulam no tecido encefálico de pacientes com AG-I. Estes fatores podem contribuir para a encefalopatia e a epileptogênese vistas na doença. Assim, o objetivo deste trabalho foi avaliar o eletroencefalograma de animais *Gcdh*$^{-/-}$ que receberam dieta com sobrecarga de lisina e injeção intraestriatal de AQ, a fim de identificar fatores associando a rota das quinureninas à neuropatologia da AG-I. Para isso, análises comportamentais e eletroencefalográficas foram conduzidas no modelo animal para AG-I (*Gcdh*$^{-/-}$-Lis) e em animais *wild type* com dieta com sobrecarga de lisina *(Gcdh*$^{+/+}$-Lis*)*, considerando os efeitos da injeção intraestriatal bilateral de AQ. Verificou-se que o modelo animal para AG-I apresenta maior susceptibilidade a crises epilépticas induzidas por AQ, apresentando maior número destas crises e menor latência para a primeira crise. Além disso, estes animais apresentam alterações significativas no perfil de oscilações cerebrais, caracterizado pela diminuição no poder das oscilações teta e gama após a injeção de AQ. Durante a administração do AQ, estes animais tiveram um aumento significativo nos valores do EEG *left index* em relação aos demais grupos, indicando um grau de encefalopatia. Estes resultados indicam que o modelo animal para AG-I é mais suscetível à ação do AQ quando diretamente administrado na região estriatal, o que pode indicar seu efeito sinérgico com os ácidos orgânicos acumulados no tecido encefálico. Estes achados podem ajudar a elucidar os efeitos neurotóxicos do acúmulo de AQ como um alvo importante no mecanismo neurodegenerativo e oxidativo do estriado em camundongos *Gcdh*$^{-/-}$-Lis. O esclarecimento do papel do AQ na neuropatogênese da AG-I pode levar ao desenvolvimento de novas estratégias terapêuticas para o tratamento de pacientes com esta doença.

Palavras-chave: Acidemia Glutárica Tipo I. Ácido Quinolínico. Estriado. Eletroencefalograma. Oscilações cerebrais.

ABSTRACT

Glutaric acidemia type I (GA-I) is an autosomal recessive metabolic disease, caused by glutaryl-CoA-dehydrogenase (GCDH) deficiency, a key enzyme from lysine, hydroxylysine and tryptophan metabolism. In GA-I, accumulation of organic acids in body fluids and tissues, mainly in the Central Nervous System (CNS) occur, characterizing GA-I as a acidemia with mainly neurological signs and symptoms, inducing neurological alterations such as dyskinesia, neurological impairment and epilepsy, commonly associated with frontotemporal atrophy and striatal degeneration. The GA-I animal model, mice deficient in GCDH activity (*Gcdh*$^{-/-}$) and receiving high lysine diet (*Gcdh*$^{-/-}$-Lys) replicates genotype and phenotype seen in GA-I patients. In spite of intense investigation, comprehension of striatal damage and GA-I neuronal features is still limited. One of the proposed hypotheses brings quinolinic acid (QA), an intermediate of kynurenine pathway, the main tryptophan catabolic pathway, as responsible for neurological symptoms seen in this disease. QA is an NMDA agonist, being excitotoxic through hyperactivation of glutamatergic system, and exerts synergic toxicity with organic acids that accumulate in GA-I patients brain tissue. These factors can contribute to the encephalopathy and epileptogenesis seen in this disease. Thereby, this work evaluated the electroencephalographic (EEG) recordings of *Gcdh*$^{-/-}$-Lys mice receiving bilateral intrastriatal QA injection, aiming factors implying a kynurenine pathway role in GA-I neurological features. To test this hypothesis, behavioral and electroencephalographic analysis were conducted in GA-I animal model (*Gcdh*$^{-/-}$-Lys) and wild type mice with high lysine diet intake (*Gcdh*$^{+/+}$-Lys), considering intrastriatal QA injection effects. The GA-I animal model showed increased susceptibility to QA-induced seizures, with increased number and decreased latency for the first seizure. Furthermore, *Gcdh*$^{-/-}$-Lys mice develop significant alterations in EEG oscillation patterns, with marked decrements in theta and gamma oscillation power following QA injection. During QA administration, these animals showed significant increases in EEG left index values when compared to other groups, suggesting a certain degree of encephalopathy. These results indicate that GA-I animal model is more susceptible to QA effects when directly administrated in the striatum, suggesting its synergic action with the organic acids built up in the brain. These findings can help elucidating the neurotoxic effects of QA buildup for neurodegenerative and oxidative damage of striatum in *Gcdh*$^{-/-}$-Lys mice. Investigating the QA role in GA-I neurological features could lead to new therapeutic strategies for the treatment of GA-I patients.

Keywords: Glutaric Acidemia Type I. Quinolinic Acid. Striatum. Electroencephalogram. Brain oscillations.

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LISTA DE ABREVIATURAS

AG-I – Acidemia glutárica tipo I

AQ – Ácido quinolínico

ECoG - Eletrocorticograma

EEG – Eletroencefalograma

EROs – Espécies reativas de oxigênio

GABA – Ácido γ-aminobutírico

GAD – Glutamato descarboxilase

GCDH – Glutaril-CoA desidrogenase

LIS - Lisina

NMDA – N-metil-D-aspartato

RAGE – Receptores de produtos finais de glicação avançada

SNC – Sistema Nervoso Central

SUMÁRIO

1 INTRODUÇÃO COMPREENSIVA

1.1 ERROS INATOS DO METABOLISMO

O termo “erros inatos do metabolismo” foi introduzido pela primeira vez por Sir Archibald Garrod, médico inglês que estudava doenças como a alcaptonúria e a cistinúria. Garrod, ao observar a incidência em famílias da principal característica da alcaptonúria, a urina escurecida, e perceber a influência da consanguinidade no aparecimento dos sinais e sintomas da doença, pôde concluir que:

> Há boas razões para pensar que alcaptonúria não é a manifestação de uma doença, mas sim da natureza de uma rota alternativa do metabolismo, [...] geralmente congênita e vitalícia. (Garrod, 1902, p. 1616).

Essa conclusão, aliada ao conhecimento sobre as leis mendelianas da hereditariedade, levou Garrod a descrever um padrão autossômico recessivo para a alcaptonúria e propor que o acúmulo de ácido homogentísico na urina, um achado comum em pacientes com a doença, era provocado por uma alteração congênita em uma rota metabólica existente no corpo humano, a partir da falha de funcionamento de uma enzima. Assim, a descrição destas “individualidades do metabolismo” foi publicada por Garrod no início do século XX (GARROD, 1902).

Com base nas ideias de Garrod e dos avanços no conhecimento dos mecanismos patológicos de diversas doenças, definiu-se que os erros inatos do metabolismo resultariam da alteração patológica de determinada reação química pertencente ao metabolismo normal do indivíduo, sendo doenças de cunho multifatorial, pois resultariam da interação entre fatores genéticos e ambientais (LANPHER; BRUNETTI-PIERRI; LEE, 2006; SCRIVER et al., 1995). Apesar de raros quando considerados de forma individual, em conjunto afetam cerca de 1-2% da população geral (BARIĆ; FUMIĆ; HOFFMANN, 2001). Assim, considerando seu impacto na saúde pública e seus índices de morbidade e mortalidade para os indivíduos afetados, sua importância clínica não pode ser ignorada (WILCKEN et al., 2003).

1.2 ACIDEMIAS ORGÂNICAS

Acidemias orgânicas compreendem um grupo heterogêneo de erros inatos do metabolismo causados geralmente pela deficiência da atividade de uma enzima da rota metabólica de carboidratos, aminoácidos ou lipídeos, provocando o acúmulo de ácidos orgânicos nos tecidos corporais e a excreção destes na urina (WAJNER et al., 2002). São consideradas as doenças metabólicas hereditárias mais comuns entre crianças gravemente doentes e possuem alta prevalência em populações de alto risco (WAJNER et al., 2002).

As principais manifestações clínicas das acidemias orgânicas ocorrem no Sistema Nervoso Central (SNC), podendo ser agudas ou progressivas devido ao acúmulo dos ácidos orgânicos (DIONISI-VICI et al., 2006). Estas manifestações são bastante diversas e surgem mais frequentemente no início da vida, incluindo hipotonia, letargia, ataxia, comportamentos anormais, crises epilépticas, coma e atraso no desenvolvimento psicomotor (DIONISI-VICI et al., 2006; OZAND; GASCON, 1991). Os mecanismos que levam ao dano cerebral ainda não estão totalmente elucidados, mas sugere-se que grande parte da sintomatologia se deva à hiperamonemia (SEASHORE, 2015), que leva à hiperexcitabilidade ao prejudicar a função astrocítica e a neurotransmissão inibitória no SNC (RANGROO THRANE et al., 2013).

1.3 ACIDEMIA GLUTÁRICA TIPO I

A acidemia glutárica tipo I (AG-I) é um erro inato, de caráter autossômico recessivo, do metabolismo de aminoácidos, causado por mutações no gene que codifica a enzima glutaril-CoA-desidrogenase (GCDH), causando a deficiência de sua atividade (HOFFMANN; ZSCHOCKE, 1999; KOELLER et al., 2002). Esta enzima da matriz mitocondrial é responsável por duas etapas intermediárias do metabolismo dos aminoácidos lisina, hidroxilisina e triptofano: a desidrogenação do glutaril-CoA e a descarboxilação do glutaconil-CoA a crotonil-CoA (HÄRTEL et al., 1993; KÖLKER et al., 2011) (Figura 1). Estas duas reações, em indivíduos com AG-I, estão bloqueadas (LIESERT et al., 1999), o que leva ao acúmulo dos ácidos

glutárico, 3-hidroxiglutárico e glutacônico, além da carnitina, em fluidos corporais, principalmente no SNC (KÖLKER et al., 2006, 2011).

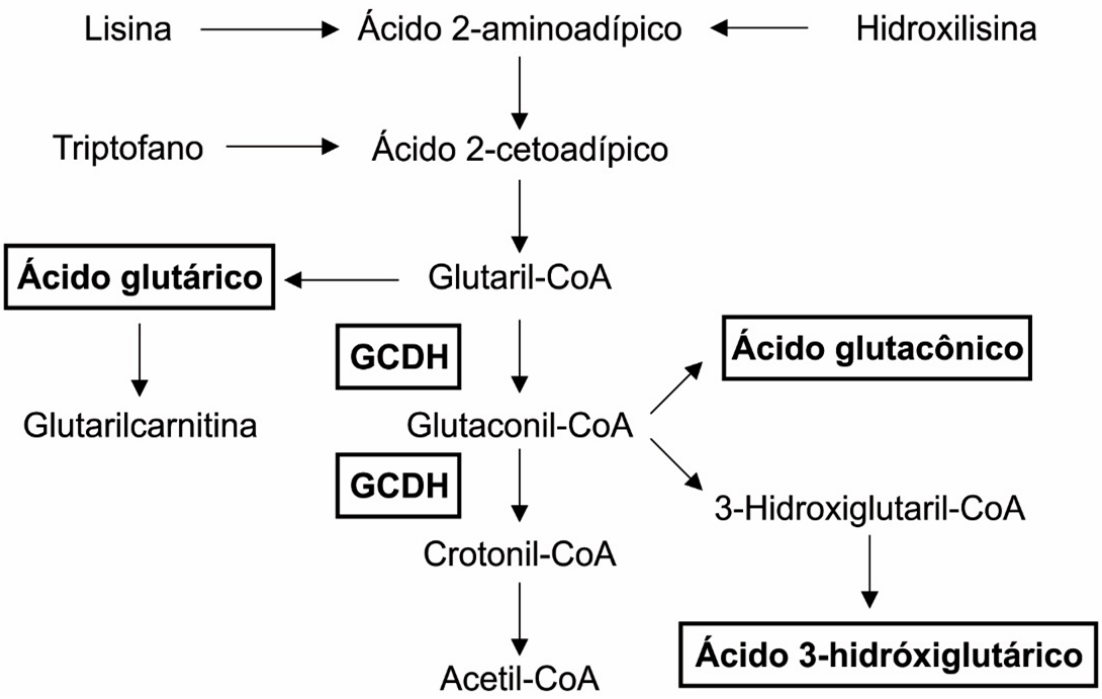

Figura 1. Rota catabólica dos aminoácidos lisina, hidroxilisina e triptofano. Retirado de: Vendramin Pasquetti, 2015

Até o momento, mais de 200 mutações foram relacionadas à deficiência na atividade da GCDH, consequentemente levando aos sinais e sintomas da AG-I (KÖLKER et al., 2011). Estas mutações afetam a atividade enzimática de forma distinta, podendo a enzima manter até 40% de atividade residual (HEDLUND; LONGO; PASQUALI, 2006). Há uma correlação entre o fenótipo bioquímico e o tipo de mutação presente, porém não há correlação entre a severidade das manifestações clínicas e a mutação envolvida, uma vez que indivíduos com a mesma mutação apresentam diversidade de manifestações (CHRISTENSEN et al., 2004; HEDLUND; LONGO; PASQUALI, 2006).

Os primeiros casos de AG-I descritos na literatura datam de 1975, quando Goodman e colaboradores reportaram achados bioquímicos de irmãos que apresentavam altos níveis de ácido glutárico na urina e identificaram alterações neurológicas progressivas que seriam compatíveis com a deficiência de GCDH (GOODMAN et al., 1975). Estima-se que a prevalência mundial da AG-I é de aproximadamente 1 a cada 100000 recém-nascidos, podendo ser mais elevada em

populações mais restritas, com grande número de indivíduos que carreguem mutações responsáveis por provocar a doença (LINDNER et al., 2004).

A AG-I é considerada uma acidemia orgânica com má formação cerebral, uma vez que apresenta alteração estriatal e má formação cortical frontal e temporal como manifestações neurológicas (HOFFMANN et al., 1994; HOFFMANN; ZSCHOCKE, 1999). Clinicamente, as primeiras evidências da AG-I são vistas anteriormente ao nascimento, como a atrofia frontotemporal e a macrocefalia (GOODMAN, 2004; HOFFMANN; ZSCHOCKE, 1999). A neuropatologia da doença desenvolve-se a partir de episódios que promovem um estado catabólico, como infecções, imunização ou episódios febris, havendo uma janela de susceptibilidade a estas crises (KÖLKER et al., 2006, 2011). A principal sequela neurológica destas crises agudas é a degeneração estriatal bilateral, seguida de uma desordem motora complexa, com o desenvolvimento de distonia, discinesia, hipotonia e espasticidade (KÖLKER et al., 2011; STRAUSS et al., 2003). Outras manifestações incluem crises epilépticas, crises de encefalopatia hepática semelhantes à Síndrome de Reye e comprometimento intelectual progressivo (HOFFMANN; ZSCHOCKE, 1999; STRAUSS et al., 2003). Outros achados patológicos incluem alargamento dos ventrículos, atrofia da massa branca, gliose, edema e lesões nos gânglios da base (AMIR et al., 1989; FUNK et al., 2005; GOODMAN et al., 1977).

O diagnóstico da AG-I é realizado principalmente por cromatografia gasosa e espectrometria de massas, que detectam o aumento da excreção de ácidos orgânicos nos fluidos corporais, principalmente urina (KÖLKER et al., 2011). Alguns pacientes, porém, podem não apresentar excreção dos ácidos orgânicos na urina (BARIĆ et al., 1999). Atualmente, as alternativas para o diagnóstico destes pacientes incluem o *screening* genético para identificação de mutações no gene *GCDH* e a avaliação da atividade da enzima GCDH (KÖLKER et al., 2011).

As intervenções terapêuticas em pacientes com AG-I buscam principalmente prevenir o desenvolvimento de desordens motoras e, durante episódios de manifestação da doença, promover intervenções emergenciais (HERINGER et al., 2010; KÖLKER et al., 2011). O tratamento de manutenção compreende a administração de uma dieta com baixo teor de lisina, a fim de reduzir o acúmulo de ácidos orgânicos, e a reposição de carnitina, para evitar a sua depleção secundária. O manejo de emergência inclui a administração de uma dieta com baixo ou nenhum teor proteico durante episódios com potencial para indução a um estado catabólico,

a fim de impedir o dano estriatal (HERINGER et al., 2010). A eficiência do tratamento de manutenção está intimamente ligada ao diagnóstico precoce da AG-I em crianças, quando os primeiros sinais começam a surgir (HOFFMANN et al., 1996). Agentes farmacológicos também podem ser utilizados para o manejo da sintomatologia neurológica já desenvolvida, com o uso de benzodiazepínicos sendo eficiente nestes casos (HOFFMANN et al., 1996).

1.3.1 Modelo animal de acidemia glutárica tipo I

Para a condução de investigações adequadas a respeito da patogênese da AG-I e de como os danos neurológicos desenvolvem-se com o curso da doença, fez-se necessário o desenvolvimento de um modelo animal de AG-I, que reproduzisse tanto o genótipo (deficiência na atividade da enzima GCDH) quanto o fenótipo (danos neurológicos e degeneração estriatal) vistos em pacientes que apresentam este erro inato do metabolismo e desenvolvem os sinais e sintomas da doença.

Para isso, Koeller e colaboradores desenvolveram, em 2002, um modelo animal de deficiência da atividade da enzima GCDH em camundongos. Por meio da recombinação homóloga em células-tronco e cruzamentos de animais heterozigotos, foi possível obter camundongos com completa perda da atividade da GCDH (*Gcdh*$^{-/-}$). Esses animais também apresentam altos níveis de excreção dos ácidos glutárico e 3-hidroxiglutárico na urina, semelhante ao visto em pacientes com o erro inato do metabolismo, além de níveis elevados destes ácidos no tecido cerebral. Assim, os animais *Gcdh*$^{-/-}$ apresentam fenótipo bioquímico semelhante ao visto em pacientes com AG-I (KOELLER et al., 2002).

Segundo os próprios autores, os animais *Gcdh*$^{-/-}$ falham em reproduzir o fenótipo neurológico visto em pacientes com AG-I, uma vez que não apresentam alterações cerebrais estruturais ou degeneração estriatal. O fenótipo comportamental destes animais também não apresenta compatibilidade com as manifestações clínicas vistas em humanos, o que os autores relacionam a uma possível diferença de metabolismo estriatal entre humanos e camundongos (KOELLER et al., 2002). Assim, o modelo foi aperfeiçoado por Zinnanti e colaboradores no ano de 2006 (ZINNANTI et al., 2006). Através da administração, a partir da quarta semana pós-natal, de uma dieta com sobrecarga de lisina (4,7% de

lisina, ao contrário dos 0,9% presentes na dieta normal) em animais com deficiência da atividade da GCDH (*Gcdh*$^{-/-}$-Lis), foi possível replicar o fenótipo neurológico visto em pacientes com a doença. Os animais submetidos a este esquema de dieta apresentam dano estriatal bilateral, perda da arquitetura cortical, gliose em várias regiões cerebrais e desenvolvem crises epilépticas espontâneas (VENDRAMIN PASQUETTI et al., 2017; ZINNANTI et al., 2006). Portanto, o modelo *Gcdh*$^{-/-}$-Lis é mais adequado para o estudo dos mecanismos responsáveis pelas manifestações neurológicas na AG-I, uma vez que replica com maior fidedignidade genótipo e fenótipo vistos em pacientes com a doença (ZINNANTI et al., 2006).

1.4 DANOS ESTRIATAIS – HIPÓTESE DO ÁCIDO QUINOLÍNICO

Embora a degeneração estriatal aguda seja uma das principais causas de morbidade em pacientes com AG-I, a compreensão das causas da susceptibilidade desta região após as crises encefalopáticas permanece incompreendida, apesar de intensa investigação (GOODMAN, 2004). Muitas teorias já foram postuladas para responder a estas perguntas, porém elas falharam em justificar a vulnerabilidade frontotemporal, a estrita janela de prejuízo estriatal durante os primeiros anos de vida e as razões pelas quais alguns indivíduos com deficiência na atividade da GCDH não apresentam danos estriatais (GOODMAN, 2004).

Uma das hipóteses, primeiramente sugerida em 1987 por Melvyn Heyes, é que a AG-I induziria, através do bloqueio da rota catabólica da GCDH, o aumento da formação do ácido quinolínico (AQ), o qual se acumularia no tecido cerebral e seria responsável pelos sinais e sintomas neurológicos apresentados por pacientes com a doença (HEYES, 1987).

O AQ é um dos compostos formados através da rota das quinureninas, a principal rota catabólica do aminoácido triptofano, e está presente fisiologicamente no tecido cerebral em pequenas concentrações, na faixa micromolar (VARADKAR; SURTEES, 2004; WHETSELL; SCHWARCZ, 1989). Esta rota é relevante devido à formação de metabólitos neuroativos, conhecidos por suas funções neurotóxicas e também neuroprotetoras (VARADKAR; SURTEES, 2004). O AQ, em especial, é neurotóxico em altas concentrações, provocando perda neuronal tanto *in vivo* quanto *in vitro*, perda dendrítica e anormalidades sinápticas, além de ser relacionado

também à patogênese de doenças neurodegenerativas e inflamatórias, como HIV, epilepsia, doença de Huntington e Alzheimer (GUILLEMIN et al., 2005; HEYES et al., 1994, 1992; SCHWARCZ et al., 2010).

Sabe-se que o AQ é um agonista endógeno e seletivo dos receptores N-metil-D-aspartato (NMDA) (STONE, 1993; STONE; PERKINS, 1981) e induz excitotoxicidade devido ao aumento do influxo de Ca^{+2}, o que por sua vez ativa rotas metabólicas de lise enzimática, aumenta a produção de espécies reativas de oxigênio (EROs) e culmina na morte celular por necrose ou apoptose (LA CRUZ; CARRILLO-MORA; SANTAMARÍA, 2012). O AQ também diminui o conteúdo de ácido γ-aminobutírico (GABA) e induz lesões na região do estriado, afetando a organização do citoesqueleto na região (BEAL et al., 1986; PIEROZAN et al., 2014). Alterações na neurotransmissão inibitória foram vistas em estudo do nosso laboratório, com diminuição da atividade da enzima glutamato descarboxilase (GAD) e diminuição da liberação de GABA em animais *Gcdh*$^{-/-}$Lis (VENDRAMIN PASQUETTI et al., 2017). Além disso, o AQ exerce toxicidade sinérgica em sinaptossomas isolados de ratos adultos com os ácidos orgânicos que normalmente se acumulam nos tecidos e fluidos de pacientes com AG-I, como os ácidos 3-hidroxiglutárico e glutárico, contribuindo para acentuar lesões mitocondriais e a produção de EROs *in vitro* (COLÍN-GONZÁLEZ et al., 2015; PIEROZAN et al., 2018).

Produtos da rota das quinureninas são intermediários também da rota do glutarato, cuja enzima chave é a GCDH (Figura 2). A inibição da atividade da GCDH levaria a um aumento do catabolismo do triptofano através da rota das quinureninas, o que culminaria no aumento da formação de AQ e em seu acúmulo em fluidos e tecidos, principalmente no SNC, em pacientes com AG-I (HEYES, 1987; VARADKAR; SURTEES, 2004). Assim, evidências sugerem papel importante do AQ na neuropatologia da AG-I, estando este composto implicado em diversos mecanismos que poderiam influenciar nas manifestações neurológicas na doença. Estudos previamente publicados na literatura mostram que o AQ, quando injetado no estriado de camundongos *Gcdh*$^{-/-}$-Lis, induz alterações no metabolismo oxidativo, prejudica a homeostase redox, induz vacuolizações e edema e leva à resposta inflamatória e disfunção astrocitária (AMARAL et al., 2018; SEMINOTTI et al., 2016).

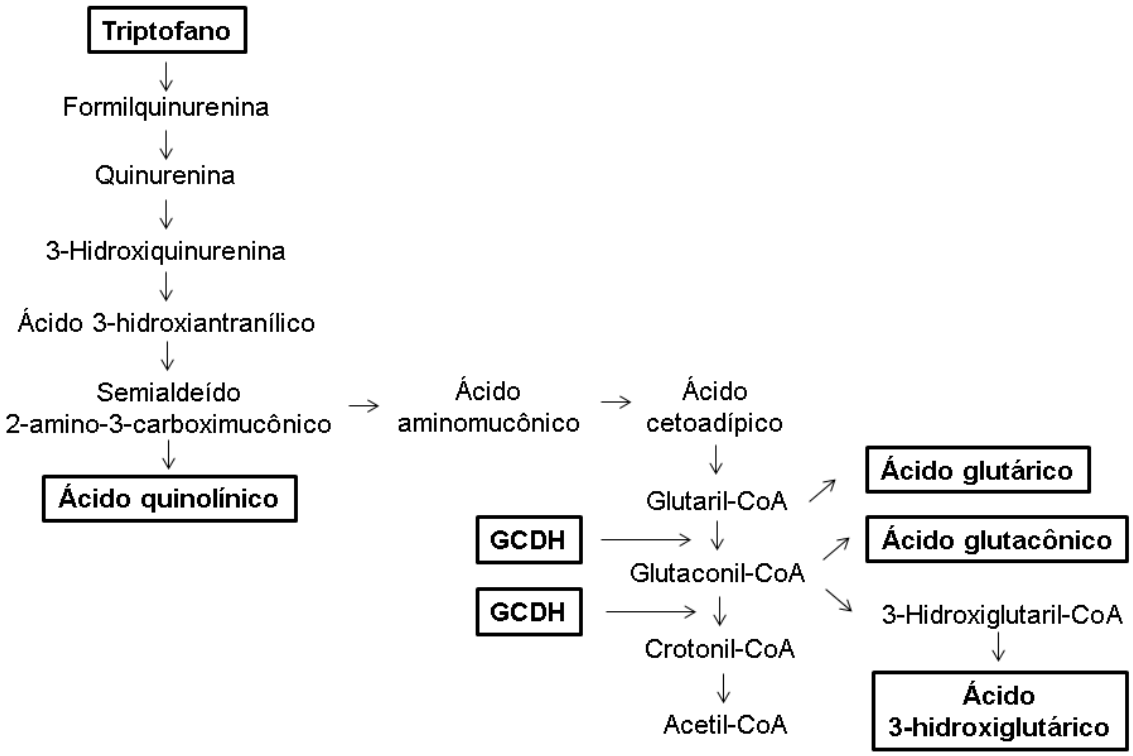

Figura 2. Rotas catabólicas das quinureninas e do glutarato, evidenciando a relação entre ambas e a formação do ácido quinolínico e de ácidos orgânicos. Adaptado de: Schuck, 2005.

A associação do AQ a pontos chave da neuropatologia da AG-I, como a janela de susceptibilidade estriatal, pode estar associada ao padrão de expressão dos receptores NMDA nos cérebros em desenvolvimento e adulto. Em roedores, o cérebro em desenvolvimento apresenta predominantemente subunidades NR2B, sendo mais suscetível à excitotoxicidade que o cérebro adulto (MCDONALD; SILVERSTEIN; JOHNSTON, 1988; VARADKAR; SURTEES, 2004). A predominância na expressão deste tipo de subunidade é vista particularmente na região estriatal durante o período de vulnerabilidade (VARADKAR; SURTEES, 2004). Além disso, o AQ também está associado à maior expressão de receptores de produtos finais de glicação avançada (RAGE) logo após sua injeção intraestriatal em roedores, o que pode estar relacionado à desregulação metabólica e ao dano oxidativo visto nesta região do cérebro durante o período de vulnerabilidade (CUEVAS et al., 2010).

1.5 CRISES EPILÉPTICAS

Crises epilépticas são manifestações clínicas resultantes de descargas paroxísticas, sincrônicas e anormais dos neurônios no cérebro. Conforme o foco de origem no cérebro, as manifestações clínicas variam, podendo ser motoras, sensoriais, sem perda da consciência (crises focais) ou com perda da consciência (generalizadas) (SCHEFFER et al., 2017).

Erros inatos do metabolismo estão incluídos como causa importante de crises epilépticas principalmente durante a infância, incluindo a AG-I. A presença de crises epilépticas espontâneas em pacientes e modelos animais da doença em virtude de alterações neurológicas já está descrita na literatura (FUNK et al., 2005; HAWORTH et al., 1991; PÖGE et al., 1997; VENDRAMIN PASQUETTI et al., 2017; ZINNANTI et al., 2006), embora a etiologia destas alterações ainda não esteja bem esclarecida.

O AQ é um composto que também induz crises epilépticas através da sua ação agonista dos receptores NMDA e do bloqueio à recaptação de glutamato pelos astrócitos, o que estimula de sobremaneira o sistema glutamatérgico (TAVARES et al., 2002; TORRES et al., 2010). A injeção de AQ de forma intraventricular ou em estruturas cerebrais específicas é utilizada como modelo animal de crises epilépticas induzidas em uma variedade de estudos (LAPIN, 1981; SCHWARCZ et al., 1984; TORRES et al., 2010). Além disso, ele parece estar envolvido na etiologia da epilepsia em humanos (NAKANO et al., 1993; SCHWARCZ et al., 1984).

1.6 ANÁLISE DO ELETROENCEFALOGRAMA

Uma das formas de investigar possíveis alterações nas oscilações cerebrais é realizar a análise do eletroencefalograma em pacientes e em modelos animais. A atividade cerebral, tanto excitatória quando inibitória, é coordenada por um sistema organizado de ritmos, aos quais pertencem as distintas oscilações cerebrais; portanto, o perfil destas diferentes oscilações traz informações importantes sobre o funcionamento neuronal e a atividade cortical (BUZSÁKI, 2006). Cada oscilação, distinguida por distintas faixas de frequência, é encontrada fisiologicamente no cérebro em diferentes estados comportamentais, momentos do ciclo circadiano ou

estruturas específicas, variando sua função e seu significado conforme o estado do indivíduo e a região cerebral considerada.

As oscilações cerebrais são registradas pelos eletroencefalograma (EEG) e eletrocorticograma (ECoG) (BUZSÁKI; ANASTASSIOU; KOCH, 2012). Uma vez obtidos, registros eletroencefalográficos podem ser filtrados e processados para a obtenção de uma série de informações, como a identificação de padrões anormais de oscilações cerebrais, que estão diretamente relacionados a alterações nos mecanismos geradores das oscilações e na circuitaria neuronal na região que está sendo registrada. A análise qualitativa do EEG nos permite identificar alterações focais, multifocais ou generalizadas, como no caso de lesões, estados alterados de consciência, a presença de crises epilépticas e de atividade interictal, ou mesmo mudanças fisiológicas, como estados de sono ou de vigília (BLUM; RUTKOVE, 2007). Para um estudo mais detalhado dos ritmos cerebrais, pode se realizar análises quantitativas através da decomposição das oscilações, que informarão as faixas de frequência predominantes em determinado período de tempo (denominado “poder” de uma oscilação) e a presença de alterações em determinada frequência no tempo (BUZSÁKI, 2006).

Análises eletroencefalográficas em camundongos *Gcdh*$^{-/-}$-Lis demonstraram que estes animais possuem atividade cortical alterada, uma vez que apresentam crises epilépticas espontâneas e mudanças no padrão de oscilações corticais, com maior predominância de oscilações delta em relação a oscilações teta e gama (VENDRAMIN PASQUETTI et al., 2017). Estas alterações podem estar diretamente relacionadas às alterações neuronais e comportamentais vistas tanto em pacientes com AG-I quanto no modelo animal para a doença, como encefalopatia (VENDRAMIN PASQUETTI et al., 2017).

Também foi possível, através do EEG, identificar a ação do AQ em diferentes estruturas cerebrais de modelos animais. Um estudo realizado há alguns anos mostrou que o AQ, quando injetado no hipocampo, foi capaz de induzir crises epilépticas e alterar as oscilações cerebrais, levando a um predomínio de oscilações de baixa frequência (SCHWARCZ et al., 1984). Em outro estudo, animais que receberam AQ por via intraventricular apresentaram crises epilépticas e diminuição no poder da oscilação teta durante períodos interictais (TORRES et al., 2010). Assim, a análise das oscilações cerebrais pode fornecer informações importantes sobre patologias que envolvam algum grau de comprometimento cerebral.

1.7 JUSTIFICATIVA

Diversas hipóteses já propostas na literatura falharam em justificar alguns aspectos ainda obscuros do desenvolvimento de sinais e sintomas neurológicos na AG-I, como a vulnerabilidade frontotemporal ainda durante a gestação, a presença de uma estreita janela temporal para o desenvolvimento da lesão estriatal após crises encefalopáticas e a variabilidade fenotípica entre pacientes com AG-I. A hipótese do ácido quinolínico apresenta-se como uma justificativa plausível para estes fatores, já que ele é capaz de induzir modelo animal para AG-I quando administrado no estriado. A análise detalhada dos registros eletroencefalográficos pode contribuir para a compreensão do aumento da susceptibilidade aos danos cerebrais e às crises epilépticas dos animais *Gcdh*$^{-/-}$-Lis expostos ao AQ.

1.8 OBJETIVOS

1.8.1 Objetivo geral

No presente trabalho, pretendemos avaliar parâmetros do perfil eletroencefalográfico do modelo animal de AG-I, animais deficientes na atividade da enzima GCDH que receberam dieta com sobrecarga de lisina (*Gcdh*$^{-/-}$-Lis) e injeção intraestriatal de ácido quinolínico, a fim de identificar possíveis fatores relacionando a rota das quinureninas à neuropatologia da AG-I.

1.8.2 Objetivos específicos

1) Avaliar a susceptibilidade às crises induzidas pela injeção intraestriatal de AQ em animais *Gcdh*$^{-/-}$ e controle (*Gcdh*$^{+/+}$), observando a presença, duração e latência das crises epilépticas;

2) Analisar o perfil eletroencefalográfico de animais *Gcdh*$^{-/-}$ e *Gcdh*$^{+/+}$ após a injeção intraestriatal bilateral de AQ e quatro dias após o início da dieta com sobrecarga de lisina;

3) Verificar a existência de efeitos da administração de dieta com sobrecarga de lisina e da injeção intraestriatal de AQ em animais *Gcdh*$^{-/-}$ e *Gcdh*$^{+/+}$, através da avaliação da taxa de mortalidade e da presença de crises epilépticas espontâneas.

2 ARTIGO CIENTÍFICO

O artigo intitulado "***IN VIVO* ELECTROPHYSIOLOGICAL EVALUATION OF AN ANIMAL MODEL OF GLUTARIC ACIDEMIA TYPE I FOLLOWING INTRASTRIATAL QUINOLINIC ACID INJECTION**" foi formatado conforme as normas para publicação junto ao periódico *Epilepsia*.

Tipo de artigo: Research article

In vivo electrophysiological evaluation of an animal model of glutaric acidemia type I following intrastriatal quinolinic acid injection

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Summary

Objectives: Glutaric acidemia type I (GA-I) is an inborn error of metabolism of lysine, hydroxylysine and tryptophan aminoacids, caused by glutaryl-CoA-dehydrogenase (GCDH) deficiency and characterized by the buildup of organic acids in body fluids and brain. After acute catabolic states, patients develop striatal degeneration. However, the mechanisms behind this damage are still unknown. Quinolinic acid (QA), a metabolite of tryptophan catabolism, is excitotoxic and acts synergically with organic acids, thus it is likely to have a role in neurological features of GA-I. The aim of this study was to investigate whether GA-I animal model, *Gcdh*$^{-/-}$ mice exposed to high lysine diet (*Gcdh*$^{-/-}$-Lys), exhibits EEG profile alteration and increased susceptibility to seizures induced by intrastriatal QA injection. **Methods:** The characteristics of QA-induced seizures and changes in brain oscillatory patterns in *Gcdh*$^{+/+}$-Lys and *Gcdh*$^{-/-}$-Lys mice were evaluated by video-EEG recordings. **Results:** *Gcdh*$^{-/-}$-Lys mice exhibited increased susceptibility to QA-induced seizures, with decreased latency for the first seizure and increased number of animals with seizures. *Gcdh*$^{-/-}$-Lys-QA mice also presented disrupted brain oscillations, with decreases in theta and gamma power and predominance of lower frequencies, as well as increased mortality rate. **Significance:** As several key points of GA-I neuropathogenesis are not yet elucidated, many hypotheses have been proposed. However, many of them failed to explain the main aspects of GA-I neurological features. The QA hypothesis is feasible, as *Gcdh*$^{-/-}$-Lys mice exhibits increased susceptibility to seizures induced by this metabolite when directly injected in the striatum. Increased knowledge about the role of QA in GA-I could yield the development of new therapeutic strategies for patients with GA-I.

Key words: Glutaric acidemia type I, quinolinic acid, striatum, electroencephalogram, brain oscillations

Introduction

Glutaric acidemia type I (GA-I) is an inborn error of the metabolism of aminoacids, caused by the deficiency of the enzyme glutaryl-CoA-dehydrogenase (GCDH)[1]. This mitochondrial enzyme has a key role on lysine, hydroxylisine and tryptophan metabolism, and the lack of its activity leads to accumulation of organic acids, such as glutaric, 3-hydroxyglutaric and glutaconic acids, and glutarylcarnitine in body fluids and tissues, mainly in the Central Nervous System (SNC)[2]. GA-I has an estimated prevalence of 1 in 100,000 newborns[3]. Patients with this disease develop mainly neurological signs and symptoms, including frontotemporal atrophy, striatal degeneration, progressive dystonia, epilepsy and intellectual impairment, which develop as consequences of acute catabolic states provoked by either fever, infections or immunization, leading to episodes of encephalopathy[2,4].

In 2002, Koeller and coworkers developed a murine model of GA-I, using molecular biology techniques to knockout *Gcdh* gene and promote total loss of GCDH activity[1]. The so called *Gcdh*$^{-/-}$ were able to replicate the genotype of GA-I patients, but not the complete phenotype, as they lacked some of the main neurological findings of GA-I, such as striatal damage[1]. This model was further improved in 2006 by Zinnanti and coworkers[5]. Beginning with a high lysine diet intake four weeks after birth, this murine model, now called *Gcdh*$^{-/-}$-Lys, was able to show many of the common neurological characteristics to GA-I in humans, such as striatal damage and neuronal loss, thus being reliable for GA-I neuropathology studies[5].

Although striatal degeneration is a key finding in GA-I patients, little is known about the susceptibility of this structure after episodes of acute encephalopathy, and so far many of the studied hypotheses failed to explain crucial aspects of GA-I neuropathology, such as frontotemporal vulnerability before birth and the existence

of a development window for striatal damage[4]. One of these hypotheses, first proposed in 1987 by Melvyn Heyes, indicates the buildup of quinolinic acid (QA), a metabolite from the kynurenine pathway, the main tryptophan catabolic pathway, in the CNS as one of the responsible factors for the GA-I neurological features[6]. The QA is an N-methyl-D-aspartate (NMDA) receptor agonist, causing excitotoxicity through Ca^{+2} influx[7]. The QA also disrupts the GABAergic system, lowering GABA content and causing striatal damage[8], and acts synergically with organic acids that normally increment in GA-I patients[9]. As GCDH pathway is impaired by the deficient activity of one of its key enzymes, tryptophan metabolism through the kynurenine pathway increases, leading to an increment in QA in the brain of GA-I patients[10]. Thus, growing evidence suggest an important role of QA in the GA-I neurological features, as this metabolite, when injected directly in *Gcdh*$^{-/-}$-Lys mice striatum, leads to disruption of redox homeostasis, edema and inflammatory response[9,11].

Electroencephalogram (EEG) analysis in *Gcdh*$^{-/-}$-Lys mice can bring new information concerning QA effects on GA-I neuropathology, as patterns of brain oscillations are common to specific brain activities or structures and may reflect pathological states[12]. A previously published work from our group showed that *Gcdh*$^{-/-}$-Lys mice have altered EEG power spectrum parameters, with increased delta waves and decreased theta and gamma waves and spontaneous recurrent seizures, which could be related to behavioral and neurological alterations seen both in GA-I patients and *Gcdh*$^{-/-}$-Lys mice, such as encephalopathy[13]. Studies concerning the effect of QA in EEG power spectrum in other experimental models have shown that this metabolite is responsible for induced seizures and increments in lower frequency oscillations[14,15]. Thus, here we use *Gcdh*$^{-/-}$-Lys mice receiving intrastriatal QA

injection to evaluate electroencephalographic and behavioral parameters and further investigate the contribution of QA on GA-I neurological features.

Material and Methods

All procedures were approved and strictly performed in accordance with the Brazilian legal guidelines (Law #11.794/2008) and the Ethical Committee for the Care and Use of Laboratory Animals of Hospital de Clínicas de Porto Alegre (HCPA) #14-0544. All efforts were made to minimize animal suffering, discomfort and the number of animals needed for reliable data production.

Animals

Gcdh$^{+/+}$ and *Gcdh*$^{-/-}$ male and female mice (P28-34 days old) were obtained from HCPA Animal Facility. Animals were housed in 22 x 37 x 18cm Plexiglass cages (up to five animals per cage before the surgery and one per cage after the electrode and cannulae implantation) in an acclimatized room (22-26°C) with a 12-h light/dark cycle. The animals had free access to water and either standard diet (20% protein, 0.9% lysine) or high lysine diet (20% protein, 4.7% lysine) *ad libitum*, according to the experiment timeline. All animals received standard diet up to P29. At P30 all *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ mice begun the high lysine diet intake and, at P32, were further separated in four groups, as explained in detail below.

Electrodes and cannulae implantation

Mice (P28) were anesthetized with ketamine:xylazine (80-120 mg/kg: 10-16 mg/kg, i.p.) for electrode and cannulae implantation. Two recording stainless steel subdural electrodes were bilaterally implanted in parietal cortex (-2.0mm AP from Bregma and

±1.2mm LL). The reference electrode and one screw for fixation were placed in the occipital bone. Two intrastriatal cannulae were implanted bilaterally (0mm AP from Bregma, 2.5mm DV, ±2.5mm LL) for posterior injection of either quinolinic acid (QA) or vehicle (V). Dental cement was used to secure the electrodes and cannulae in place. After surgery, each animal was placed individually in a Plexiglass cage for recovery.

Video-EEG recordings

At P30 each animal had its home cage transferred to an observation box, where the electrodes were connected to an amplifier (MAP-32, Plexon, Inc.) and the baseline video-EEG recording for each animal was performed for 20min-period. Afterwards all *Gcdh*$^{-/-}$ and *Gcdh*$^{+/+}$ mice started taking the high lysine diet as stated above. At P32, each animal received, through each intrastriatal cannulae, 1 µL injection of either vehicle (saline, 0.9%) or 50 mM QA solution (50 nmol) dissolved in saline. A 10 µL Gastight® syringe (Hamilton Company) was used to measure the volumes injected and to slowly diffuse the solutions through the cannulae into the striatum of both hemispheres. The QA dose corresponds to 4.55 nmol/g. After the administration, the syringe was left in place for additional 30 seconds and then slowly withdrawn. The doses of QA used were based on previous studies[11,16]. According to the injection received, the animals were divided in four final groups as following: *Gcdh*$^{+/+}$-Lys-V group (*Gcdh*$^{+/+}$ mice receiving high lysine diet and vehicle injection); *Gcdh*$^{+/+}$-Lys-QA group (*Gcdh*$^{+/+}$ mice receiving high lysine diet and QA injection); *Gcdh*$^{-/-}$-Lys-V group (*Gcdh*$^{-/-}$ mice receiving high lysine diet and vehicle injection); and *Gcdh*$^{-/-}$-Lys-QA group (*Gcdh*$^{-/-}$ mice receiving high lysine diet and QA injection).

Immediately after the injection, a 1h-period of video-EEG recording was performed for each animal, to evaluate the susceptibility to QA-induced seizures. Seizure development, latency and duration were evaluated by revising the videos and analyzing the EEG recordings. At P34 a 1h-period of video-EEG was further recorded to confirm whether *Gcdh*$^{-/-}$-Lys mice were able to present spontaneous recurrent seizures (SRS) as described before[13] and to evaluate the possible long-term effects of QA injection on brain oscillations.

EEG analysis

The EEG data analysis was first carried out using the pClamp 10.3 software (Molecular Devices) to identify the QA-induced seizures right after injection in all animals tested and the occurrence of SRS in *Gcdh*$^{-/-}$-Lys mice. The detailed analysis of power frequencies of EEG recordings was carried out using built-in routines in MATLAB software (Mathworks Inc.). EEG signals were filtered at 0.1-500Hz followed by digitalization at 1 kHz for posterior analysis. The time-frequency decomposition of all EEG recordings was performed using the spectrogram function that uses a Fourier transform. The decomposed signal was quantified in seven frequency bands: delta (1-4 Hz), theta (4-12 Hz), slow gamma (30-50 Hz), middle gamma (50-90 Hz), fast gamma (90-150 Hz), ripples (160-235 Hz) and fast ripples (250-500 Hz). The analysis was performed in two 20-seconds epochs from baseline recordings (P30) and four 30-seconds epochs from recordings obtained immediately after the injection (P32) and four days after the baseline recordings (P34). Those epochs were chosen, according to the video observation, from moments where the mice were not moving, in order to prevent muscular artifacts on the EEG recordings. The four epochs from EEG recordings taking place immediately after the injection were also chosen based

on an hour quadrant in order to identify the progression of EEG alterations induced by QA injection (P32 0min, 15min, 30min and 45min, respectively). All epochs were chosen at least three minutes far from electroencephalographic seizures. Quantification values were normalized by the ratio from P32 or P34 epochs per baseline epochs. The spectral power data was normalized to the baseline values.

For EEG left index analysis, the power spectral density was obtained using a *pwelch* function. The EEG left index was calculated as the logarithm of the ratio between the power of the lower frequencies (1-7.4 Hz) and the higher frequencies (13.5-26.5 Hz). Based on values stated by Vogels et al. (1996)[17], a left index of 0.9 or above (or comparable linear values above 8) was considered abnormal. Lower left index values indicate a left shift of the EEG, that means the predominance of lower frequencies on the EEG[18], and are indicative of encephalopathy or coma.

Cannulae identification

At the end of the experiments, each animal was euthanized and, using a 10 µL Gastight® syringe (Hamilton Company), 1 µL of methylene blue dye was slowly diffused through the cannulae. The brains were removed, left overnight in 4% paraformaldehyde solution and then stored in 30% sucrose solution in low temperature. Coronal brain slices (150 µm thick) were cut in a vibratome (VTS-1000, Leica) in a 4°C Phosphate Buffered Saline (PBS) solution and mounted in slides. The position of the cannulae implanted was confirmed by light microscopy, according to methylene blue dye location. Figure 1 shows a brain slice, with methylene blue dye evident in the site of cannulae implantation for intrastriatal QA injection.

Statistical analysis

The effect of QA on seizures induction and mortality rate were expressed as percentage and they were analyzed using Chi-square test. The number of QA-induced seizures was expressed as mean ± SEM and it was analyzed using unpaired *t* test. Seizure duration was expressed as median ± interquartile range and it was analyzed using Mann Whitney test. The survival curves for latency for the first QA-induced seizure and mortality up to P34 were analyzed using Log-rank (Mantel-Cox) Test. Data from EEG power spectral analysis and left index were expressed as mean ± SEM and they were analyzed using One-way ANOVA followed by Tukey's post-hoc test or Repeated measures ANOVA followed by Tukey's post-hoc test. The significance level (α) considered was 0.05. However, for the EEG power spectral analysis the significance level (α) of 0.01 was used since some data did not fit in a normal distribution. Differences were considered significant when $p<0.05$.

Results

Gcdh$^{-/-}$-Lys mice exhibited increased susceptibility to QA-induced seizures

QA is a proconvulsive agent[19,20] and its buildup concentration has neurotoxic effects that could act synergically with organic acids that accumulate in body fluids and brain in GA-I, contributing to the physiopathology of this disease[9]. Therefore, we evaluated whether *Gcdh*$^{-/-}$-Lys mice, the animal model of GA-I, exhibited increased susceptibility to QA-induced seizures. To address this issue, we performed intrastriatal injection and video-EEG recordings in the four groups of animals described above. We observed that the number of *Gcdh*$^{-/-}$-Lys-QA animals (11/13, 85%) that had QA-induced seizures was significantly higher than *Gcdh*$^{+/+}$-Lys-QA animals (5/10, 50%), whereas no *Gcdh*$^{+/+}$-Lys-V or *Gcdh*$^{-/-}$-Lys-V animals had

seizures (Chi-square; *p<0.0001; Fig. 2A). We also compared the number, duration and latency of QA-induced seizures between *Gcdh*$^{-/-}$-Lys-QA and *Gcdh*$^{+/+}$-Lys-QA groups. The number of seizures per animal and duration of QA-induced seizures were similar for all groups (*Gcdh*$^{+/+}$-Lys-QA: 6±3 seizures; *Gcdh*$^{-/-}$-Lys-QA: 5±1 seizures; unpaired t test, p=0.379; Fig. 2B; *Gcdh*$^{+/+}$-Lys-QA: median 11 s, interquartile range: 5 – 24.5 s; *Gcdh*$^{-/-}$-Lys-QA: median 15 s, interquartile range: 7 – 23.25 s; Mann-Whitney U Test, p=0.1425; Fig. 2C). Therefore, the behavioral and EEG analysis revealed no significant differences between groups. However, *Gcdh*$^{-/-}$-Lys-QA animals had significantly shorter latencies to the first QA-induced seizure when compared to *Gcdh*$^{+/+}$-Lys-QA animals (*Gcdh*$^{-/-}$-Lys-QA: 9.5 min, interquartile range: 3.3 – 28.0 min; *Gcdh*$^{+/+}$-Lys-QA: 45.5 min, interquartile range: 13.3 – 60.0 min; Log-rank (Mantel-Cox) Test, p=0.0067; Fig. 2D). Representative ictal and interictal EEG recordings from *Gcdh*$^{+/+}$-Lys-QA and *Gcdh*$^{-/-}$-Lys-QA groups are shown in Figure 2E$_1$ and 2E$_2$, respectively.

Gcdh$^{-/-}$-Lys-QA mice show decreased theta and gamma oscillations

In order to investigate differences in the brain activity between *Gcdh*$^{+/+}$-Lys and *Gcdh*$^{-/-}$-Lys animals, as well as address the effect of intrastriatal QA injection on electroencephalographic parameters, the EEG power spectral analysis was performed. After intrastriatal QA injection, *Gcdh*$^{-/-}$-Lys-QA animals showed significant decreases in theta and gamma oscillations when compared to both *Gcdh*$^{+/+}$-Lys-V and *Gcdh*$^{-/-}$-Lys-V groups (Table 1). We observed that *Gcdh*$^{-/-}$-Lys-QA animals showed decreased delta, theta, slow gamma, middle gamma and fast gamma oscillations immediately after the injection (P32 0min) when compared to *Gcdh*$^{+/+}$-Lys-V animals. They also showed decreased theta, slow gamma and middle gamma

oscillations when compared to *Gcdh*$^{-/-}$-Lys-V animals (Table 1). The decrease in theta and slow gamma seen in *Gcdh*$^{-/-}$-Lys-QA animals persisted up to the end of recording period, whereas the decrease in middle gamma lasted up to 30 minutes after the QA injection (P32 30min). The decrease in fast gamma was only seen immediately after QA injection. Finally, decreases in theta and slow gamma, when *Gcdh*$^{-/-}$-Lys-QA animals were compared to *Gcdh*$^{-/-}$-Lys-V group, were noted up to 30 minutes after injection. There were no significant differences in the frequency spectral power analyzed between *Gcdh*$^{+/+}$-Lys-QA and *Gcdh*$^{+/+}$-Lys-V animals (Table 1).

When the same analysis was performed at P34, no significant differences were observed between groups (Table 1). Therefore, *Gcdh*$^{-/-}$-Lys-QA animals had their brain activity mostly affected at the day of QA injection when compared to both *Gcdh*$^{+/+}$-Lys-V and *Gcdh*$^{-/-}$-Lys-V animals.

Gcdh$^{-/-}$-Lys-QA mice show increased EEG left index and mortality rate

The EEG left index analysis is used to determine the level of encephalopathy or coma[17,18]. Therefore, we measured the EEG left index to evaluate the effect of QA in GA-I animals. Figure 3A-F shows scatter plots of left index values for each animal group at different time-periods, from baseline (P30) up to 4 days after high lysine diet intake (P34). The respective mean values are shown on Table 2.

Immediately after the injection (P32 0min, Fig. 1B), *Gcdh*$^{-/-}$-Lys-QA mice exhibit significantly higher left index values (above 0.9) when compared to *Gcdh*$^{+/+}$-Lys-V and *Gcdh*$^{-/-}$-Lys-V groups. All groups had significantly higher left index values when compared to their baseline values (Table 2). At 15, 30 and 45 minutes after the injection, however, this increment in the EEG left index values persisted only for

Gcdh$^{-/-}$-Lys-QA mice (Table 2, Fig. 3C-E), and it was significantly different from the other groups.

At P32 30min, left index values for *Gcdh*$^{+/+}$-Lys-V were significantly lower than *Gcdh*$^{+/+}$-Lys-QA mice and both decreased to normal values when compared to P32 0min time-period. These levels remained at the normal range up to end of the evaluation period (Table 2). Significant differences between *Gcdh*$^{-/-}$-Lys-V and *Gcdh*$^{+/+}$-Lys-V animals were also seen at P32 30min and P32 45min (Fig. 3D, E).

At P34, although left index values of *Gcdh*$^{-/-}$-Lys-QA mice remained higher when compared to *Gcdh*$^{+/+}$-Lys-QA or V mice (Fig. 3F), the levels of all groups were at the normal range (Table 2).

We also evaluated the mortality rate of *Gcdh*$^{+/+}$-Lys and *Gcdh*$^{-/-}$-Lys mice up to P34, in order to address the effect of QA injection and persistent high lysine diet intake. The mortality rate of *Gcdh*$^{-/-}$-Lys-QA mice was significantly higher when compared to all other groups (*Gcdh*$^{-/-}$-Lys-QA: 4/13 deaths, 31%; *Gcdh*$^{+/+}$-Lys-V: 0/11 deaths, 0%; *Gcdh*$^{+/+}$-Lys-QA: 1/10 deaths, 10%; *Gcdh*$^{-/-}$-Lys-V: 1/9 deaths, 11%; Chi-square test; *p <0.0001; Figure 3G). One *Gcdh*$^{+/+}$-Lys-QA animal died right after QA injection at two days of high lysine diet (Fig. 3H). At P34, only *Gcdh*$^{-/-}$-Lys-QA and *Gcdh*$^{-/-}$-Lys-V mice presented SRS (1 animal for each group). None of *Gcdh*$^{+/+}$-Lys-QA or *Gcdh*$^{+/+}$-Lys-V developed SRS.

Taken together, our results show that *Gcdh*$^{-/-}$-Lys-QA mice exhibit predominantly lower frequencies on EEG and increased mortality rate

Discussion

Since the first report regarding individuals with signs and symptoms compatible to GCDH deficiency[21], many hypothesis have been postulated to explain the

neuropathology of this disease. Many of them, however, failed to explain some crucial aspects of GA-I, such as the striatal vulnerability during the first years of life, and, in spite of intense investigation, there is little evidence supporting any of the theories[4]. Based on a previously proposed hypothesis of QA-increment contribution to GA-I neurological features[6], here we performed *in vivo* electrophysiological study to investigate the role of QA in neurological alterations in GA-I, using GCDH deficient mice taking high lysine diet (*Gcdh*$^{-/-}$Lys), and receiving intrastriatal QA injection (*Gcdh*$^{-/-}$Lys-QA). Our main findings in this animal model include increased susceptibility and decreased latency to QA-induced seizures, changes in EEG power spectrum, with predominance of lower frequency oscillations and decrease in theta and gamma oscillations, and increased mortality rate after four days of high lysine diet intake.

Excitotoxicity through the glutamatergic system is a key point to explain the mechanisms underlying the neurological features of GA-I, as the buildup of organic acids in body fluids and tissues in GA-I patients, such as glutaric and 3-hidroxyglutaric acids, can induce neurotoxicity through NMDA receptors and subsequent modulation of glutamatergic neurotransmission[22,23]. The QA also induces excitotoxicity through the glutamatergic system, as a selective NMDA receptor agonist[24], and by inducing striatal GABA depletion when applied locally[25]. However, the evidence for excitotoxicity through a buildup of organic acids or QA in brain tissue as the only mechanism underlying neurotoxicity in GA-I is still controversial[26]. Therefore, it has been postulated that organic acids and QA could act synergically, having additive effects on excitation and inhibition imbalance and neurological dysfunction[9].

In this study, we were able to further investigate the effect of QA and its possible synergism with the buildup of organic acids in GA-I using electrophysiological techniques to evaluate EEG pattern and seizure activity in *Gcdh*$^{-/-}$-Lys mice. Our data demonstrate that, although duration and number of seizures were not different between *Gcdh*$^{+/+}$-Lys-QA and *Gcdh*$^{-/-}$-Lys-QA groups, the latency for the first induced seizure was significantly shorter for *Gcdh*$^{-/-}$-Lys mice. This may indicate the increased susceptibility to QA-induced seizures in GA-I animal model, as no other factors but the development of the disease could be considered as differences between *Gcdh*$^{+/+}$-Lys and *Gcdh*$^{-/-}$-Lys mice.

Gcdh$^{-/-}$-Lys-QA mice, when compared to *Gcdh*$^{+/+}$-Lys-V and *Gcdh*$^{-/-}$-Lys-V groups, presented significant decreases in theta and gamma power at the time of QA injection, two days after high lysine diet intake. Since theta oscillations are well characterized during cognitive processes[12], the decrease in theta power could be correlated to the cognitive impairment seen in GA-I patients[27] and in the animal model[1]. Theta and gamma rhythms are also markedly regulated by the GABAergic system[12], which is known to be disrupted in *Gcdh*$^{-/-}$Lys mice[13] and impaired in the striatum following QA injection[25]. Thus, our findings suggest that acute intrastriatal QA administration could contribute to disturbances in normal brain oscillations in *Gcdh*$^{-/-}$-Lys mice, probably through disruption of GABAergic system.

The EEG left index analysis is useful to represent left shifts in the EEG power spectrum, as increases in lower frequencies power, together with decreases in higher frequencies power, are representative of neurological damage, encephalopathy and coma, thus leading to increased left index values[17,18]. As shown in Figure 3 and Table 2, *Gcdh*$^{-/-}$-Lys-QA mice show significant increase in EEG left index when compared to all groups at P32, whereas *Gcdh*$^{-/-}$-Lys-V mice only showed

this increase 30 minutes after the injection. As no differences were found between *Gcdh*$^{+/+}$-Lys groups, it is possible to suggest an additive neurotoxic effect of QA with other mechanisms underlying GA-I neurological features. All groups have shown increases in left index at P32 0min when compared to their baseline values, what may be an effect of the volume injected though the cannulae. This was quickly reverted for *Gcdh*$^{+/+}$-Lys and *Gcdh*$^{-/-}$-Lys-V animals, but not for *Gcdh*$^{-/-}$-Lys-QA mice (Table 2).

At P34, the EEG left index values for *Gcdh*$^{-/-}$-Lys-QA mice remained significantly increased when compared to *Gcdh*$^{+/+}$-Lys-V and *Gcdh*$^{+/+}$-Lys-QA groups. However, the mean left index value from *Gcdh*$^{-/-}$-Lys-QA group remained at normality, with only one mouse from this group presenting a left index value above 0.9, according to previously established values[17] (Figure 3F). It is evident that *Gcdh*$^{-/-}$-Lys-QA mice present significant changes in brain oscillations when compared to *Gcdh*$^{+/+}$-Lys-V and *Gcdh*$^{+/+}$-Lys-QA groups.

Regarding the presence of SRS in *Gcdh*$^{-/-}$-Lys mice four days after high lysine diet intake, as shown previously[13], one *Gcdh*$^{-/-}$-Lys-V and one *Gcdh*$^{-/-}$-Lys-QA mice from each group presented SRS during the time of evaluation. As video-EEG was recorded for only 1h-period for each animal at P34, it is possible that more *Gcdh*$^{-/-}$-Lys presented SRS and the short recording duration was not enough to demonstrate this finding.

We found a significant increase in mortality rate of *Gcdh*$^{-/-}$-Lys-QA mice four days after high lysine diet intake when compared to other groups (Fig. 3G, H). It is possible that the mortality at P34 could be attributed to the effect of both high lysine diet and QA injection, as the diet itself was not able to induce the mortality rate seen

in *Gcdh*$^{-/-}$-Lys-QA mice. Thus, this finding reinforces the effect of QA on GA-I pathophysiology.

In conclusion, our data reports behavioral and electrophysiological findings regarding intrastriatal injection of quinolinic acid in a murine model of glutaric aciduria type I (*Gcdh*$^{-/-}$-Lys mice). The increased susceptibility to QA-induced seizures, changes in EEG power spectrum patterns and increased mortality rate following high lysine diet intake and QA injection support the hypothesis of a synergism between organic acids and QA on the development of GA-I neurological features. Our findings could help us understand the underlying mechanisms of GA-I neurological deficits, therefore shed light to new therapeutic strategies for GA-I patient management and striatal damage prevention.

Key point box

- *Gcdh*$^{-/-}$-Lys mice are more susceptible to QA-induced seizures, as latency for the first seizure decreases and number of mice with seizures increases.
- Brain rhythms are disrupted in *Gcdh*$^{-/-}$-Lys-QA mice, with predominance of lower frequency oscillations.
- Mortality rate is increased in *Gcdh*$^{-/-}$-Lys-QA mice as a result of both QA injection and high lysine diet intake.

Disclosure of Conflicts of Interest

None of the authors has any conflict of interest to disclosure.

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Figure and table legends

Figure 1. Coronal brain slice showing the sites of cannulae implantation identified by the methylene blue dye in the striatum. Scale bar: 1 mm.

Figure 2. *Gcdh*$^{-/-}$-Lys mice exhibit increased susceptibility to QA-induced seizures following intrastriatal injection. (A) Plots of percentage of animals with QA-induced seizures (Sz). **(B)** Scatter plot of number of QA-induced seizures per animal. **(C)** Scatter plot of QA-induced seizure duration. **(D)** Curve of the latency for the first QA-induced seizure. **(E)** Representative ictal (above) and interictal (below) EEG recordings from the left parietal electrode of *Gcdh*$^{+/+}$-Lys-QA (**E$_1$**) and *Gcdh*$^{-/-}$-Lys-QA (**E$_2$**) animals.

Figure 3. *Gcdh*$^{-/-}$-Lys-QA mice exhibit increased EEG left index and mortality rate. (A-F) Scatter plots showing left index values for all groups analyzed at different time-periods. **(G)** Mortality rate of all groups evaluated up to P34. **(H)** Survival of *Gcdh*$^{+/+}$-Lys and *Gcdh*$^{-/-}$-Lys mice when days of high lysine diet intake were considered. The arrow shows when intrastriatal injection took place. *p<0.05; **p<0.005; ***p<0.0001.

Table 1: Frequency band power at different time-periods

Time*	Groups	Delta	Theta	Slow Gamma	Middle Gamma	Fast Gamma	Ripples	Fast Ripples
P32 0 min	*Gcdh*$^{+/+}$-Lys-V (n=11)	1.11±0.09	0.77±0.08	0.53±0.06	0.69±0.09	0.85±0.11	0.77±0.13	0.76±0.17
	Gcdh$^{+/+}$-Lys-QA (n=9)	0.47±0.1	0.36±0.12	0.24±0.07	0.45±0.07	0.59±0.10	0.48±0.13	0.30±0.06
	Gcdh$^{-/-}$-Lys-V (n=9)	1.08±0.19	0.76±0.16	0.52±0.1	0.77±0.12	0.81±0.12	0.86±0.19	0.81±0.19
	Gcdh$^{-/-}$-Lys-QA (n=13)	0.52±0.11[a]	0.16±0.05[a,c]	0.14±0.02[a,c]	0.30±0.04[a,c]	0.40±0.04[a]	0.38±0.05	0.36±0.08
P32 15 min	*Gcdh*$^{+/+}$-Lys-V (n=11)	1.00±0.09	0.94±0.05	0.74±0.06	0.77±0.05	0.91±0.08	0.92±0.16	0.93±0.22
	Gcdh$^{+/+}$-Lys-QA (n=9)	0.57±13.6	0.62±0.13	0.42±0.08	0.6±0.16	0.78±0.27	0.84±0.46	0.92±0.57
	Gcdh$^{-/-}$-Lys-V (n=9)	1.09±0.16	1.01±0.16	0.70±0.1	0.95±0.13	1.10±0.24	1.43±0.39	1.43±0.40
	Gcdh$^{-/-}$-Lys-QA (n=13)	0.63±0.12	0.26±0.06[a,c]	0.19±0.03[a,c]	0.37±0.04[a,c]	0.46±0.07	0.65±0.26	0.78±0.42
P32 30 min	*Gcdh*$^{+/+}$-Lys-V (n=11)	0.88±0.07	1.00±0.06	0.89±0.08	0.89±0.07	0.95±0.08	1.02±0.17	1.12±0.25
	Gcdh$^{+/+}$-Lys-QA (n=9)	0.61±0.09	0.73±0.11	0.63±0.12	0.91±0.22	1.39±0.45	1.97±0.80	2.01±0.83
	Gcdh$^{-/-}$-Lys-V (n=9)	1.23±0.17[b]	1.09±0.18	0.75±0.11	0.84±0.11	0.82±0.11	0.76±0.15	0.66±0.15
	Gcdh$^{-/-}$-Lys-QA (n=13)	0.74±0.08	0.49±0.08[a,c]	0.32±0.04[a,c]	0.53±0.06	0.61±0.07	0.60±0.11	0.46±0.08
P32 45 min	*Gcdh*$^{+/+}$-Lys-V (n=11)	1,47±0,56	1.14±0.13	0.92±0.07	1.05±0.14	1.56±0.53	2.46±1.41	3.14±2.2
	Gcdh$^{+/+}$-Lys-QA (n=9)	0.64±0.10	0.77±0.10	0.74±0.14	1.04±0.19	1.43±0.34	2.09±0.71	2.15±0.88
	Gcdh$^{-/-}$-Lys-V (n=9)	1.08±0.12	1.00±0.13	0.75±0.13	0.28±0.08[a,b]	0.96±0.25	1.26±0.57	1.21±0.57
	Gcdh$^{-/-}$-Lys-QA (n=13)	0.67±0.10	0.57±0.08[a]	0.40±0.04[a]	0.60±0.08	0.66±0.1	0.77±0.25	0.77±0.34
P34	*Gcdh*$^{+/+}$-Lys-V (n=11)	1.04±0.10	1.03±0.05	1.01±0.06	1.35±0.17	2.08±0.45	2.76±0.55	2.47±0.47
	Gcdh$^{+/+}$-Lys-QA (n=9)	1.05±0.09	1.26±0.1	0.98±0.11	1.33±0.18	2.03±0.55	2.62±1.01	1.84±0.65
	Gcdh$^{-/-}$-Lys-V (n=8)	1.29±0.15	1.23±0.11	1.09±0.09	1.31±0.11	1.71±0.25	2.77±0.79	3.05±1.0
	Gcdh$^{-/-}$-Lys-QA (n=8)	1.12±0.16	1.06±0.18	0.84±0.15	1.04±0.21	1.26±0.31	1.57±0.55	1.88±0.88

[a] difference from *Gcdh*$^{+/+}$-Lys-V group; [b] difference from *Gcdh*$^{+/+}$-Lys-QA group; [c] difference from *Gcdh*$^{-/-}$-Lys-V group. Time*=Different time-periods recorded at P32 after injection of either V or QA and at P34. One-way ANOVA followed by Tukey post hoc test, $p<0.05$, with $\alpha = 0.01$. Data is shown as mean ± SEM.

Table 2: Left index at different time-periods

Time	*Gcdh*$^{+/+}$-Lys-V (n=10)	*Gcdh*$^{+/+}$-Lys-QA (n=7)	*Gcdh*$^{-/-}$-Lys-V (n=8)	*Gcdh*$^{-/-}$-Lys-QA (n=13)
P30 Baseline	0.53±0.03	0.61±0.02	0.61±0.03	0.64±0.02
P32 0min	0.72±0.04^{d}	0.82±0.1^{d}	0.76±0.04^{d}	1.04±0.07^{a,c,d}
P32 15min	0.48±0.06^{e}	0.65±0.05	0.63±0.03	1.01±0.04^{a,b,c,d}
P32 30min	0.40±0.05^{e}	0.57±0.05^{e}	0.63±0.05^{a}	0.87±0.04^{a,b,c,f}
P32 45min	0.39±0.04^{e}	0.53±0.04^{e}	0.63±0.04^{a}	0.79±0.03^{a,b,c,d,f}
P34	0.48±0.02^{e}	0.50±0.03^{e}	0.61±0.02^{e}	0.64±0.05^{a,b,e,f,g}

$^{a\text{-}c}$ difference between groups (One-way ANOVA followed by Tukey post hoc test, $p<0.05$): ^{a} difference from *Gcdh*$^{+/+}$-Lys-V group; ^{b} difference from *Gcdh*$^{+/+}$-Lys-QA group; ^{c} difference from *Gcdh*$^{-/-}$-Lys-V group;
$^{d\text{-}g}$ difference at different time points for the same group (Repeated measures ANOVA followed by Tukey post hoc test, $p<0.05$): ^{d} difference from P30 Baseline; ^{e} difference from P32 0min; ^{f} difference from P32 15min; ^{g} difference from P32 45min. Data is shown as mean ± SEM.

Figure 1

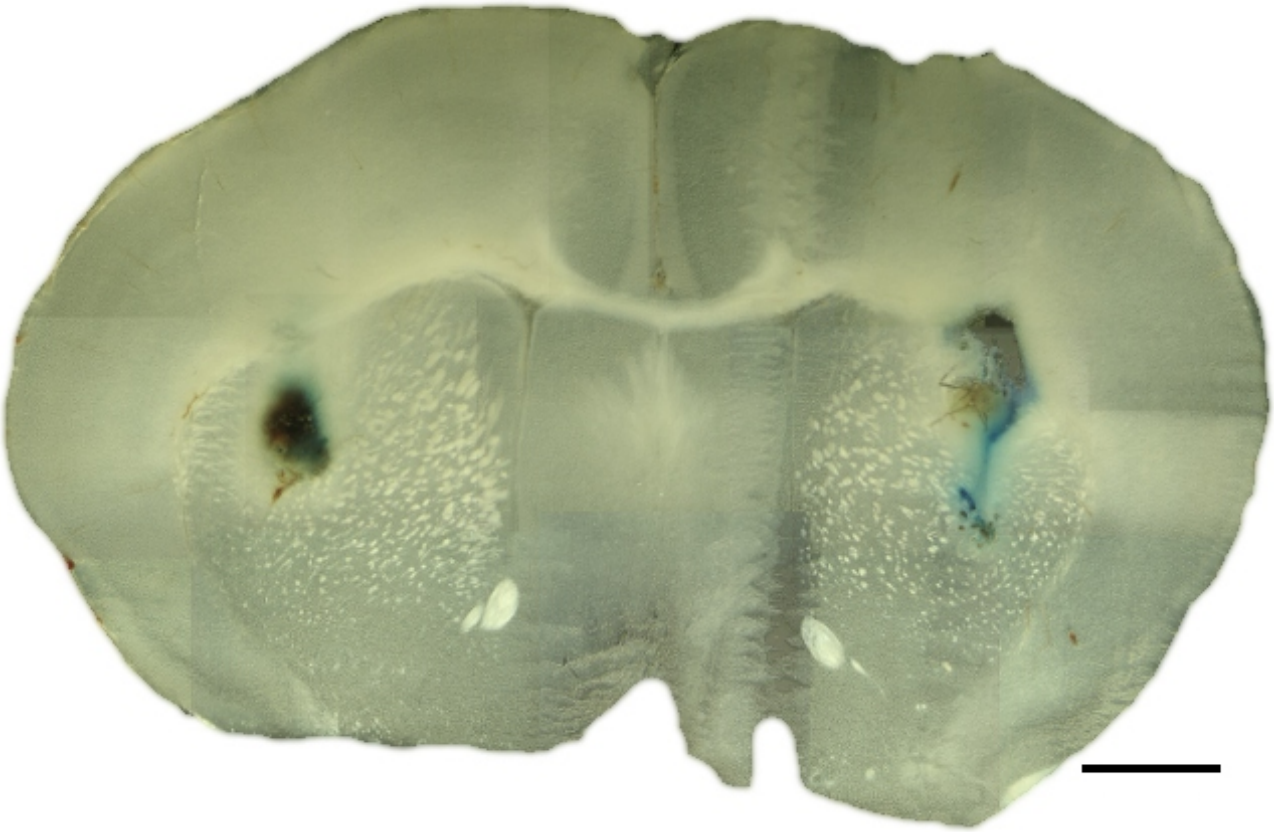


Figure 2

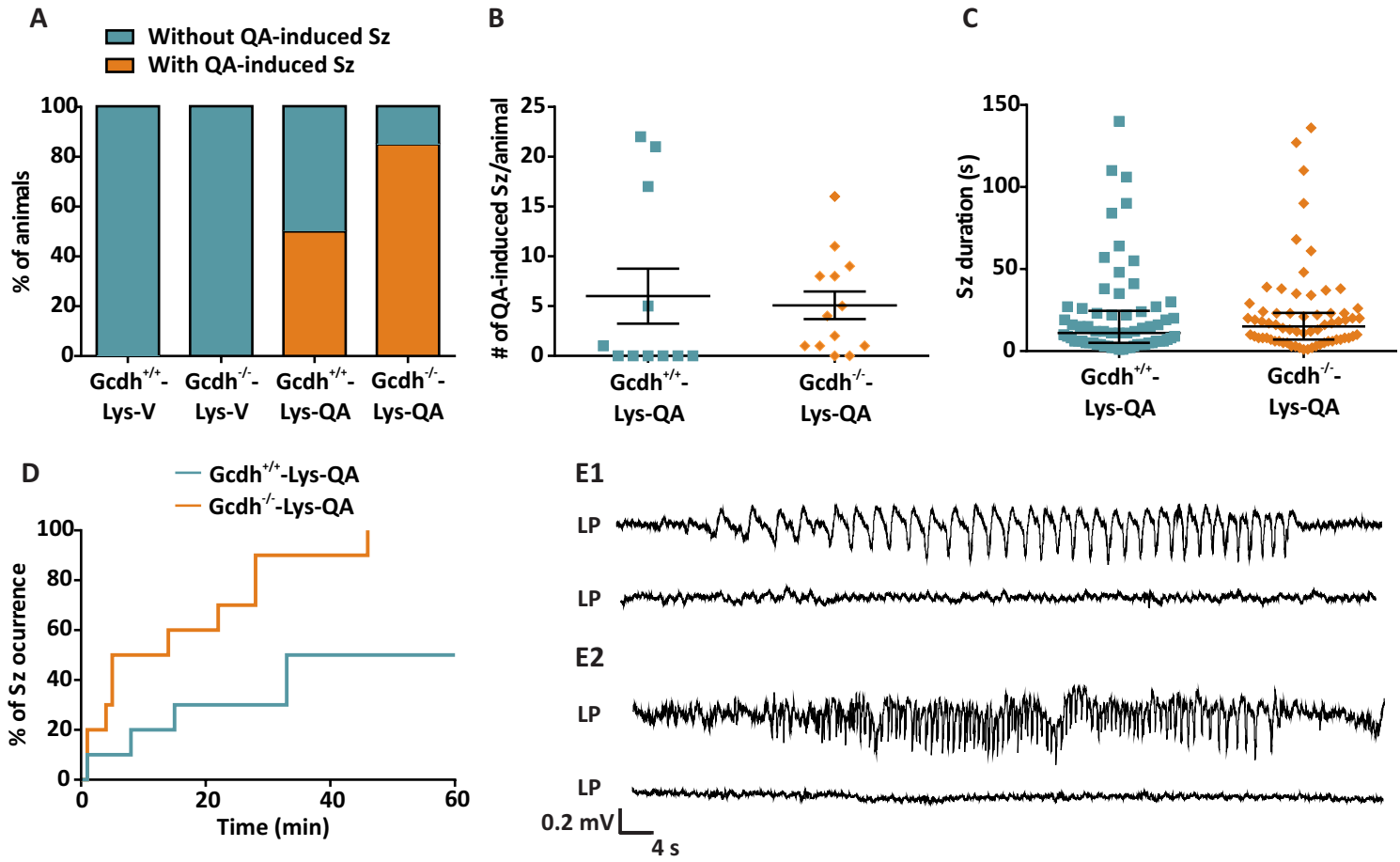
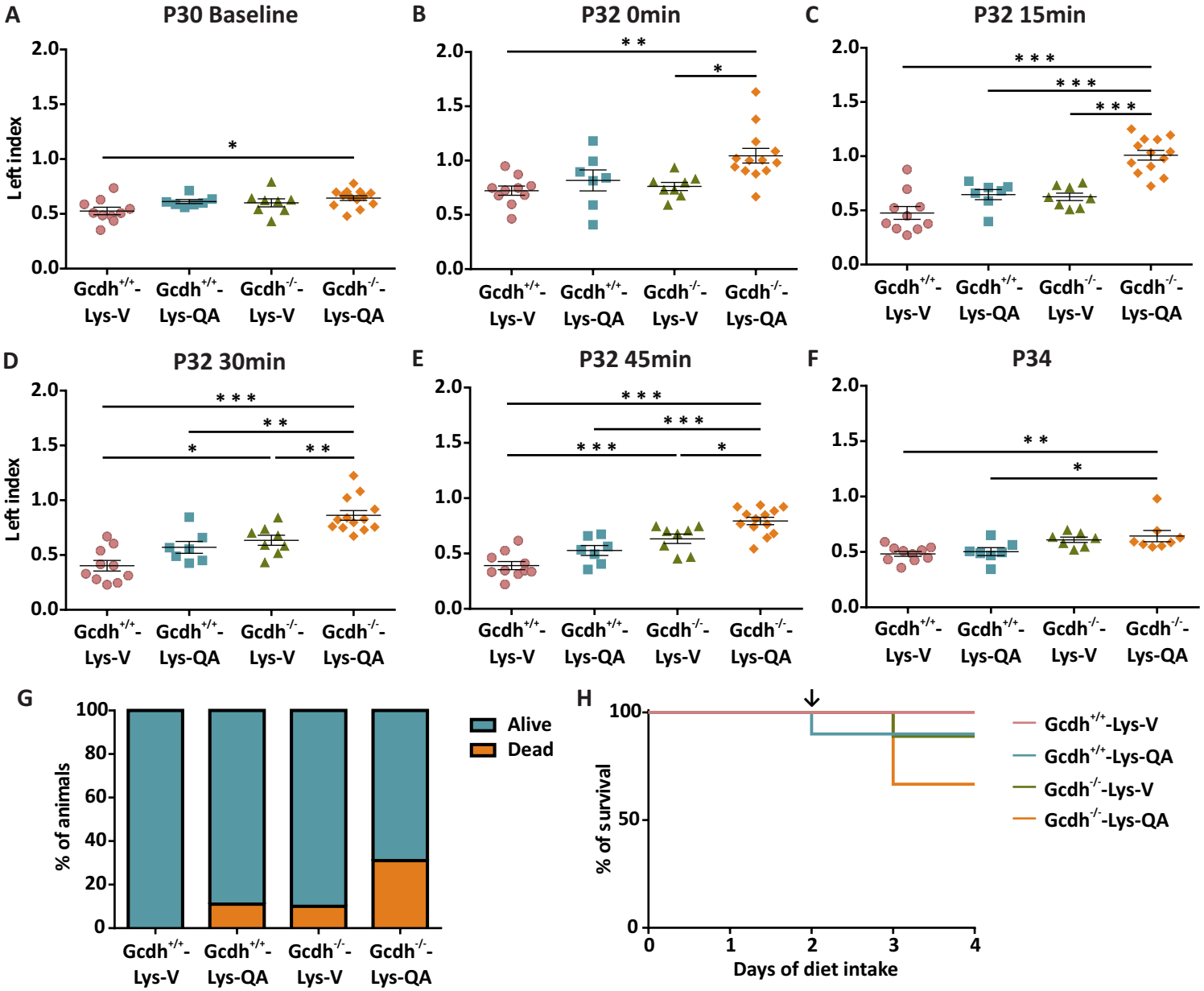

A
Without QA-induced Sz
With QA-induced Sz
% of animals
100
80
60
40
20
0
Gcdh+/+- Lys-V
Gcdh-/- Lys-V
Gcdh+/+- Lys-QA
Gcdh-/- Lys-QA
B
# of QA-induced Sz/animal
25
20
15
10
5
0
Gcdh+/+- Lys-QA
Gcdh-/- Lys-QA
C
Sz duration (s)
150
100
50
0
Gcdh+/+- Lys-QA
Gcdh-/- Lys-QA
D
Gcdh+/+-Lys-QA
Gcdh-/-Lys-QA
% of Sz ocurrence
100
80
60
40
20
0
0
20
40
60
Time (min)
E1
LP
LP
E2
LP
LP
0.2 mV
4 s

Figure 3


A
P30 Baseline
B
P32 0min
C
P32 15min
D
P32 30min
E
P32 45min
F
P34
Left index
2.0
1.5
1.0
0.5
0.0
Gcdh+/+-Lys-V
Gcdh+/+-Lys-QA
Gcdh-/--Lys-V
Gcdh-/--Lys-QA

G
% of animals
100
80
60
40
20
0
Alive
Dead
Gcdh+/+-Lys-V
Gcdh+/+-Lys-QA
Gcdh-/--Lys-V
Gcdh-/--Lys-QA

H
% of survival
100
50
0
0
1
2
3
4
Days of diet intake
Gcdh+/+-Lys-V
Gcdh+/+-Lys-QA
Gcdh-/--Lys-V
Gcdh-/--Lys-QA

3 CONCLUSÕES E PERSPECTIVAS

O presente trabalho demonstrou maior susceptibilidade a crises epilépticas induzidas pela injeção intraestriatal de AQ no modelo animal de AG-I (*Gcdh*$^{-/-}$-Lis), uma vez que estes animais apresentaram aumento no número de crises e menor latência para o desenvolvimento da primeira crise epiléptica. Além disso, animais do grupo *Gcdh*$^{-/-}$-Lis-AQ apresentaram alterações eletroencefalográficas, como diminuição no poder das oscilações teta e gama e predominância de oscilações de baixa frequência, como demonstrado pelo aumento no valor de *left index* no EEG. Além disso, verificou-se aumento significativo na taxa de mortalidade nos animais do grupo *Gcdh*$^{-/-}$-Lis-AQ em comparação aos demais grupos (*Gcdh*$^{+/+}$-Lis-V, *Gcdh*$^{+/+}$-Lis-AQ e *Gcdh*$^{-/-}$-Lis-V). Estes dados reforçam a hipótese da contribuição do AQ na neuropatogênese da AG-I.

Uma vez analisado em detalhe o efeito do AQ em parâmetros eletrofisiológicos *in vivo* de animais *Gcdh*$^{-/-}$-Lis, pretendemos aumentar o n dos grupos, para melhorar a robustez de nossas análises. Também pretendemos ampliar as análises eletroencefalográficas realizadas até então, iniciando pela razão teta/delta, que trará maiores informações sobre alterações do poder das oscilações cerebrais e corroborará com os achados a respeito do EEG *left index* dos animais *Gcdh*$^{-/-}$-Lis-AQ. Outras análises incluem espectrogramas, que demonstram de forma qualitativa e quantitativa o perfil de oscilações cerebrais de um modelo animal, e também a coerência entre as oscilações, trazendo informações sobre a interação entre sistemas que operam em diferentes frequências (SRINIVASAN et al., 2007).

Assim, através da compreensão das causas dos déficits neurológicos da AG-I, é possível investigar novas estratégicas terapêuticas para a doença, uma vez que os tratamentos atualmente disponíveis não são totalmente eficazes em impedir os danos estriatais e a progressão da doença após o desenvolvimento dos sinais e sintomas neurológicos.

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ANEXO A – NORMAS DE PUBLICAÇÃO DA REVISTA “EPILEPSIA”

Epilepsia, **59**(1):5–15, 2018
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Official Journal of the International League Against Epilepsy

INSTRUCTIONS FOR AUTHORS

Epilepsia is the official journal of the **International League Against Epilepsy (ILAE)**. The Journal publishes original articles on all aspects of epilepsy, clinical and experimental, especially of an International importance. Manuscripts should be the work of the author(s), must not have been previously published elsewhere, and must not be under consideration by another journal.

If you have a question not addressed in these pages then contact the journal at epilepsia@epilepsia.com.

Editorial Policies

(1) The Editors-in-Chief of *Epilepsia* invite manuscripts in all areas of epilepsy-related research, especially if useful for an international audience. Manuscript submission is free. As a general guide, manuscripts will be considered for publication if they contribute significant new findings to the field. The primary aim of *Epilepsia* is to publish innovative and high quality papers that provide clinical and/or basic science insights.

The Editors will make an initial evaluation of all manuscripts to determine whether they provide new important information and in the field, are in the proper format, and are appropriate for the Journal (editorial review). Reports are unlikely to be accepted for publication if they are not based on sound science and/or they provide only incremental knowledge of limited general usefulness. To assist authors in deciding whether to submit a manuscript to *Epilepsia*, we provide the following commonly encountered examples of reports which we are unlikely to publish:

(a) Papers that describe clinical features or epidemiology in a given region of the world that do not provide new insights into epilepsy not already published;
(b) Correlative studies where the sample size is too low to provide statistically sound findings;
(c) Genetic association studies in which the association has already been confirmed;
(d) Investigatory articles describing the application of a new technical variation which is not likely to have clinical utility or impact;
(e) Correlative clinical studies, which are conceived without clear hypotheses and the results of which are of little clinical utility;
(f) Basic research studies that are not grounded in epilepsy relevant hypotheses;
(g) Single group, before-after evaluations of therapeutic interventions and programs that do not include a control group;
(h) Small case series which largely replicate what is already known;
(i) Case reports (highly unlikely to be accepted unless they provide novel findings of theoretical or clinical importance).

Epilepsia will accept, review, and publish studies with negative results, provided that appropriate controls have been used, the study is adequately powered, and the results are important and or useful to others in their search community.

(2) Manuscripts describing original research, and passing the initial editorial screen, will be subject to external peer review. Publication of the data before submission to *Epilepsia* as preprints on servers external to the authors' institution such as arXiv, bioRxiv, PeerJ, and figshare are not allowed; these manuscripts will not be accepted. An abstract of the work may have been published, however, if the material in the manuscript has been presented at meetings and the abstract has been published as part of meeting proceedings. At least two reviews are generally obtained for these submissions; additional reviews may be sought at the discretion of the Editors. Appeals of rejection decisions will be considered by the Editors-in-Chief; decisions of the Editors-in-Chief are final.

(3) In the cover letter, authors should indicate that the material described in the manuscript is the work of the author(s), has not been previously published including as preprint on servers. The authors should also specify that the material included in the manuscript is not simultaneously under consideration by any other journal.

(4) As a condition of publication, *Epilepsia* requires authors to transfer copyright to the ILAE. Authors will be asked to login into Author Services and complete the appropriate license agreement via Wiley Author Licensing Service.

(5) *Epilepsia* complies with recommendations of the International Committee of Medical Journal Editors (http://www.ICMJE.org). Authors are required to include a statement at the end of their manuscript affirming that the work described is consistent with the Journal's guidelines for ethical publication (see below). *Epilepsia* is a member of the

Committee on Publication Ethics (COPE), and we adhere to its principles (http://publicationethics.org/).

(6) Data reporting should follow appropriate checklists and guidelines (e.g., STROBE for observational trials; CONSORT for clinical trials), and other checklists should be consulted for other reports including diagnostic accuracy (STARD), systematic reviews and/or meta-analyses (PRISMA, with systematic review protocol registered on PROSPERO) or neuroepidemiological studies (STROND). Checklists can be downloaded from the following:
STROBE – http://strobe-statement.org
CONSORT – http://www.consort-statement.org/consort-statement/
STARD – http://www.stard-statement.org/
PRISMA – http://www.prisma-statement.org/
PROSPERO – https://www.crd.york.ac.uk/prospero/

Epilepsia encourages authors to share the data and other artefacts supporting the results in the paper by archiving it in an appropriate public repository. Authors should include a data accessibility statement, including a link to the repository they have used, in order that this statement can be published alongside their paper. A global registry, re3data.org, is available to help authors identify relevant research data repositories. *Epilepsia* requires authors to cite data in the format proposed by the Joint Declaration of Data Citation Principles: authors; year; dataset title; data repository or archive; version (if any); persistent identifier (e.g., doi). Source: Data Citation Synthesis Group: Joint Declaration of Data Citation Principles. Martone M. (ed.) San Diego CA: FORCE11; 2014 [https://www.force11.org/group/joint-declaration-data-citation-principles-final].

(7) For animal experiments, the authors need to state that the experiments have been performed in accordance with all applicable national and/or international guidelines/laws. The authors should also provide their allowance number for performing animal experiments when available and should add a statement indicating that the principles outlined in the ARRIVE guidelines and the Basel declaration (http://www.basel.declaration.org) including the 3R concept have been considered when planning the experiments.

(8) Authors are also required to provide full disclosure of any conflict of interest as a part of the submitted manuscript (see Disclosure of Conflicts of Interest in the Manuscript Format section under Manuscript Preparation). Manuscripts that do not conform to these guidelines will not be considered for publication. Discovery of or failure to comply will result in rejection of the manuscript, retraction of the published article, and/or a ban on future submissions by the author(s).

(9) In submitting a manuscript, the submitting/corresponding author must acknowledge that: a) all co-authors have been substantially involved in the study and/or the preparation of the manuscript; b) no undisclosed groups or persons have had a primary role in the study and/or in manuscript preparation (i.e., there are no "ghost-writers"); and c) all co-authors have seen and approved the submitted version of the paper and accept responsibility for its content. The Editors reserve the right to require authors to submit their original data for comparison with the manuscript's illustrations, tables, and results.

(10) Sometimes editors make mistakes. If an author believes an editor has made a decision in error we welcome an appeal. Please contact the editor and in your appeal letter, clearly state why you think the decision is a mistake and set out specific responses to any comments related to the rejection. An appeal does not guarantee a re-review.

Types of Manuscripts

The following types of material may be considered for publication:

(1) Peer-reviewed papers (to be submitted by uploading online via Scholar One Manuscript Central http://mc.manuscriptcentral.com/epilepsia).

a. Critical Reviews and Commentaries. The Editors-in-Chief encourage submission of reviews and commentaries on topical and controversial issues. Authors planning/proposing such papers should contact the Editors-in-Chief at epilepsia@epilepsia.com before submitting their manuscripts. Authors can also approach one of *Epilepsia's* Associate Editors about possible reviews. While there are no strict length limits on this type of paper, manuscripts generally should be around 5000 words and include a maximum of 100 references. Ample figures and tables are encouraged. Longer manuscripts will be considered at the discretion of the Editors-in-Chief, but justification should be provided by the authors.

b. Full-length Original Research Articles. These articles should be limited in length to 4000 words, 50 references and no more than 6 figures and tables (combined). Additional figures and tables will be permitted at the discretion of the Editors or can be submitted as online only Supporting Information (which will be linked to the online version of the published article). Authors should aim for presenting material clearly and completely, in the most concise and direct form possible; the Introduction should be brief (typically less than 600 words), and the Discussion should be restricted to issues directly relevant to the Results (typically less than 1200 words).

c. Brief Communications. These articles including short studies, small series, case reports, etc. should describe previously unpublished material, including original research and/or clinical observations. The papers are limited generally to 1800 words (excluding the summary), 18 references, and no more than 2 figures and tables (combined). Please note that the Editors may use their discretion to request that brief communications be shortened to a length that they feel is appropriate, and may provide for a larger number of figures and tables if justified.

Brief Communications may be published online only (not in the print version of the journal) depending on their impact. They will appear in a specific issue in the electronic (online) version, and will be identified and described (Short Summary) in the Table of Contents of the printed version of that issue. The online versions will be dealt with by PubMed/ Medline and other indexing/citation systems, exactly the same way as print articles; they will be referenced by their DOI number and date of online publication.

d. Controversy in Epilepsy: For emerging areas related to epilepsy care and research for which there is more opinion than high quality data, *Epilepsia* uses the Controversy series as a venue. Authors can propose a pro- and con- position each limited to 2000 words. Contact the edi- tors at epilepsia@epilepsia.com before submitting in this series.

(2) Editorially-reviewed material (to be submitted by email to the Editors-in-Chief at epilepsia@epilepsia.com, except letters and commentaries which should be submitted online at http://mc.manuscriptcentral.com/epilepsia)

Other contributions that do not report original research will be published at the discretion of the Editors-in- Chief, with only editorial review. Such material includes: workshop reports and conference summaries, obituaries, letters/commentary to the Editors (500 word limit, and only exceptionally figures or tables), special (brief) reports from ILAE Commissions or other working groups, and announcements. Such material will usually be published in **Gray Matters**.

(3) Supplements (to be submitted as directed by the Editors-in-Chief)

Supplements, including meeting abstracts, will be published only after advance arrangements are made with the Editors-in-Chief. Guidelines for preparing supplements are given below. Proposal for, and questions about supplements should be directed to one of the Editors-in-Chief (epilepsia@epilepsia.com). Such proposals must be explicitly approved by the Editors-in-Chief, who will also confirm the page rate charge for the proposed supplement.

(4) Special reports: In some cases, special reports from ILAE Commissions or other broadly constituted working groups will be published after peer review. The corresponding author of such papers should confer with the Editors-in-Chief to determine if the full manuscript will be peer-reviewed, or whether only a short version will be considered for publication in *Epilepsia's* Gray Matters (see below).

MANUSCRIPT PREPARATION

General Style Guidelines

Manuscripts are to be submitted (and will be published) in English. Writers not fluent in English should seek assistance to ensure proper grammar and syntax, and to help generate a manuscript organization that facilitates reader understanding. Authors for whom English is a second language may choose to have their manuscript professionally edited before submission, to improve the English. A list of independent suppliers of editing services can be found at http://wileyeditingservices.com/en/. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication. The Editors will not re-write papers submitted in unacceptable English, and will return such manuscripts for revision before sending them out for review.

Use international non-proprietary (generic) names when referring to drugs; avoid proprietary (brand) names. All acronyms should be spelled out at first mention. Spell out numbers below 10 and all numbers that are used to begin a sentence; use Arabic numerals for numbers above 10 and for units of measure. Manuscript text should be double spaced with at least 1 inch margin on all sides using size 12 font. Word limits for each type of submission will generally be enforced unless there are good reasons not to do so. If manuscripts exceed these guidelines, authors should submit a cover letter explaining why the additional length is necessary.

Authors are encouraged to use the most recent terminology of seizures and epilepsy (Fisher et al., 2014) and epilepsy classification of the ILAE (Berg et al., 2010). Studies involving treatments should adhere to ILAE's classification of medically refractory epilepsy (Kwan et al., 2011).

Manuscript Format

a. Critical Reviews and Invited Commentaries

- ❑ **Title Page** (see Full-Length Original Research below)
- ❑ **Summary and Key Words**

  Reviews and commentaries should generally begin with a summary (less than 300 words) of the content. The unstructured summary should provide the reader an outline of the main points of the paper. The Summary should be followed by a list of 3-6 Key Words; please provide Key Words that will assist in the indexing of your article (i.e., make it easy for individuals who are searching PubMed to find your paper). Do not use words already incorporated into your title (those words are picked up automatically by the indexing service).

- ❑ **Body of review**

  There is no designated structure for the body of Reviews or Commentaries. Authors are encouraged, however, to use sub-headings to separate major sections and to facilitate clarity and to use figures and tables to illustrate the key issues of the document.

  Tables, figures, figure legends, references, acknowledgements, statement of compliance with the Journal's guidelines for ethical standards in publishing, disclosure of conflicts of interest, and Supplementary material as for *Full-Length Original Research* (see below)

b. Full-Length Original Research, Special Reports, and Brief Communications

- **Title Page**

  Include the following information: Full title of the manuscript which generally should be as concise and precise as possible; authors' names (first and last names, middle initial when commonly used by that author); institutional affiliation for each author (use superscripted numbers after each author's name, and a corresponding superscripted number before each institutional affiliation); contact information for the corresponding author (name, address, telephone number, fax number, e-mail address); Key Words for use by abstracting services (same as following summary); number of text pages; number of words; number of references; number of figures; number of tables.

- **Summary and Key Words**

  Provide a summary of no more than 300 words (200 words for Brief Communications). The summary for Full Length Original Research reports should consist off our sections, labeled: Objective; Methods; Results; Significance. This structured summary should concisely and specifically describe why and how the study was performed, the essential results, and what the authors conclude from the results. To promote brevity, authors may use phrases rather than complete sentences. The summary for Special Reports, Invited Commentaries, and Brief Communications is not structured, but should cover the same topics as the structured summary. The summary (structured or unstructured) should be followed by 3-6 Key Words (see above). A second short summary (less than 100 words) is required for Brief Communications that can be used in the print issue Table of Contents. Submit the second short summary as a Supporting Document.

- **Key Point Box**

  Include 3 to 5 key bullet points that summarize your article after the main body of text. Please ensure each bullet point is no longer than 140 characters. (Brief Communications do not have a key point box). An example of a key point box can be found on the *Epilepsia* Scholar One Manuscripts website (http://mc.manuscriptcentral.com/Epilepsia); please click 'Instructions and Forms' at the top right-hand corner of the homepage.

- **Introduction**

  State the objectives of the study clearly and concisely, and provide a context for the study by referring judiciously to previous work in the area. Do not attempt to present a comprehensive view of the field. Provide a statement about the significance of this research for understanding and/or treating epilepsy.

- **Methods**

  Describe the research methods in sufficient detail that the work can be duplicated; alternatively, give references (if they are readily accessible) to previous comprehensive descriptions. Identify the statistical procedures that were used and the rationale for choosing a particular method, especially if it is not standard.

  Reports of experimental studies on humans must explicitly certify that the research received prior approval by the appropriate institutional review body and that informed consent was obtained from each volunteer or patient. Studies involving animals must include an explicit statement that animal care and use conformed to institutional policies and guidelines. When animals are subjected to invasive procedures, details must be provided regarding the steps taken to eliminate/minimize pain and suffering, including the specific anesthetics, analgesics, or other drugs used for that purpose (amounts, mode of delivery, frequency of administration).

  If extensive descriptions of methods are needed, provide basic information with in the text and submit supplementary information for online Supporting Information.

- **Results**

  Results should be reported fully and concisely, in a logical order. Do not repeat methodological details from the Methods section. Where possible, use figures and/or tables to present the data in a clear and concise format. Do not repeat data in the text that are given in a table, but refer to the table. Provide textual explanations for all figures, with clear reference to the figure(s) under discussion. Descriptive information provided in figure legends need not be repeated in the text; use the text, however, to describe key features of the figures. When appropriate, give sample numbers, the range and standard deviation (or mean error) of measurements, and significance values for compared populations.

- **Discussion**

  Provide an interpretation of the results and assess their significance in relation to previous work in the field. Do not repeat the results. Do not engage in general discussion beyond the scope of the experimental results. Conclusions should be supported by the data obtained in the reported study; avoid speculation not warranted by experimental results, and label speculation clearly. Discuss the significance of the data for understanding and/or treating epilepsy.

- **Statistical Methods**

  The following guidelines assume familiarity with common statistical terminology and methods. We recommend that authors consult a biostatistician during the planning stages of their study, with further consultations during the analytical and interpretational stages.

  1. Analysis guidelines:

  - Use robust analytic methods when data are skewed.
  - Use Kaplan Meier methods, Cox Proportional Hazards, and mixed models analyses for longitudinal data.

- Account properly for statistical outliers.
- Use exact methods as much as possible in analyses of categorical data.
- Use appropriate correction procedures to account for multiple comparisons, and conduct post-hoc comparisons with statistically appropriate methods.

2. Presentation guidelines:

- Report means accompanied by standard deviations; standard errors should not be used.
- Present results with only as much precision as is appropriate.
- Present confidence intervals, whenever possible, including in figures.
- Describe quantity of missingness and methods used for handling such missingness.
- In general, present two-sided p-values. P-values larger than 0.01 should be reported to two decimal places, those between 0.01 and 0.001 to three decimal places, and those smaller than 0.001 should be reported as $p<0.001$.
- In reporting clinical trials, include a flow diagram, a completed trial checklist, and trial registration information. The CONSORT flow diagram and checklist are recommended (http://www.consort-statement.org/).

❑ **Acknowledgments**

Acknowledge sources of support (grants from government agencies, private foundations, etc.); including funds obtained from private industry. Also acknowledge (consistent with requirements of courtesy and disclosure) participation of contributors to the study who are not included in the author list.

❑ **Disclosure of Conflicts of Interest**

In addition, each author should provide full disclosure of any conflicts of interest. One of the following sentences must be included at the end of the paper: either "Author A has received support from, and/or has served as a paid consultant for Author B has received support from. The remaining authors have no conflicts of interest." Or "None of the authors has any conflict of interest to disclose." Note: Disclosure is needed for financial income/payment from commercial sources, the interests of which are relevant to this research activity. Please identify sources from which financial assistance/ income was obtained during the period of the research activity and generation of the current report. Grants from government and/or private agencies should be identified in the Acknowledgments section.

❑ **Ethical Publication Statement**

All papers must include the following statement to indicate that the authors have read the Journal's position on issues involved in ethical publication (see below) and affirm that their report is consistent with those guidelines: "We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines."

❑ **References**

Authors are responsible for the accuracy of their references. References should follow a modified Vancouver style format. Citation of references in the text should be in superscript numbers (including those in figure legends and tables). Cite the end references in numerical order. The first three authors should be listed and followed by et al. Use journals' PubMed abbreviations in the reference list at the end of the paper (as opposed to journals' names being written out in full). Reference program patches are available on the *Epilepsia* Scholar One Manuscripts website (http://mc.manuscriptcentral.com/Epilepsia); please click 'Instructions and Forms' at the top right-hand corner of the homepage.

Number of references is limited to the following:
Full Length Original Research Paper – 50
Brief Communications – 18
Reviews – 100
Special Reports – 100

Sample References:

Journal Article

Berg AT, Berkovic SF, Brodie MJ, et al. Revised terminology and concepts for organization of seizures and epilepsies: report of the ILAE Commission on Classification and Terminology, 2005-2009. Epilepsia 2010; 51: 676-685.

Journal article published electronically ahead of print version

Reilly C, Atkinson P, Das KB et al. Academic achievement in school-aged children with active epilepsy: A population-based study. Epilepsia Epub 2014 Oct 20.

Journal article In Press

Battino D, Tomson T, Bonizzoni E, et al. Seizure control and treatment changes in pregnancy: Observations from the EURAP epilepsy pregnancy registry. Epilepsia (in press 2013)

Letter

Marucci G. Commentary on the new ILAE classification system for focal cortical dysplasias. Epilepsia 2012; 1:219-220. Letter

Published Abstract

Noe K, Drazkowski J. Safety of Long-Term Video EEG Monitoring. Epilepsia 2008; 59(suppl 7):1.125. Abstract

Book

Shorvon S. Handbook of the treatment of epilepsy. Oxford: Blackwell Publishing; 2005

Chapter in a Book

Fraser RT, Gumnit RJ, Thorbecke R, et al. Psychosocial rehabilitation: A pre- and postoperative perspective. In Engel J (Ed) Surgical treatment of the epilepsies. 2nd Ed. New York: Raven, 1993:669-667

Online

Russo CA, Elixhauser A. Hospitalizations for Epilepsy and Convulsions, 2005: Statistical Brief #46. Available at:http://www.hcup-us.ahrq.gov/reports/statbriefs/sb46.jsp. Accessed February 12, 2011.

- **Figure legends**

Number each legend sequentially to conform to the figure number (e.g., Figure 1, Figure 2...). The legend should provide a brief description of the figure, with explanation of all symbols and abbreviations. Written permission to use non-original material must be obtained (from the original authors (where possible) and publishers) by the authors. Credit for previously published material (author(s), date, journal/book title, and publisher) must be included in the legend. A figure legend should be listed at the end of the manuscript following the list of references.

- **Tables**

Tables should be formatted as the authors wish the table to appear in print. Present all tables together at the end of the manuscript, with each table on a separate manuscript page. Each table should be given a number and a descriptive title. Provide notes and explanations of abbreviations below the table, and provide clear headings for each column and row. Do not duplicate data given in the text and/or in figures. Written permission to use non-original material must be obtained (from the original authors (where possible) and publishers) by the authors. Credit for previously published material (author(s), date, journal/book title, and publisher) must be included in the table notes.

- **Figures**

All figures should be prepared with care and professionalism. Submissions that do not comply with the following formatting requirements will be returned for correction and re-submission. Figures should be submitted as TIF files in the size expected for final publication– approximately 3 inches (7-8 cm) for half column and 6 to 7 inches (15-17 cm) for double columns. Submit black and white figures with a minimum of 300 dpi (MRI scans) and for line drawings or figures that included imbedded text (bar graphs with numbers) at least 600 dpi. Complex figures (including photographs, micrographs, and MR-related images), either in color, in half-tones, or in black and white, should also be submitted in TIF format with a resolution of at least 600 dpi. We recommend saving the TIF files with LZW compression (an option when you 'save as' in packages like Photoshop), which will make the files smaller and quicker to upload without reducing the resolution/quality. Save each TIF file with a name that includes the first author's last name and the figure number as referenced in the text (e.g., Smith-fig1.tif). Provide clear labels on the ordinate and abscissa. Figures with more than one part should be combined by the authors in the correct orientation and labeled with A, B, C etc. When relevant, include calibration information. Label figures use Calibri font and the authors should make sure that all labels are large enough to be clearly legible when the figure is reduced to fit onto a journal page. The maximum size of any figure is 7x9 in (17×22.5 cm) and 40 mega pixels; the total number of pixels for each figure (i.e., height×width) must be less than 40 megapixels otherwise the image will not convert to PDF for review. There is no charge for color figures. We strongly encourage authors to generate figures in color (to enhance clarity of presentation and aesthetic appeal), using the following color palette:

Color #	RGB Definition	CMYK Definition
#e4b8b4	228/184/180	0/25/15/9
#ce8080	206/128/128	0/50/30/18
#a30234	163/2/52	0/100/60/37
#511d24	81/29/36	42/85/67/60
#f1b682	241/182/130	0/29/50/4
#e37c1d	227/124/29	0/58/100/8
#ffde76	255/223/118	0/11/64/0
#abb47d	171/180/125	13/0/47/27
#67771a	103/119/26	27/0/94/55

Color #	RGB Definition	CMYK Definition
#a1c5cb	161/197/203	25/0/7/16
#5698a3	86/152/163	50/0/14/32
#00545f	0/84/95	100/0/28/64
#002f30	0/47/48	87/34/47/77
#bacfec	186/207/236	25/11/0/0
#0076c0	0/118/192	100/46/0/0
#002157	0/33/87	100/75/0/60
#7a5072	122/80/114	50/73/30/18

Photographs or videos of patients should not reveal patient identity; masking eyes and/or other identifiers is compulsory unless the eyes are essential to the meaning of the photograph or video. In addition, such photographs and videos must be accompanied by a letter saying that signed consent forms authorizing publication have been obtained for all identifiable patients, and that the consents will be maintained by the author for seven years or until the patient reaches 21 years of age, which ever is longer. Do not send *Epilepsia* the consent forms; U.S. Federal privacy rules prohibits ending signed consent forms to *Epilepsia* or Wiley Publishing without written permission from the patient to do so. A sample signed consent form can be found on the *Epilepsia* Scholar One Manuscripts website (http://mc.manuscriptcentral.com/Epilepsia); please click 'Instructions and Forms' at the top right-hand corner of the homepage.

- **Supporting Information**

Supporting information, to be published online only, can be submitted for review. Such material may include: additional figures, large tables, videos, etc. that cannot be accommodated within the normal printed space allocation for an article–but provide important complementary information for the reader. As determined by the reviewers and Editors, supporting information will be posted on the Wiley Online Library *Epilepsia* server and directly integrated into the full-text HTML article. Explicit reference to the supporting information in the main body of the text of the article is recommended, and the material must be captioned at the foot of the text, below the reference list. Supporting information will be published as submitted and will not be corrected or checked for scientific content, typographical errors or functionality. Although hosted on Wiley Online Library, the responsibility for scientific accuracy and file functionality remains entirely with the authors. A disclaimer will be displayed to this effect with any supporting information published.

Supporting Information files should be accompanied by detailed information (if relevant) about what they are and how they were created (e.g., a native dataset from a specific piece of apparatus). Acceptable formats for supporting information include:

General – Standard MS Office format (Word, Excel, PowerPoint, Project, Access, etc.); PDF

Graphics – GIF; TIF (or TIFF); EPS; PNG; JPG (or JPEG); BMP; PS (postscript); embedded graphics (e.g. a GIF pasted into a Word file) are also acceptable.

Video–QuickTime; MPEG; AVI. All video clips must be created with commonly-used codecs, and the codec used should be noted in the supplementary material legend. Video files should be tested for playback before submission, preferably on computers not used for its creation, to check for any compatibility issues. Video clips are likely to be large; try to limit their size to less than 10 MB.

c. Gray Matters

- **Title**

Letters, workshop reports, etc. should be given a brief title. Letters should start with the opening *To the Editors*:

- **Authors and affiliations**

Provide authors' names (first and last names, middle initial when commonly used by that author); institutional affiliation for each author (use superscripted numbers after each author's name, and a corresponding superscripted number for each institutional affiliation); e-mail contact address for the corresponding author.

- **Body of submission**

Letters and commentaries should be restricted to 500 words or less, unless other wise allowed by the Editors. Figures and tables will be included only in exceptional cases. Gray Matters will not be used to publish case reports. Tables, figures, figure legends, references, acknowledgements, disclosure of conflicts of interest, ethical publication statement and Supporting Information–as for *Full Length Original Research* (see above).

(3) Details of Preparation

Detailed instructions for all aspects of electronic manuscript submission (including useful information on image files) is available on the *Epilepsia* Scholar One Manuscripts website (http://mc.manuscriptcentral.com/Epilepsia); please click 'Instructions and Forms' at the top right-hand corner of the home page; then click on the link 'Instructions to Authors'.

a. Text

Manuscripts should be prepared using a word processing program. Save text and tables as a Microsoft Word document. Place the lead author's name and the page number in the upper right hand corner of each page. Begin numbering with the Title Page as #1, and number pages consecutively including references, figure legends, and tables. Text (including acknowledgements, disclosure statement, and figure legends) and references should be double-spaced, and be composed in 12 point font (preferably Times New Roman). When generating a revised manuscript, identify the altered portions of the manuscript with highlighted text, underlined, colored or bold font to indicate where changes to the original version of the text have been made.

b. Tables, Figures, and Supporting Information

See above.

Manuscript Submission

(1) Online submission via Manuscript Central

Manuscripts should be submitted via the Journal's website on Scholar One Manuscripts at http://mc.manuscriptcentral.com/epilepsia. Instructions at the site will guide the author through the submission process.

Separate files should be submitted for: Cover letter to editors, manuscript text, tables, each figure, supplemental material, permissions to use previously-published material, patient consent declaration.

(2) Cover letter

All manuscripts should be submitted with a cover letter, addressed to the Editors-in-Chief, which explains why the manuscript should be published in *Epilepsia*. In particular, authors should identify novel findings, innovative approaches, and important insights that would make the manuscript of particular value to the broad readership of *Epilepsia*.

(3) Text, table and figure files

All files should be given a label that includes the first author's last name and the nature of the file (e.g., Smith-manuscripttext.doc; Smith-Fig1.tif).

(4) Other materials/forms

At the time of submission, all other materials (e.g., permission forms, supplemental material, patient consent) must be uploaded onto Manuscript Central, faxed to the editorial office (Fax: +1-702-548-0706) or emailed to epilepsia@epilepsia.com.

(5) Questions/Contacts

Questions and request for assistance should be addressed to the Journal at epilepsia@epilepsia.com. The Managing Editor, Ms. Laurie Beninsig will in most cases be able to provide direction, or will contact the Editors-in-Chief for further assistance.

Manuscript Publication

(1) The Editors may approach authors to provide one or two of their figures as possible cover material for the printed journal. These figures will need to be large enough and with the appropriate dpi.

(2) Online tracking of your article

Online production tracking of your article is available through Wiley's Author Services. Author Services enables authors to track their article once it has been accepted through the production process to publication online and in print. Authors can check the status of their articles online and choose to receive automated e-mails at key stages of production. The corresponding author will receive an e-mail with a unique link that enables him/her to register and have the article automatically added to the system. To facilitate this service, please ensure that you provide a complete e-mail address when submitting the manuscript. Visit http://authorservices.wiley.com/bauthor/ for more details on online production tracking and for other publication resources (including FAQs and tips on article preparation, submission and more).

(3) Proofs

Proofs are sent electronically in a PDF format, and must be returned within 48 hours of receipt. Late returns of proofs will cause substantial delay in article publication. It is the corresponding author's responsibility to see that the proof is accurately checked and corrected, and to return the proofs promptly to avoid publication delays. Please check the spelling of coauthors' names and affiliations, text, tables, legends, and references carefully. It is the authors' responsibility to make sure that the information is accurate. Indicate corrections either using the PDF editor function (so as to return proofs electronically to eeeproofs@aol.com), or with clear hard-copy indications which should be faxed to +1 508-586-4024. The proof corrections stage is not the time for fine-tuning language or making any other substantive changes. Confine corrections to errors in printing; authors may be charged for major author-initiated changes.

(4) Early View

The publication-ready PDF of an article will be published initially online. Early View publication will precede print publication by a variable time period. The online publication date will be considered the official publication date. Early View published material will be indexed by PubMed, and can be cited by DOI number. In general, manuscripts will be published on Early View within 28 days of the publisher's receipt of the complete accepted manuscript (including CAF and permission forms).

(5) Print issue publication

Publication of an article in a print issue will typically occur after Early View publication. Print issue articles carry their electronic publication date.

(6) Public access of accepted/published articles

Prior to acceptance, articles may be shared (print or electronic copies) with colleagues; at this time the article may be posted on the author's personal website, on his/her employer's website, and/or on free public servers in the author's subject area - with the acknowledgement that the article has been submitted to *Epilepsia*. After an article has been accepted, authors may share print or electronic copies of the article (accepted and revised to address peer review) with colleagues, and may use the material in personal compilations, other publications of his/her own work, and for educational/research purposes. Articles published in *Epilepsia* are freely accessible to the public via the Wiley Online Library website one year after publication. *Epilepsia* will automatically upload NIH-supported studies to PubMed Central after a 12 month moratorium (provided the appropriate funding acknowledgement has been

provided). Similarly, at this time authors may post an electronic version of the article on their own personal websites, on their employer's website/repository, and on free public servers in the relevant subject area. Electronic versions of the accepted (or published) article must include a link to the published version of the article, together with the following text: "The definitive version is available at http://www3.interscience.wiley.com/journal/117957420/home." Authors can also choose to make their articles open access and available free for all readers through the payment of an author fee. This facility allows authors to fulfill the requirements for studies supported by agencies requiring open access before 12 months. For full details visit http://authorservices.wiley.com/Bauthor/onlineopen.asp

(7) Reprints

An order form for reprints will be included with the electronic transmission of initial proofs. For pricing of quantities in excess of 500 copies, please contact Helane Silverman (email: hsilverman@wiley.com).

Supplement Publication

(1) Policy

A decision to publish a supplement is based on the topic, Guest Editor, proposed table of contents and contributing authors, and availability of necessary funding. Supplement topics must be of importance to *Epilepsia* readers, and supplements will be published only if there is scientific or educational rationale for combining papers on a given theme within one publication. The number and quality of the articles must be sufficient to constitute a body of important information. Each supplement will have a Guest Editor who is an expert on the theme of the supplement. The Guest Editor is responsible for compiling articles and assisting with the editorial process, and is responsible for the overall quality and integrity of the supplement. The publication of a supplement usually incurs charges, payable to Wiley Publishing.

(2) Publishing guidelines

Articles in a supplement are subject to the same copyright regulations and ethical publishing guidelines that apply to articles published in regular issues of *Epilepsia*. All supplement articles are peer-reviewed; the first level of review is carried out by the Guest Editor and his/her designates. The second level of review will include the articles being sent out for peer review.

(3) Online only and print supplements

Abstract supplements, from meetings or congresses sponsored by the ILAE or its chapters, will generally be published online only. Longer articles will be published in print supplements (these articles will also appear online). Print supplements may be generated from proceedings of symposia organized by an independent body of professionals in which the funding organization does not have a controlling voice on scientific content. The Guest Editor and/ororganizers of such symposia should be members of ILAE chapters. Supplements from other sources including invited supplements initiated by the Editors-in-Chief will also be considered.

(4) Supplement content

The content of supplements must not be biased in the interest of any sponsor. *Epilepsia* does not permit presentations that extol a commercial product, and supplements should not be perceived as endorsing a particular product. Publication of supplements does not constitute product or sponsor endorsement by *Epilepsia* or ILAE. In most cases, supplements should not focus on a single product; however, when a new product is introduced, a single product focus will be considered by the Editors-in-Chief. In all cases, the content of a supplement must be determined by a body of professionals working independently of the sponsor. The Guest Editor is charged with assuring that the material presented in the supplement is not biased toward the interests of the product manufacturer.

(5) Supplement sponsorship

Most supplements require external sponsorship. When a supplement proposal is presented to the Editors-in-Chief, they will fix appropriate fees. Supplement costs may be negotiated with the Editors-in-Chief and the publisher's supplement representative. The Editors- in-Chief may choose to publish a supplement of particular academic and clinical value without external sponsorship.

(6) Instructions for submitting supplements

Agreement to publish a supplement must be obtained from the Editors-in-Chief prior to submission. Proposals for supplements should be submitted to the Editors-in-Chief (Epilepsia@epilepsia.com) well in advance of desired publication date, so that the proposal can be evaluated and discussed. Timing is especially critical if the supplement is linked to asymposium or congress, since rapid publication is often important to assure that the information is current. The proposals should identify the Guest Editor and include a list of topics and potential authors. The proposal should include an estimate of supplement length so that the Editors-in-Chief can provide reasonable information about the cost of publication. The cost of any supplement, and related financial issues, should be discussed with Joann Mitchell at Wiley Publishing (jmitchell@wiley.com). Collection of manuscripts, as well as initial editing and reviewing should be

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(7) Format of supplement articles

In general, articles should follow the format described above for Critical Reviews (in regular issues of the Journal). Contact the Editors-in-Chief for additional information and special instructions.

Epilepsia's Position on Issues Involved in Ethical Publication

(1) Authorship/Credit

Epilepsia follows the guidelines of the International Committee of Medical Journal Editors regarding criteria for authorship (http://www.icmje.org/). The author list should include those who have made substantial intellectual/conceptual contributions to the work. Such contributions should include participation in: (a) experimental design, data acquisition, and analysis and interpretation of data; (b) drafting and/or critically revising the article with respect to intellectual content; and (c) final approval of the manuscript version to be published. We strongly discourage the inclusion of "honorary" authors (individuals who are listed as authors but have not contributed to the work/manuscript - e.g., heads of departments) and "ghost" authorship (individuals who have substantively contributed to the work and/or manuscript but are not listed as authors or contributors). In cases where writing support is necessary, the writer(s)should be acknowledged in the Acknowledgments section, and the source of funding for writing support should be provided under Disclosure of Conflicts of Interest. The corresponding/submitting author must, when submitting a manuscript, give assurance that all authors have read and approved the submitted manuscript. The corresponding/submitting author should also give assurance that all authors have seen and approved the final (accepted) manuscript, and that the manuscript includes all conflict of interest declarations. All individuals who have contributed to the work but do not meet criteria for authorship should be cited in the Acknowledgment section.

(2) Funding

Sources of funding (for the research, data analysis, and manuscript generation) should always be disclosed in the Acknowledgements section. Sources may include government funding agencies, institutions and departments, private industry, and charitable organizations and foundations. Funding for all authors should be acknowledged.

If no funding has been provided for the research, please include the following sentence: "This research did not receive any grant from the public, commercial, or not-for-profit sector funding agencies."

(3) Procedures involving Human and Animal Subjects

The authors should include within the manuscript an explicit statement indicating that the submitted study was approved by the relevant research ethics committee or institutional review board (IRB).When the study involves human participants (including material from human subjects), authors should also provide assurance that appropriate consent was obtained. When studies involve animal subjects, authors should provide methodological details about steps taken to minimize pain/discomfort. Such papers must contain a statement that affirms that the experimental protocols were approved by the institutional animal care and use committee (IACUC).

(4) Confidentiality

In all cases, information and images derived from individual patients must be presented with assurance of appropriate consent and with details removed that might reveal identity of the individual.

(5) Disclosure

All authors are required to disclose associations which might affect their ability to present and/ or interpret data objectively, particularly financial ties to funding sources for the work under review (e.g., membership on corporate scientific boards, stock ownership, consultant arrangements, patent ownership or application, etc.). Disclosure of such associations for the Editorial personnel of *Epilepsia* (Editors-in-Chief, Associate Editors, Editorial Board members) will be published each year. Reviewers will also be asked to affirm that they have no conflict of interest when critiquing a manuscript.

(6) Research Misconduct (Data Fabrication/ Falsification)

Epilepsia will attempt to ensure that any allegations of misconduct are properly investigated. In the case of any allegations, authors will be given a right to respond. While the Journal is limited in its ability to investigate misconduct, we will seek COPE's advice and alert appropriate bodies and encourage them to investigate.

Epilepsia's Position on Issues Involved in Ethical Publication

(7) Plagiarism, Duplication, and Redundant Publication

Epilepsia requires that work submitted for publicationis the authors' own work and has not been misappropriated. When previously published material is used, appropriate credit must be given and written permission obtained (for use of copyrighted material). *Epilepsia* also explicitly discourages duplication of published material and redundant publication. All manuscripts submitted to *Epilepsia* are checked with the iThenticate software to detect instances of overlapping and similar text. In the case of apparent or substantial overlap, authors will be asked to rewrite their article.

(8) Corrections of Erroneous Information

Authors are expected to proof-read their articles carefully before returning page proofs for publication. They should make needed corrections at this time. We recognize that it is only human to err occasionally, and the Journal is committed to correcting mistakes when those errors affect the interpretation of data or information presented in an article. Such corrections will be published in the form of an Erratum, and linked to the original article electronically. Errors that result from author oversight in the proofing process, and that do not affect data interpretation, will not be corrected.

(9) Peer Review

Epilepsia is committed to a peer-review system that is fair to the author and enhances the value of the articles published in the Journal. In order to encourage qualified reviewers to offer their time and efforts to the Journal, reviewer identity is kept confidential. Reviewers are chosen for their expertise in the field; conflicts of interest are avoided whenever the Editors are aware of such issues, and reviewers are asked to affirm that they have no conflicts of interest in reviewing a given *Epilepsia* manuscript. Authors are encouraged to identify specific individuals who, they believe, cannot provide unbiased review. While the Editors-in-Chief reserve the right to make the final decision to accept or reject an article, appeals will be seriously considered. Address appeals to the Editors-in-Chief, who will examine the reviews and the author responses, consult the relevant Associate Editor, and seek additional reviewer input if deemed necessary.