Epilepsy & Behavior 71 (2017) 218–225

Contents lists available at ScienceDirect

Epilepsy & Behavior

journal homepage: www.elsevier.com/locate/yebeh

Molecular and neurochemical substrates of the audiogenic seizure strains: The GASH:Sal model

Ana I. Prieto-Martín [a], J. Daniel Aroca-Aguilar [b], Francisco Sánchez-Sánchez [b], Luis J. Muñoz [c], Dolores E. López [c], Julio Escribano [b], Carlos de Cabo [a,*]

[a] Research Department, Neuropsychopharmacology Unit, Albacete General Hospital, 37 Hermanos Falcó Street, Albacete E-02006, Spain
[b] Department of Genetics, Faculty of Medicine, University of Castilla-La Mancha, 14 Almansa Street, Albacete E-02006, Spain
[c] INCYL, University of Salamanca, 1 Pintor Gallego Street, Salamanca E-37007, Spain

ARTICLE INFO

Article history:
Revised 12 May 2015
Accepted 14 May 2015
Available online 11 June 2015

Keywords:
Reflex epilepsy
$GABA_A$ receptor subunits
KCC2
Animal models of seizures
Inferior colliculus convulsions
Genetic epilepsy

ABSTRACT

Purpose: Animal models of audiogenic epilepsy are useful tools to understand the mechanisms underlying human reflex epilepsies. There is accumulating evidence regarding behavioral, anatomical, electrophysiological, and genetic substrates of audiogenic seizure strains, but there are still aspects concerning their neurochemical basis that remain to be elucidated. Previous studies have shown the involved of γ-amino butyric acid (GABA) in audiogenic seizures. The aim of our research was to clarify the role of the GABAergic system in the generation of epileptic seizures in the genetic audiogenic seizure-prone hamster (GASH:Sal) strain.
Material and methods: We studied the K^+/Cl^- cotransporter KCC2 and β2-GABAA-type receptor (GABAAR) and β3-GABAAR subunit expressions in the GASH:Sal both at rest and after repeated sound-induced seizures in different brain regions using the Western blot technique. We also sequenced the coding region for the KCC2 gene both in wild- type and GASH:Sal hamsters.
Results: Lower expression of KCC2 protein was found in GASH:Sal when compared with controls at rest in several brain areas: hippocampus, cortex, cerebellum, hypothalamus, pons–medulla, and mesencephalon. Repeated induction of seizures caused a decrease in KCC2 protein content in the inferior colliculus and hippocampus and an increase in the pons–medulla. When compared to controls, the basal β_2-$GABA_AR$ subunit in the GASH:Sal was overexpressed in the inferior colliculus, rest of the mesencephalon, and cerebellum, whereas basal β_3 subunit levels were lower in the inferior colliculus and rest of the mesencephalon. Repeated seizures increased β2 both in the inferior colliculus and in the hypothalamus and β_3 in the hypothalamus. No differences in the KCC2 gene-coding region were found between GASH:Sal and wild-type hamsters.
Conclusions: These data indicate that GABAergic system functioning is impaired in the GASH:Sal strain, and repeated seizures seem to aggravate this dysfunction. These results have potential clinical relevance and support the validity of employing the GASH:Sal strain as a model to study the neurochemistry of genetic reflex epilepsy.

This article is part of a Special Issue entitled "Genetic and Reflex Epilepsies, Audiogenic Seizures and Strains: From Experimental Models to the Clinic".

1. Introduction

The involvement of the GABAergic, glutamatergic, and monoaminergic systems in audiogenic seizures affecting diverse brain regions — largely the inferior colliculus (IC), as well as the superior colliculus (SCol), periaqueductal gray, reticular formation, substantia nigra, amygdala (AMYG), cortex (CTX), hippocampus (HIPP), and even the hypothalamus (HYP) — has been extensively studied and reviewed by Cairasco's, Faingold's, and Coleman's laboratories in audiogenic epilepsy strains [1–7]. Alterations in the GABAergic system seem to be a main feature in the neurochemical mechanisms underlying audiogenic epilepsy. Lower GABA binding sites were found in whole brain homogenates from seizure susceptible DBA/2 mice [8]. Seizure-prone BALB/c mice showed reduced striatal GABA, when compared with seizure-resistant mice [9]. In sublines of inbred Rb mice, susceptibility to audiogenic seizures has been correlated with low levels of GABA in the HIPP, whereas lower amounts of GABA in the olfactory bulb were found to be associated either with seizure-severity or seizure-diversity [10]. Also, the audiogenic absence-like seizures in the C57Bl10 sps/sps mutant have been shown to be associated with lower glutamic acid

* Corresponding author at: Complejo Hospitalario Universitario de Albacete, Unidad de Neuropsicofarmacología, Edificio de Investigación, 3 planta, c/ Hermanos Falcó, 37, E-02006, Albacete, Spain. Tel.: +34 967 597500; fax: +34 967 243952.
E-mail addresses: anaprima05@hotmail.com (A.I. Prieto-Martín), JoseDaniel.Aroca@uclm.es (J.D. Aroca-Aguilar), Francisco.SSanchez@uclm.es (F. Sánchez-Sánchez), lmp@usal.es (L.J. Muñoz), lopezde@usal.es (D.E. López), julio.escribano@uclm.es (J. Escribano), carlosd@sescam.jccm.es (C. de Cabo).

http://dx.doi.org/10.1016/j.yebeh.2015.05.025
1525-5050/

decarboxylase activity in the CTX and HIPP. Gamma-amino butyric acid binding sites are reduced in these areas, as well as in the mesencephalon (MES), cerebellum (CER), and pons–medulla (P - M) when compared with the parent strain [11]. Genetically epilepsy-prone GEPR-9s rats, however, have been demonstrated to present increased number of GAD-expressing neurons in the IC [12] as well as higher maximal ligand binding for both high and low affinity GABA sites compared to Sprague–Dawley rats that do not display audiogenic seizures [13]. The genetically epilepsy-prone hamster GPG/Vall, which exhibits generalized tonic–clonic seizures in response to sound stimulation was found to have increased levels of GABA in the SCol [14] but lower GABA concentrations in the IC [15] when compared with wild-type Syrian hamsters.

Gamma-amino butyric acid is one of the main inhibitory neurotransmitters in the adult vertebrate central nervous system. Its fast inhibitory actions take place when GABA gates postsynaptic $GABA_A$ ionotropic receptors ($GABA_ARs$). The native $GABA_AR$ is a heteropentamer typically comprised of two α (α_{1-6}), two β (β_{1-3}), and one γ (γ_{1-3}) subunit(s) that form a selective channel for chloride (Cl^-) ions [16]. Under standard circumstances, the Cl^- concentration inside the adult neuron ($[Cl^-]_i$) is lower than in the intercellular space, and the opening of the $GABA_AR$ Cl^- channel after GABA binding produces an influx of Cl^- that leads to hyperpolarization of the cell membrane [17]. Lower Cl^- levels are maintained through the actions of the KCC2 K^+/Cl^- cotransporter that extrudes Cl^- from the neuron [18]. However, during development or in pathological situations, $[Cl^-]_i$ are increased, and $GABA_AR$ actions are depolarizing. This increase in $[Cl^-]_i$ has been associated with lower KCC2 expression both during development and in pathological conditions, including epilepsy [19–23]. Conversely, KCC2 mutations that reduce neuronal Cl^- extrusion have been found to be associated with epileptic syndromes in humans [24,25]. $GABA_AR$ subunit composition determines its functionality [16,26], and it has also been shown to change during development and across brain regions [27–29]. γ-amino butyric acid receptor β2-subunit is one of the most abundant $GABA_AR$ subunits in the whole brain [30], while β_3 is the most abundant β-type subunit in the auditory pathway [29]. Both contain the main binding sites for benzodiazepines [16,30]. They often display complementary distribution and abundance across brain regions [27–29], and alterations in their expression have been widely observed in both human patients [31,32] and animal models of epilepsy [33–35].

The GASH:Sal hamster represents a novel model to study audiogenic epilepsy [36,37]. Previous published reports from our team have demonstrated the epileptic nature of their ictal electroencephalogram [38] and the similarity of the behavioral pattern of their generalized seizures with other already established models [39]. In the present work, we studied KCC2 as well as β_2-$GABA_AR$ and β_3-$GABA_AR$ subunit expressions in the GASH:Sal strain both at rest and after sound-induced repeated seizures in different brain regions to elucidate their role in the etiology of this epileptic syndrome.

2. Materials and methods

2.1. Animals

The seizure-prone hamster (GASH:Sal) strain employed in these studies was developed at the University of Salamanca. This strain derives from one original epileptic hamster which appeared spontaneously at the University of Valladolid and gave rise to the first seizure-prone hamster strain called GPG:Vall (Gómez-Palomo–Gómez genetically epilepsy-prone hamster, Valladolid). The GPG:Vall strain eventually lost fertility and is now extinct. Before that happened, some individuals were transferred to the University of Salamanca where a new strain was developed (GASH:Sal: genetic audiogenic seizure-prone hamster, Salamanca). For details, see [36,37]. The GASH:Sal strain phenotype is autosomal recessive. Animals' susceptibility to seizures starts around postnatal day 18, which is the time of hearing onset for this species. The strain has the following behavior after sound stimulation: phase 1 — post-stimulus latency period, phase 2 — wild running, phase 3 — tonic–clonic seizures, and phase 4 — stupor. Phases 1–3 last for about 30 s. Stupor (phase 4) may last for 15–20 min. The severity of seizures increases with age, reaching a peak around 2–3 months of age. After 6 months, animals only show phases 1 and 2. The epileptic behavioral syndrome was very similar between GASH:Sal and the extinct GPG:Vall strains. However, GPG:Vall showed morphological alterations in the cochlea and diverse auditory nuclei [40] not present in GASH:Sal [36].

All experimental procedures were approved by the University of Castilla-La Mancha Animal Care Committee and were in accordance with the Declaration of Helsinki and the Guidelines of the Directive 2010/63/EU of the European Parliament and of the Council.

2.2. Experimental groups and sound stimulation procedure

Sixteen adult hamsters (8 GASH:Sal + 8 wild-type Syrian hamsters, 70–80 days old) were used. Experimental groups were as follows: sound-stimulated GASH:Sal epileptic hamsters (SE, n = 4), sound-stimulated control hamsters (SC, n = 4), resting (nonstimulated) GASH:Sal epileptic hamsters (RE, n = 4), and resting (nonstimulated) control hamsters (RC, n = 4). Both SE and SC groups were exposed to white noise (1–37 KHz, 30–80 dB, 10 s) twice a day (leaving a 4–5-hour interval) for five days. The procedure was monitored by the same observer for all the animals tested. This protocol was chosen based on previous evidence from our laboratory to ensure the detection of significant variations in protein levels using Western blot techniques. In a pilot study, only 3 exposures to the abovementioned sound stimulus (once a day for 3 consecutive days) failed to alter cellular prion protein (PrPc) levels in any of the brain regions studied. However, the 10 sound-stimulation protocol employed in this paper was effective in increasing PrPc in the inferior colliculus (unpublished data), probably because of an accumulative effect. Also, the latter protocol proved to have significant effects on neuronal nitric oxide synthase expression in several brain regions without causing a kindling effect [37]. Nevertheless, the protocol employed may elicit axonal growth, since in a previous study we detected an increase in growth-associated protein 43 in the inferior colliculus (unpublished results). The resting (nonstimulated) RC and RE groups served as a basal reference for both the effects of the sound stimulation procedure and the genetic strain differences. After observing 41 GASH:Sal generations, we have determined that under standard housing conditions and in the absence of sound stimulation, GASH:Sal individuals (i.e., RE animals) do not undergo seizures. Nonetheless, animals were checked every day, and no seizures were observed.

2.3. Tissue sampling

Subjects from the sound-stimulated groups (SE and SC) were killed after the 10th stimulation (at the end of the last seizure in the case of the SE group). The RE and RC were sacrificed on the same day without any extra manipulation. Animals were anesthetized (ketamine, 75 mg/kg + xylazine, 10 mg/kg), their brains were quickly removed and dissected on ice, and the following 8 areas were collected: pons + medulla (P + M), cerebellum (CER), inferior colliculus (IC), rest of the mesencephalon (rMES), hypothalamus (HYP), striatum (STR), hippocampus (HIPP), and cerebral cortex (CTX). Brain areas were immediately weighed, frozen on dry ice, and kept in a freezer at −80 °C until assayed.

2.4. Western blot analysis

Brain regions from 16 animals were processed for Western blot analysis using a method which was previously described [37]. Briefly, brain regions were homogenized using a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland) in homogenization buffer containing protease inhibitors. Homogenates were centrifuged at 10,000 rpm for

10 min at 4 °C. Supernatants were subsequently centrifuged at 20,000 rpm for 1 h. The resulting membrane-enriched pellets were resuspended, and protein content was determined using the Bradford protein assay method. Twenty milligrams of total protein per lane were separated on reducing 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes with a tank transfer system. Western blots were probed with rabbit antisera against β_2-GABA$_A$R and β_3-GABA$_A$R subunits (1:1000, Sigma-Aldrich, St. Louis, MO) and the potassium–chloride cotransporter KCC2 (1:2000, Upstate-Millipore, Billerica, MA). Antisyntaxin (35 kDa) and anti-α-tubulin (55 kDa) monoclonal antibodies (1:5000; Chemicon-Millipore, Billerica, MA, and Sigma-Aldrich, St. Louis, MO, respectively) were used for loading control. For detection, we used a peroxidase coupled anti-IgG antibody and a chemiluminescence kit. The immunoblots were read on a luminescent image analyzer LAS-3000 UV mini (Fujifilm, Tokyo, Japan), and densitometric analysis was performed using Quantity One Analysis Software (Bio-Rad Laboratories, Hercules, CA). Band densities were normalized to the corresponding loading control densities and expressed as arbitrary density units (a.d.u.)

2.5. Statistical analysis

Data are expressed as means ± S.E.M. Two-way analysis of variance was used, where appropriate (ANOVA, factors: *strain* (levels: wild-type control and GASH:Sal) × *sound stimulation* (levels: stimulation and resting)), to assess group means followed by the multiple range Fisher's LSD test for post hoc assessment of individual means. $p < 0.05$ was taken to indicate statistically significant differences.

2.6. KCC2 gene (Slc12a5) coding region sequencing

Total RNA was extracted from brain cortex samples from GASH:Sal and wild-type hamsters using a quick gen RNeasy mini Kit (Qiagen, Venlo, Netherlands). Complementary DNA (cDNA) was synthesized from 1 μg RNA employing the reverse transcription polymerase chain reaction (RT-PCR) technique and a Revert Aid TM First strand cDNA synthesis kit (Fermentas, Burlington, ON, Canada). Since the transcriptome for *Mesocricetus auratus* was not yet available at the time of our experiments [41], primers were designed based on 100% conserved regions from mouse (AF332063) and rat (EF641113) KCC2 cDNA sequences obtained from Genbank. A Taq polymerase from Biotools (Madrid, Spain) was used for the PCR reactions. An ABI PRISM BigDye TM Terminator V3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems, Foster city, CA) was used for the sequencing reactions which were purified using a Montage SEQ96 Sequencing Cleanup kit (Montage life science kits, Millipore, Bedford, MA). All kits were used according to the instructions from the manufacturers. Sequencing reactions were analyzed by the Albacete Faculty of Medicine Sequencing Service (University of Castilla-La Mancha, Spain) using an ABI PRISM3100-avant sequencer (Applied Biosystems, Foster City, CA).

3. Results

3.1. Sound stimulation

As expected from previous studies from our team, the sound stimulation procedure employed in this study produced the abovementioned acute full 4-phase generalized seizures in 100% of the epileptic GASH:Sal hamsters (SE group), every time they were tested. However, the same sound stimulation procedure had no detectable effect on wild-type control hamsters (SC group), neither in their behavior nor in any of the biochemical parameters studied (Figs. 2 and 3).

3.2. Western blot

Fig. 1 shows typical blots for KCC2 and β_2-GABA$_A$R and β_3-GABA$_A$R subunit expressions.

3.2.1. KCC2 protein expression

Basal genetic differences between GASH:Sal and wild-type hamsters were found in all the regions studied with the exception of the IC and STR (ANOVA significances for *strain*: $F(1,15) = 16.675$, $p < 0.05$ (HIPP); $F(1,15) = 10.440$, $p < 0.01$ (rMES); $F(1,15) = 5.831$, $p < 0.05$ (CTX); $F(1,15) = 18.802$, $p < 0.01$ (CER); $F(1,15) = 11.018$, $p < 0.01$ (HYP); $F(1,15) = 48.252$, $p < 0.01$ (P + M)), with GASH:Sal showing lower KCC2 expression than wild-type control hamsters (Figs. 2 and 3A).

After repeated seizures, KCC2 levels decreased in the IC of the GASH:Sal (ANOVA significance for *strain* × *sound stimulation* interaction: $F(1,15) = 10.610$, $p < 0.01$) (Fig. 3A) and further decreased in the HIPP from the SE group (Fig. 2), whereas KCC2 expression increased in the P + M from the SE group (ANOVA significance for *sound stimulation*: $F(1,15) = 9.417$ $p < 0.01$) (Fig. 2.)

3.2.2. β_2-GABA$_A$R subunit protein expression

Genetic differences between GASH:Sal and wild-type hamsters (ER and CR groups) were found in the IC, rMES, and CER (ANOVA significances for *strain*: $F(1,15) = 72.622$, $p < 0.01$; $F(1,15) = 13.345$, $p < 0.01$; and $F(1,15) = 9.924$, $p < 0.01$, respectively). The GASH:Sal showed higher basal levels than wild-type controls.

Repeated audiogenic seizures produced an increase in β_2-GABA$_A$R subunit in both the IC (ANOVA significances for *sound stimulation*: $F(1,15) = 8.069$, $p < 0.01$ and *strain* × *sound stimulation* interaction: $F(1,15) = 12.911$, $p < 0.01$) and the HYP (ANOVA significance for *sound stimulation*: $F(1,15) = 4.939$, $p < 0.05$) in the SE group (Fig. 3B.)

3.2.3. β_3-GABA$_A$R subunit protein expression

The basal differences between the GASH:Sal and wild-type hamsters found for the β_3-GABA$_A$R subunit expression in the IC and rMES were opposite to those found for the β_2-GABA$_A$R subunit, with the GASH:Sal hamster presenting higher levels than the controls (ANOVA significances for *strain*: $F(1,15) = 10.871$, $p < 0.01$ (IC) and $F(1,15) = 16.593$, $p < 0.01$(rMES)) (Fig. 3C).

However, the effect of the repeated seizures in the HYP in the SE group was also an increase in β_3-GABA$_A$R subunit expression (Fig. 3C) (ANOVA significance for *sound stimulation*: $F(1,15) = 7.883$, $p < 0.01$) similar to that found for the β_2-GABA$_A$R subunit in this brain region (Fig. 3B).

3.3. KCC2 gene coding region sequencing

The analysis of the coding region for the KCC2 gene (Slc12a5) in the Syrian hamster (*M. auratus*) showed a sequence of 3374 pb (Supplementary Fig. 1; GenBank KT257165) coding for 1125 amino acids, with an estimated molecular weight of 140 kDa. This sequence presented great resemblance with that of the house mouse (*Mus musculus*) (Supplementary Fig. 2).

No differences were found between the coding sequence for the KCC2 gene between the wild-type and the GASH:Sal hamsters (Fig. 4).

3. Discussion

The GASH:Sal strain is a natural model of genetic audiogenic epilepsy. The animals show very reproducible generalized seizures each time the animal receives a suitable white-noise stimulus, allowing a precise control of the seizures (determined by the experimenter). Besides, their ictal EEG has been confirmed to be of epileptic nature [38] making the GASH:Sal strain a valid model for the study of the mechanisms underlying reflex audiogenic epilepsy. Generally speaking, the results

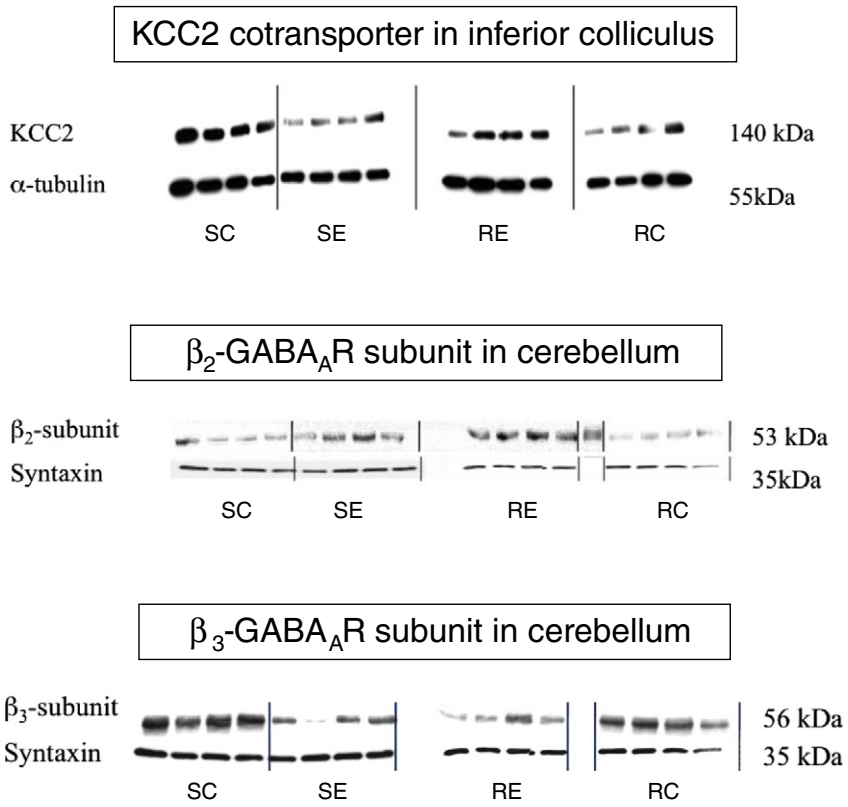

Fig. 1. Examples of the Western blots employed for data analysis. Antibody against α-tubulin was employed for loading control for KCC2 determinations. Since α-tubulin is very close in its molecular weight to $GABA_AR$ β subunits, antibody against syntaxin was used as a loading control for the β_2 and β_3 subunits experiments.

from our study are in agreement with previous literature on audiogenic seizure strains that show an impairment of GABAergic function in many of these models [1–15] and confirm the numerous brain areas that can be affected/recruited in audiogenic epilepsy.

The main finding from the present work is the extensive basal impairment of KCC2 expression in the GASH:Sal strain. The important number of brain regions where the KCC2 cotransporter has a lower basal expression in the GASH:Sal when compared with the wild-type controls is indicative of the relevance that this molecule may have in their genetic epileptic syndrome. This lower basal levels are likely to have repercussions on GABAergic neurotransmission [19,20]. Since KCC2 is responsible for maintaining a low $[Cl^-]_i$, a reduced KCC2 expression may result in an increased $[Cl^-]_i$. This situation may diminish GABA hyperpolarization when opening the $GABA_AR$ Cl^- pore, or even render the action of GABA depolarizing [21,23,26]. According to this view, the GASH:Sal brain, because of its basal lower KCC2 levels, would be in a state of hyperexcitability that could be the basis for their susceptibility to seizures. This notion is supported by earlier reports employing manipulated mice models with deletions in the KCC2 gene in which they found depolarizing actions for GABA in the motorneurons of the spinal cord [42], tactile-elicited seizures [43], and higher susceptibility to epileptogenic agents [43–45]. Besides, hypofunctional variants of KCC2 have been identified as the cause of epileptic syndromes in patients [24,25].

Another interesting result from this study is that repeated seizures produced a reduction of KCC2 levels in the GASH:Sal strain both in the IC and in the HIPP, two brain regions where KCC2 was not already basally lower than in the wild-type hamsters. This phenomenon could potentially lead to an aggravation of their syndrome. These findings agree with work from other authors using different models. Thus, both in vivo [46] and in brain slices [47,48], induction of status epilepticus has been shown to cause a sustained lowering of KCC2 mRNA and protein levels in the hippocampus. Besides, a reduction of KCC2 labeling has also been found in surgery samples from patients with intractable epilepsy [49–51]. Further, a subcellular redistribution of KCC2 labeling in the affected regions has been found in these patients, decreasing KCC2 staining in the neuropil while increasing in the somata [49,50] sometimes accompanied by GABAergic hyperinnervation [51]. These decompensations have been correlated with neuronal hyperexcitability and the origins of the epileptic foci. In fact, Cohen et al. [52] found depolarizing responses to GABA in sclerotic hippocampal slices from patients with temporal lobe epilepsy.

Recent studies have reported mutations in the KCC2 coding sequence in patients with epilepsy [24,25]. However, according to our sequencing data, the coding sequence for the KCC2 cotransporter in the GASH:Sal strain does not reveal any mutations when compared with the wild-type coding sequence. The possibility still remains that a mutation could be present in the promoter region. Mutations in other proteins regulating KCC2 expression, such as transcription factors USF (upstream stimulatory factor) −1 and −2 [53], cannot be excluded either. Brain-derived neurotrophic factor (BDNF) has been shown to downregulate KCC2 expression [46], and it appears to be overexpressed after seizures in the HIPP, AMYG, and CTX in epilepsy models [54]. On the other hand, SPAK (Ste20-related proline alanine-rich kinase) and OSR1 (oxidative stress response 1) kinases interacting with WNKs (with no lysine kinases) have also been shown to regulate KCC2 functionality [55] and have been postulated to be involved in epileptic processes [56]. Thus, mutations affecting these regulatory molecules could be implicated in the susceptibility to seizures shown by the GASH:Sal.

The differences found between the GASH:Sal strain and wild-type hamster in $GABA_ARs$ both in the IC and in the rMES seemed to be alterations in subunit composition rather than changes in the amount of receptors: lower levels of the β_3 subunit were accompanied by higher

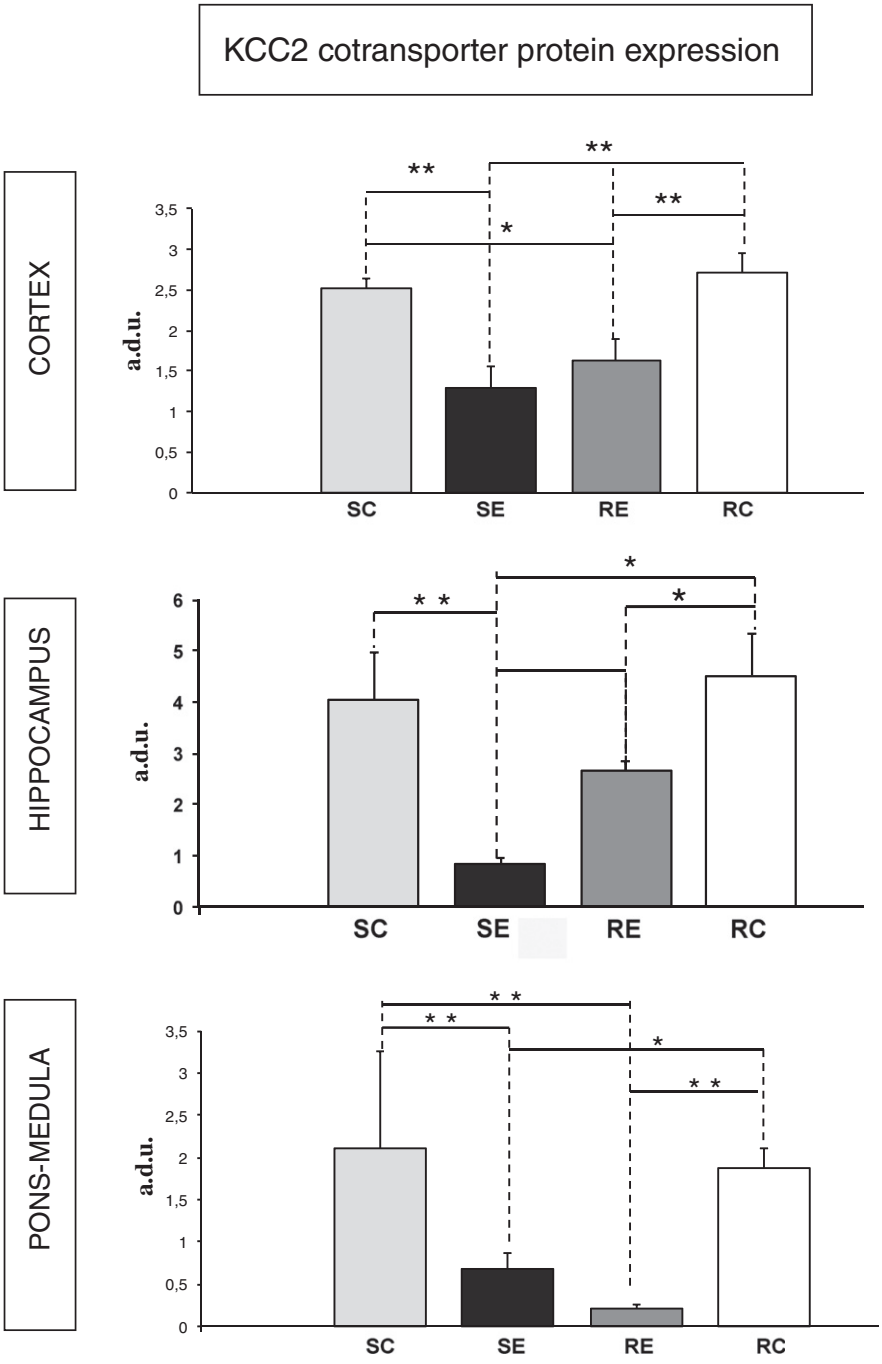

Fig. 2. KCC2 protein expression in the cortex, hippocampus, and pons medulla from control hamsters and the GASH:Sal. Data were analyzed using two-way ANOVA (factors: STRAIN and SOUND STIMULATION) followed by Fisher's test for multiple comparisons. Data are given as means ± S.E.M. from N = 4 animals. *p < 0.05, **p < 0.01 in the Fisher's test. SC = sound-stimulated control hamsters; SE = sound-stimulated epileptic hamsters; RE = resting epileptic hamsters; RC = resting control hamsters; a.d.u. = arbitrary density units.

expression of the β_2 subunit. Since, in the CER of the GASH:Sal, there were higher levels of the β_2 subunit with no significant differences in β_3 subunit expression, a net higher number of $GABA_A$Rs than in wild type in this region cannot be completely excluded. These data are in agreement with previous observations in which different $GABA_A$R subunit expressions were found between seizure-prone and seizure-resistant rat models of temporal lobe epilepsy [57]. Moreover, higher β2 levels have been associated with seizure susceptibility in BALB/cByJ mice when compared with C57BL/6J mice [35]. The presence of the β_2 subunit in the $GABA_A$R composition is associated with faster desensitization and shorter opening duration [16] becoming more sensitive to GABA. The relatively higher concentration of β_2 subunit (and, therefore, more sensitive $GABA_A$Rs) in the IC, rMES, and CER in the GASH:Sal may represent a compensatory effect to the lower levels of KCC2 in these regions.

After repeated seizures, only the IC suffered a further decompensation in the expression of the two $GABA_A$R subunits (even higher levels of β_2 and lower expression of β_3 subunit) in the GASH:Sal. Regarding the HYP, repeated sound-elicited seizures led to an increase of both β_2 and β_3 subunits, which may represent an increase in the number of $GABA_A$Rs, perhaps as an adaptive physiological mechanism to the seizures. Interestingly, morphological and functional alterations have been previously

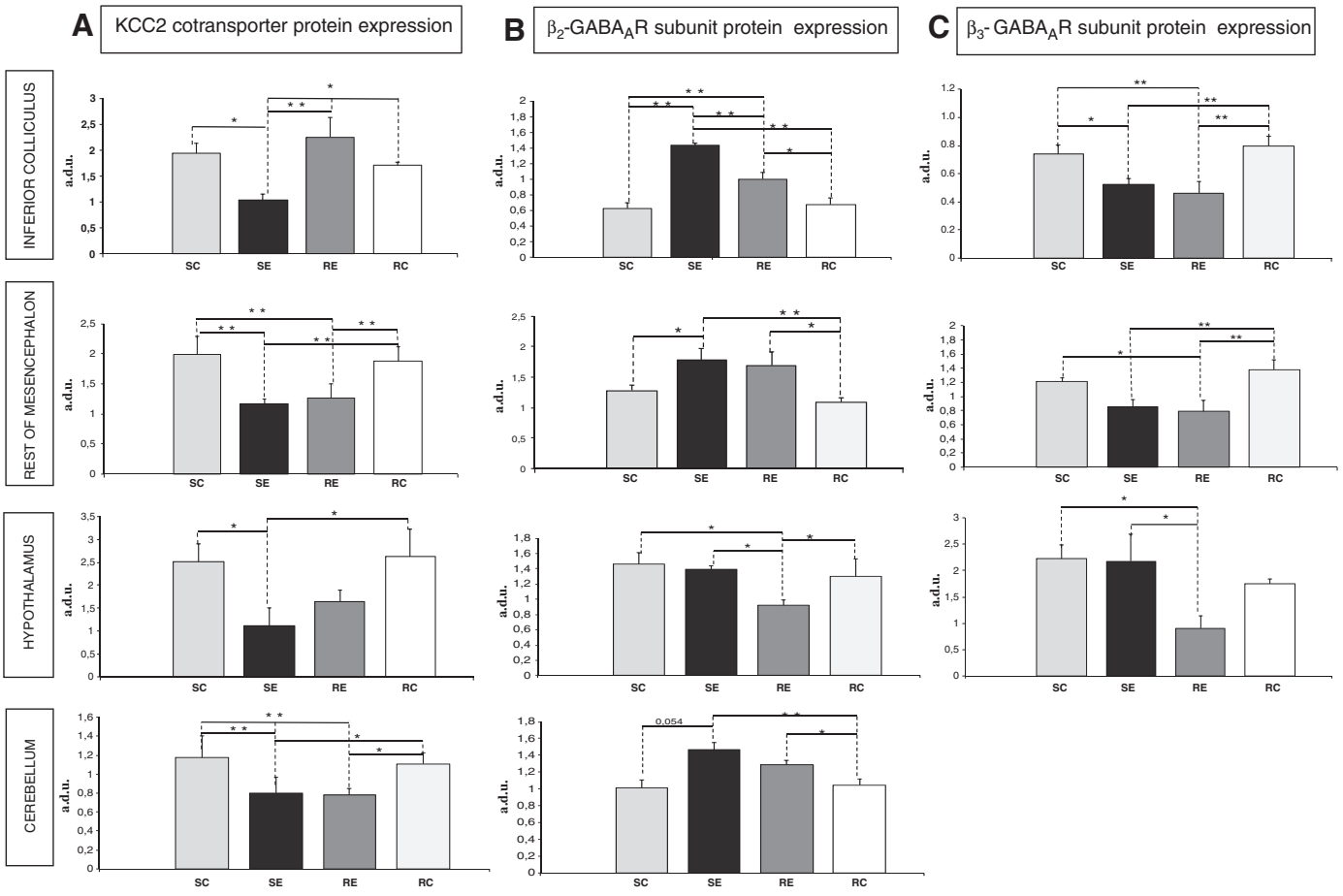

Fig. 3. KCC2, β_2-GABA$_A$R, and β_3-GABA$_A$R protein expressions in the inferior colliculus, rest of mesencephalon, hypothalamus, and cerebellum from control hamsters and GASH:Sal. Data were analyzed using two-way ANOVA (factors: STRAIN and SOUND STIMULATION) followed by Fisher's test for multiple comparisons. Data are given as means ± S.E.M. from N = 4 animals. *$p < 0.05$, **$p < 0.01$ in the Fisher's test. SC = sound-stimulated control hamsters; SE = sound-stimulated epileptic hamsters; RE = resting epileptic hamsters; RC = resting control hamsters; a.d.u. = arbitrary density units.

described in the hypothalamic–pituitary–adrenal axis of the Wistar audiogenic rat (WAR) [7]. Since this region is involved in the regulation of blood pressure and increased sympathetic tone, the GASH:Sal may also be useful to investigate events related to sudden unexplained/unexpected death in epilepsy. Put together, these results from sound-stimulated GASH:Sal hamsters agree with the available reports from other teams. Both data from hippocampal samples from patients with temporal lobe epilepsy [31,32] and animal models using induction of

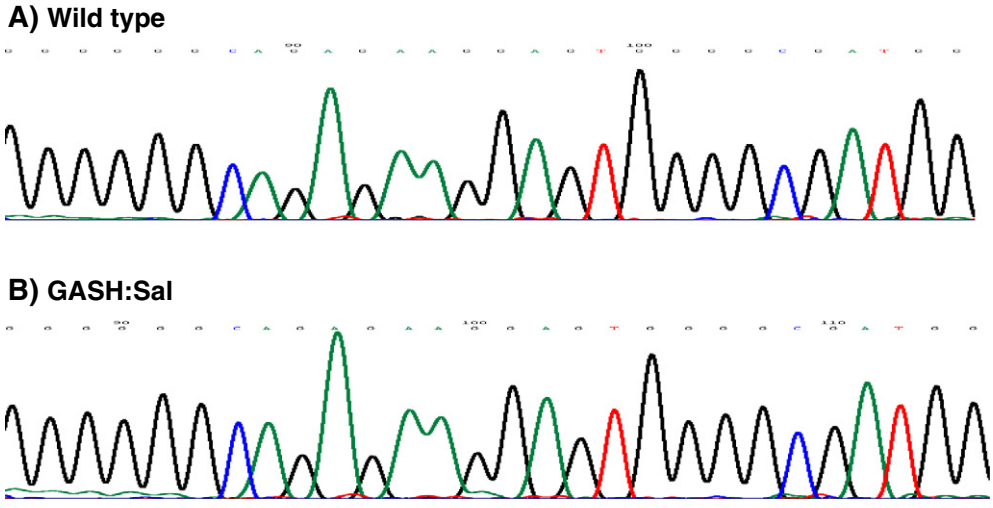

Fig. 4. Syrian hamster (*Mesocricetus auratus*) KCC2 gene (Slc12a5) coding region sequencing. Fragments from KCC2 gene sequencing for control (wild-type) hamster (A) and GASH:Sal (B). No differences were observed in the sequence obtained for the KCC2 gene coding region between wild-type hamsters and GASH:Sal.

status epilepticus [33,34] have shown an overexpression of β_2 and β_3 subunits.

3.1. Conclusions and final considerations

Overall, our results show alterations in the basal levels of the K^+/Cl^- cotransporter KCC2 and $GABA_AR$ subunit expression in various brain regions in the GASH:Sal audiogenic seizure strain. These impairments may render the GASH:Sal brain hyperexcitable and could be related to the seizure-susceptibility of this strain. Repeated seizures seemed to aggravate the alterations, supporting the notion that "seizures beget seizures". The GASH:Sal strain could be a good model to test new therapies targeting K^+/Cl^- cotransporters, perhaps using loop diuretics [22]. Further research is needed to elucidate the role of BDNF and other KCC2 regulating proteins, as well as other K^+/Cl^- cotransporters, in the audiogenic seizure syndrome of the GASH:Sal strain.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yebeh.2015.05.025.

Acknowledgments

This study was supported by the following: FIS-FEDER (Research Grants Ref.: 01/3018 and PS09/01154 to CdC) and JCCM (Consejería de Sanidad) (Research Grants Ref.: 04048-00 and GC 05011 to CdC and Predoctoral Fellowship Ref.: JI 03001 to AIPM). We thank the Albacete Faculty of Medicine Sequencing Service (University of Castilla-La Mancha, Spain) for data analysis. Our thanks also go to Fernando Andrés Pretel, BSc CS, and Beatriz Navarro Bravo, PhD, from the Clinical Research Support Unit (Albacete General Hospital, Spain) for expert statistical advice.

Conflict of interest

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. The authors disclose no conflict of interest.

References

[1] Ross KC, Coleman JR. Developmental and genetic audiogenic seizure models: behavior and biological substrates. Neurosci Biobehav Rev 2000;24:639–53.
[2] Ross KC, Waldman BC, Conejero-Goldberg C, Freed W, Coleman JR. Transplantation of M213-2O cells with enhanced GAD67 expression into the inferior colliculus alters audiogenic seizures. Exp Neurol 2002;177:338–40.
[3] Garcia-Cairasco N. A critical review on the participation of inferior colliculus in acoustic–motor and acoustic–limbic networks involved in the expression of acute and kindled audiogenic seizures. Hear Res 2002;168:208–22.
[4] Faingold CL. Emergent properties of CNS neuronal networks as targets for pharmacology: application to anticonvulsant drug action. Prog Neurobiol 2004;72:55–85.
[5] Garcia-Cairasco N. Puzzling challenges in contemporary neuroscience: insights from complexity and emergence in epileptogenic circuits. Epilepsy Behav 2009;14(Suppl. 1):54–63.
[6] Faingold CL. Brainstem networks: reticulo-cortical synchronization in generalized convulsive seizures. In: Noebels JL, Avoli M, Rogawski MA, Olsen RW, Delgado-Escueta AV, editors. Jasper's basic mechanisms of the epilepsies [Internet]. 4th ed. Bethesda (MD): National Center for Biotechnology Information (US); 2012 [Available from http://www.ncbi.nlm.nih.gov/books/NBK98187/].
[7] Kandratavicius L, Balista PA, Lopes-Aguiar C, Ruggiero RN, Umeoka EH, Garcia-Cairasco N, et al. Animal models of epilepsy: use and limitations. Neuropsychiatr Dis Treat 2014;10:1693–705.
[8] Horton RW, Prestwich SA, Meldrum BS. Gamma-aminobutyric acid and benzodiazepine binding sites in audiogenic seizure-susceptible mice. J Neurochem 1982;39:864–70.
[9] Vriend J, Alexiuk NA, Green-Johnson J, Ryan E. Determination of amino acids and monoamine neurotransmitters in caudate nucleus of seizure-resistant and seizure-prone BALB/c mice. J Neurochem 1993;60:1300–7.
[10] Simler S, Ciesielski L, Clement J, Rastegar A, Mandel P. Involvement of synaptosomal neurotransmitter amino acids in audiogenic seizure-susceptibility and -severity of Rb mice. Neurochem Res 1992;17:953–9.
[11] Ortiz JG, Negrón AE, Thomas AP, Heimer H, Moreira JA, Cordero ML, et al. GABAergic neurotransmission in the C57BL/10 sps/sps mouse mutant: a model of absence seizures. Exp Neurol 1991;113:338–43.
[12] Ribak CE, Morin CL. The role of the inferior colliculus in a genetic model of audiogenic seizures. Anat Embryol (Berl) 1995;191:279–95.
[13] Booker JG, Dailey JW, Jobe PC, Lane JD. Cerebral cortical GABA and benzodiazepine binding sites in genetically seizure prone rats. Life Sci 1986;39(9):799–806.
[14] Fuentes-Santamaría V, Alvarado JC, Herranz AS, García-Atarés N, López DE. Morphologic and neurochemical alterations in the superior colliculus of the genetically epilepsy-prone hamster (GPG/Vall). Epilepsy Res 2007;75:206–19.
[15] Fuentes-Santamaría V, Alvarado JC, Herranz AS, García-Atarés N, López DE. Decreased levels of GABA in the inferior colliculus of the epilepsy-prone hamster (GPG/Vall). Epilepsy Res 2008;79:224–7.
[16] Conley EC. Inhibitory receptor-channels gated by extracellular gamma-aminobutyric acid. In: Conley EC, Brammar WJ, editors. The ion channel facts book: extracellular ligand-gated channels, vol. I. San Diego: Academic Press; 1996. p. 293–365.
[17] Lerma J, Mora F, Sánchez Prieto J. Sinapsis aminoacidérgicas y peptidérgicas. In: Delgado JM, Ferrús A, Mora F, Rubia FJ, editors. Manual de Neurociencia. Madrid: Síntesis; 1998. p. 231–48.
[18] Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lamsa K, et al. The K+/Cl− co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature 1999;397:251–5.
[19] Ben-Ari Y. Basic developmental rules and their implications for epilepsy in the immature brain. Epileptic Disord 2006;8:91–102.
[20] Ben-Ari Y, Khalilov I, Kahle KT, Cherubini E. The GABA excitatory/inhibitory shift in brain maturation and neurological disorders. Neuroscientist 2012;18:467–86.
[21] Cossart R, Bernard C, Ben-Ari Y. Multiple facets of GABAergic neurons and synapses: multiple fates of GABA signalling in epilepsies. Trends Neurosci 2005;28:108–15.
[22] Dzhala VI, Talos DM, Sdrulla DA, Brumback AC, Mathews GC, Benke TA, et al. NKCC1 transporter facilitates seizures in the developing brain. Nat Med 2005;11:1205–13.
[23] Staley K. Molecular mechanisms of epilepsy. Nat Neurosci 2015;18:367–72.
[24] Kahle KT, Merner ND, Friedel P, Silayeva L, Liang B, Khanna A, et al. Genetically encoded impairment of neuronal KCC2 cotransporter function in human idiopathic generalized epilepsy. EMBO Rep 2014;15:766–74.
[25] Puskarjov M, Seja P, Heron SE, Williams TC, Ahmad F, Iona X, et al. A variant of KCC2 from patients with febrile seizures impairs neuronal Cl− extrusion and dendritic spine formation. EMBO Rep 2014;15:723–9.
[26] de Cabo-de la Vega C, Villanueva-Hernández P, Prieto-Martín A. The neurochemistry of epilepsy, inhibitory neurotransmission and experimental models: new perspectives. Rev Neurol 2006;42:159–68.
[27] Laurie DJ, Wisden W, Seeburg PH. The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J Neurosci 1992;12:4151–72.
[28] Wisden W, Laurie DJ, Monyer H, Seeburg PH. The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J Neurosci 1992;12:1040–62.
[29] Campos ML, de Cabo C, Wisden W, Juiz JM, Merlo D. Expression of GABA(A) receptor subunits in rat brainstem auditory pathways: cochlear nuclei, superior olivary complex and nucleus of the lateral lemniscus. Neuroscience 2001;102:625–38.
[30] Möhler H, Fritschy JM, Vogt K, Crestani F, Rudolph U. Pathophysiology and pharmacology of GABA(A) receptors. Handb Exp Pharmacol 2005:225–47.
[31] Loup F, Wieser HG, Yonekawa Y, Aguzzi A, Fritschy JM. Selective alterations in GABAA receptor subtypes in human temporal lobe epilepsy. J Neurosci 2000;20(14):5401–19.
[32] Pirker S, Schwarzer C, Czech T, Baumgartner C, Pockberger H, Maier H, et al. Increased expression of GABA(A) receptor beta-subunits in the hippocampus of patients with temporal lobe epilepsy. J Neuropathol Exp Neurol 2003;62:820–34.
[33] Brooks-Kayal AR, Shumate MD, Jin H, Rikhter TY, Coulter DA. Selective changes in single cell GABAA receptor subunit expression and function in temporal lobe epilepsy. Nat Med 1998;4:1166–72.
[34] Nishimura T, Schwarzer C, Gasser E, Kato N, Vezzani A, Sperk G. Altered expression of GABAA and GABAB receptor subunit mRNAs in the hippocampus after kindling and electrically induced status epilepticus. Neuroscience 2005;134:691–704.
[35] Verleye M, Dumas S, Heulard I, Krafft N, Gillardin JM. Differential effects of etifoxine on anxiety-like behaviour and convulsions in BALB/cByJ and C57BL/6 J mice: any relation to overexpression of central GABAA receptor beta2 subunits? Eur Neuropsychopharmacol 2011;21:457–70.
[36] Muñoz LJ, López DE. Establecimiento y caracterización de una línea de hámsters sirios propensos a padecer convulsiones audiógenas. Salamanca (Spain): Muñoz de la Pascua Publisher84-609-5027-1; 2005.
[37] Prieto-Martín AI, Llorens S, Pardal-Fernández JM, Muñoz LJ, López DE, Escribano J, et al. Opposite caudal versus rostral brain nitric oxide synthase response to generalized seizures in a novel rodent model of reflex epilepsy. Life Sci 2012;90:531–7.
[38] Carballosa-Gonzalez MM, Muñoz LJ, López-Alburquerque T, Pardal-Fernández JM, Nava E, de Cabo C, et al. EEG characterization of audiogenic seizures in the hamster strain GASH:Sal. Epilepsy Res 2013;106:318–25.
[39] Barrera-Bailón B, Oliveira JA, López DE, Muñoz LJ, Garcia-Cairasco N, Sancho C. Pharmacological and neuroethological studies of three antiepileptic drugs in the Genetic Audiogenic Seizure Hamster (GASH:Sal). Epilepsy Behav 2013;28(3):413–25.
[40] Fuentes-Santamaría V, Cantos R, Alvarado JC, García-Atares N, López DE. Morphologic and neurochemical abnormalities in the auditory brainstem of the genetically epilepsy-prone hamster (GPG/Vall). Epilepsia 2005;46:1027–45.
[41] Tchitchek N, Safronetz D, Rasmussen AL, Martens C, Virtaneva K, Porcella SF, et al. Sequencing, annotation and analysis of the Syrian hamster (*Mesocricetus auratus*) transcriptome. PLoS One 2014;9:e112617.
[42] Hübner CA, Stein V, Hermans-Borgmeyer I, Meyer T, Ballanyi K, Jentsch J. Disruption of KCC2 reveals an essential role of K–Cl cotransport already in early synaptic inhibition. Neuron 2001;30:515–24.

[43] Woo N-S, Lu J, England R, McClellan R, Dufour S, Mouny DB, et al. Hyperexcitability and epilepsy associated with disruption of the mouse neuronal-specific K–Cl cotransporter gene. Hippocampus 2002;12:258–68.
[44] Tornberg J, Voikar V, Savilahti H, Rauvala H, Airaksinen MS. Behavioural phenotypes of hypomorphic KCC2-deficient mice. Eur J Neurosci 2005;21:1327–37.
[45] Zhu L, Polley N, Mathews GC, Delpire E. NKCC1 and KCC2 prevent hyperexcitability in the mouse hippocampus. Epilepsy Res 2008;79:201–12.
[46] Rivera C, Li H, Thomas-Crusells J, Lahtinen H, Viitanen T, Nanobashvili A, et al. BDNF induced TrkB activation downregulates the K^+–Cl^- cotransporter KCC2 and impairs neuronal Cl^- extrusion. J Cell Biol 2002;159:747–52.
[47] Rivera C, Voipio J, Thomas-Crusells J, Li H, Emri Z, Sipila S, et al. Mechanism of activity-dependent downregulation of the neuron-specific K–Cl cotransporter KCC2. J Neurosci 2004;24:4683–91.
[48] Li X, Zhou J, Chen Z, Chen S, Zhu F, Zhou L. Long-term expressional changes of Na^+–K^+–Cl^- co-transporter 1(NKCC1) and K^+–Cl co-transporter 2 (KCC2) in CA1 region of hippocampus following lithium–pilocarpine induced status epilepticus (PISE). Brain Res 2008;1221:141–6.
[49] Aronica E, Boer K, Redeker S, Spliet WGM, Van Rijen C, Troost D, et al. Differential expression patterns of chloride transporters, $Na^+/K^+/2Cl^-$ cotransporter and K^+/Cl^--cotransporter, in epilepsy-associated malformations of cortical development. Neuroscience 2007;145:185–96.
[50] Munakata M, Watanabe M, Otsuki T, Nakama H, Arima K, Itoh M, et al. Altered distribution of KCC2 in cortical dysplasia in patients with intractable epilepsy. Epilepsia 2007;48:837–44.
[51] Muñoz A, Mendez P, DeFelipe J, Alvarez-Leefmans FJ. Cation-chloride cotransporters and GABA-ergic innervation in the human epileptic hippocampus. Epilepsia 2007; 48:663–73.
[52] Cohen I, Stéphane VN, Baulac CM, Miles R. On the origin of interictal activity in human temporal lobe epilepsy in vitro. Science 2002;298:1418–21.
[53] Markkanen M, Uvarov P, Airaksinen MS. Role of upstream stimulating factor in the transcriptional regulation of the neuron-specific K–Cl cotransporter KCC2. Brain Res 2008;1236:8–15.
[54] Scharfman HE. Brain/derived neurotrophic factor and epilepsy — a missing link? Epilepsy Curr 2005;3:83–8.
[55] de Los Heros P, Alessi DR, Gourlay R, Campbell DG, Deak M, Macartney TJ, et al. The WNK-regulated SPAK/OSR1 kinases directly phosphorylate and inhibit the K^+–Cl^- co-transporters. Biochem J 2014;458:559–73.
[56] Kahle KT, Deeb TZ, Puskarjov M, Silayeva L, Liang B, Kaila K, et al. Modulation of neuronal activity by phosphorylation of the K–Cl cotransporter KCC2. Trends Neurosci 2013;36:726–37.
[57] Poulter MO, Brown LA, Tynan S, Willick G, William R, McIntyre DC. Differential expression of alpha1, alpha2, alpha3, and alpha5 GABAA receptor subunits in seizure-prone and seizure-resistant rat models of temporal lobe epilepsy. J Neurosci 1999;19:4654–61.