DISRUPTION OF ClC-2 EXPRESSION IS ASSOCIATED WITH PROGRESSIVE NEURODEGENERATION IN AGING MICE

M. A. CORTEZ,[a,d1] C. LI,[b1] S. N. WHITEHEAD,[c]
S. U. DHANI,[b,e] C. D'ANTONIO,[b,e] L. J. HUAN,[b]
S. A. L. BENNETT,[c] O. C. SNEAD III[a,d] AND
C. E. BEAR[b,e*]

[a]*Programmes in Brain and Behaviour Research Institute, Hospital for Sick Children, Toronto, ON, Canada*

[b]*Programmes in Molecular Structure and Function Research Institute, Hospital for Sick Children, Toronto, ON, Canada*

[c]*Department of Biochemistry, Microbiology, and Immunology (BMI), University of Ottawa, Ottawa, ON, Canada*

[d]*Department of Pediatrics, University of Toronto, Toronto, ON, Canada*

[e]*Department of Physiology, University of Toronto, Toronto, ON, Canada*

Abstract—Heterozygous mutations in ClC-2 have been associated in rare cases with increased susceptibility to generalized, idiopathic epilepsy. Initially, it was hypothesized that mutations in ClC-2 may be associated with epilepsy due to a direct role for ClC-2 in the modification of hippocampal neuronal excitability. However, the absence of an overt seizure-susceptibility phenotype in young ClC-2 knockout (KO) mice rendered this hypothesis- implausible. A recent study of older ClC-2 KO mice (>6 months) revealed abnormalities in the myelin of central axons and a subtle defect in the neuronal function in the central auditory pathway. These findings prompted us to re-examine hippocampal neuron morphology and excitability in older ClC-2 KO mice. Interestingly, electrocorticographic recordings obtained in older mice revealed spontaneous interictal spikes which are a marker of perturbed hippocampal neurotransmission with a resultant increase in excitation. This electrophysiological defect was associated with astrocyte activation and evidence of neuronal degeneration in the CA3 region of the hippocampus of these older mice. Together, these findings raise the possibility that ClC-2 expression plays a subtle neuroprotective role in the aging hippocampus. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: chloride channel, interictal spikes, hippocampus, electrocorticographic, astrocyte activation, inflammation.

Idiopathic, generalized epilepsies exhibit a heterogeneous clinical phenotype and genetic complexity (Lu and Wang, 2009; Macdonald and Kang, 2009). Variants in ion channels which mediate excitatory and inhibitory neurotransmission, including the voltage gated sodium channel, SCN8A (Papale et al., 2009) and the GABA$_A$ receptor

γ2subunit (Kang et al., 2009) have been previously associated with rare cases of generalized epilepsy. In addition, certain mutations in the chloride channel CLCN2 have recently been associated with idiopathic generalized epilepsy (Combi et al., 2009; Saint-Martin et al., 2009). Of these three novel missense mutations (R235Q, R577Q and S719L), the two arginine mutations lead to faster channel deactivation kinetics when studied in a heterologous expression system, consistent with loss of function (Saint-Martin et al., 2009). However, the authors of these studies highlight the complex inheritance of the above variants, pointing to incomplete segregation among affected family members and transmission by unaffected patients suggesting that CLCN2 mutations alone may not be sufficient to confer disease.

Initially, a physiological role for ClC-2 in neurotransmission was implicated on the basis of immunolocalization and transfection studies. For instance, electron microscopic studies of rodent hippocampal pyramidal neurons showed that ClC-2 protein is localized to synaptic and perisynaptic regions within GABAergic neurons, as well as sub-synaptic vesicles proximal to the GABAergic synapse (Sik et al., 2000). On the basis of electrophysiological studies of transfected neurons several years ago, it was at first suggested that ClC-2 expression may act to regulate GABA$_A$-mediated currents through its effect on modifying transmembrane chloride gradients (Staley et al., 1996). Hence, this model would predict that mutations in ClC-2, which impair functional expression, may be associated with neuronal excitation and seizure activity because local anion gradients and GABA$_A$ mediated currents are altered. Hence, disruption of ClC-2 could result in epilepsy due to disruption of GABA$_A$-mediated inhibition.

Clcn2 deficient mice were generated by the research groups of Jentsch and Melvin and rather unexpectedly, studies of these mice failed to reveal any overt seizure activity or enhanced susceptibility to the volatile seizure-inducing drug, flurothyl (Bosl et al., 2001). Furthermore, there was no morphological evidence of brain pathology in young adult mice. These findings failed to support a primary role for ClC-2 in GABA$_A$ mediated inhibitory neurotransmission in mice although, the potential compensation by a related chloride channel protein was not investigated.

Interestingly, more recent and detailed analyses of the CNS of *Clcn2* knockout mice revealed a phenotype in older mice (greater than 6 months). Blanz et al. (2007) detected a progressive spongiform vacuolation of the white matter of the brain and spinal cord in mice of this age. Defective conduction of central auditory neuronal pathways, consistent with impaired oligodendrite function and the loss of

---

myelin integrity was also observed. In these older mice, the authors also detected upregulation of protein biomarkers of inflammation, consistent with astrocyte and glial activation. ClC-2 protein has been shown to be localized to the endfeet of astrocytes, the subcellular site for interaction of astrocytes with other cell types. Given the physical association of astrocytes and oligodendrites, the defect in myelin integrity observed in *Clcn2* knockout mice could result from the detrimental effect of astrocyte-mediated inflammation on oligodendrite function. These findings suggest that ClC-2 expression be protective with respect to oligodendrocyte viability in the aging brain.

Since the morphological and functional defects of the cerebellum of *Clcn2*−/− mice described by Blanz and colleagues were progressive with age and subtle in nature, we re-examined hippocampal morphology and neurotransmission in older mice. We found that *Clcn2*−/− mice (>6 months) exhibited neurodegeneration of the CA3 region and this lesion was associated with an abnormal electrocorticographic signal, revealing an altered hippocampal function. Consistent with previous studies of the cerebellum (Blanz et al., 2007), these changes in hippocampal function were associated with astrocyte activation. These findings support a role for ClC-2 in maintaining hippocampal neuron viability.

EXPERIMENTAL PROCEDURES

Mice

Clcn2+/− breeding pairs were obtained from Dr. J.E. Melvin, University of Rochester and *Clcn2 (−/−)* and *Clcn2 (+/+)* siblings were generated at the Hospital for Sick Children. Two age groups were compared including young adults (1–3 months of age) and older mice (6–10 months of age).

Quantitative analysis of cell death and degeneration

Mice (Wt, $n=3$; ClC-2 KO, $n=3$) were euthanized by lethal injection with sodium pentobarbital and transcardially perfused with 10 mM phosphate buffered saline (PBS, 10 mM sodium phosphate, 154 mM NaCl) followed by 4% paraformaldehyde in PBS. For histological analysis and immunofluorescence, brains were removed and post-fixed for 24 h in 4% paraformaldehyde followed by 48 h cryoprotection in 20% sucrose. Serial coronal sections (10 μm) were cryostat-cut (Leica Microsystems Inc.) and stained with Methyl Green Pyronine Y (MGPY) as described in (Moffitt, 1994), Hematoxylin and Eosin (H&E) or Cresyl Violet as described in (Bennett et al., 1995). In MGPY-stained sections, DNA is stained blue and RNA is stained pink. Cells defined as viable exhibited a light blue nucleus, confirming nuclear integrity, and a pink cytoplasm, indicative of RNA transcription. Cells with hyperchromatic nucleus and/or lack of cytoplasmic (RNA) staining were identified as damaged cells (Al-Hazzaa and Bowen, 1998). In H&E-stained sections, pyknotic, eosinophilic or hyperchromatic cells with amorphous or fragmented nuclei were defined as damaged cells. Cells with oval nuclei, prominent nucleoli lacking eosinophilic cytoplasm and homogenously stained by Hematoxylin or Cresyl Violet were defined as healthy cells (Bennett et al., 1995; Bennett et al., 1998). Quantitative analyses were performed using the Advanced Measurement Module of Openlab 3.1.7 software (Improvision). Four measurements were taken from each area and averaged to yield a single value per animal. Data are expressed as the percentage of damaged cells/0.1 mm^2 Neuronal number was established by immunofluorescence using monoclonal anti-neuron-specific nu-

clear antigen (NeuN) (1:100, Chemicon) detected with an anti-mouse IgG tagged with a Cy-3 fluorophore (1:800, Jackson). Data are expressed as number of cells/0.1 mm^2 Neurons dying through an apoptotic-like mechanism were identified by double-labeling for anti-NeuN and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL). Coronal sections (bregma −2.06) were permeabilized by a 5 min incubation in 0.1% Triton-X/0.1% sodium citrate on ice and a 1.5 min incubation in ethanol: acetic acid (2:1) on ice. Sections were rinsed for 2 min in 10 mM PBS and reacted for 1 h at 37 °C with FITC–labeled dUTP in TdT buffer (Roche). Negative controls included sections incubated with FITC–labeled dUTP in the absence of TdT. Degenerating neurons were identified by fluorojade B labeling. Slides were incubated in a solution of 1% NaOH in 80% alcohol for 5 min, followed by 2 min washes in 70% alcohol and distilled water and a 10 min incubation in a 0.06% potassium permanganate solution. Slides were rinsed in distilled water and stained in a 0.0004% Fluorojade B staining solution for 20 min. Data are expressed as the percentage of NeuN+ neurons/0.1 mm^2 exhibiting TUNEL or fluorojade reactivity.

Electrocorticography (ECoG)

Mice were implanted with electrodes for recording of the Electrocorticogram (ECoG) under pentobarbital anaesthesia. Electrode implants consisted of four electrodes over the frontal and parietal cortices and two depth electrodes indwelled into the thalamus and hippocampus. The electrodes were placed 1 mm deep, 2 mm anterior to bregma and 2 mm lateral from midline. Recordings were made 1, 24 and 48 h after recovery from anaesthesia. Each animal was placed in an individual Plexiglass chamber (Harvard Apparatus, Holliston, MA, USA) for a 20 min adaptation period prior to ECoG recordings in order to minimize movement artifact. ECoG recordings were made on paper using a Grass Polysomnograph machine (Grass Instruments, Quincy, MA, USA) ECoG recordings were obtained between 10:00 and 14:00 h to minimize circadian variations (Loscher and Fiedler, 1996; Stewart et al., 2006).

Immunoblotting

Whole brain or hippocampal tissues were obtained from anaesthetized and decapitated mice (*Clcn2*−/− or their wild type siblings) of 6–10 months of age for comparative analysis by immunoblotting. The ClC-2 protein was detected in Westerns using a polyclonal, peptide-purified, C-terminal directed antibody (Dhani et al., 2003). The following commercially available antibodies were used to probe for the presence of the GABA$_A$ receptor subunits by immunoblotting: α1 (1:500, Upstate Biotechnology) β2/3 (1:1000, Upstate Biotechnology) and γ2 (1:1000, Chemicon, Pittsburg, PA, USA). The anti-ClC-3 antibody was obtained from Chemicon, Pittsburg, PA, USA. The antibody used to detect glial fibrillary acidic protein (GFAP) was obtained from DAKO North America Inc. (LA, USA).

Statistics

Data are presented as the mean+standard error of measurement (SEM) and were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests. In each analysis, α was set at $P<0.05$.

RESULTS

Disruption of ClC-2 expression leads to neurodegeneration in the CA3 region of the hippocampus of older mice

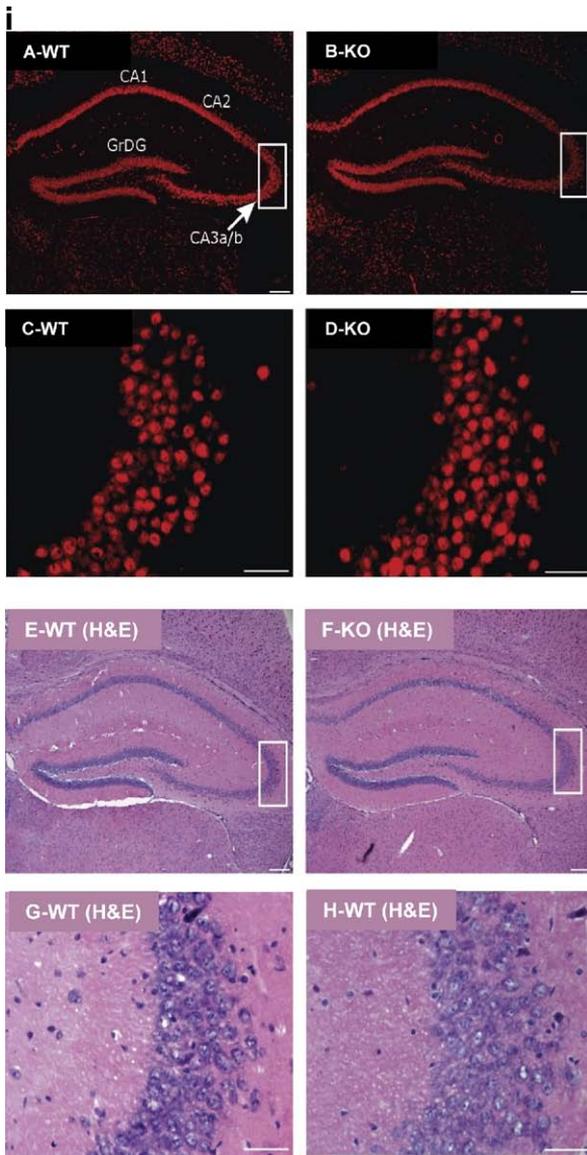The consequences of Clcn2 disruption on brain morphology and neuronal viability in various regions was deter-

Fig. 1. (i) Normal hippocampal morphology in young adult mice (1–3 mon). (A–D). NeuN+ neuronal number is similar in ClC-2 KO mice and Wt siblings. CA3a/b region is indicated with a white box in (A) and (B) and details are shown in (C) and (D). (E–H). Morphometry. H&E stain shows that overall cell number and histology is not affected by disruption of ClC-2. Scale bars, 100 μm (E, F). White box (CA3a/b region) expanded in (G, H) with scale bars. Abbreviations: CA1, 2, 3a/b=CA pyramidal cell fields; FJ=Fluorojabe B; GrDG=granule cell layer of the dentate gyrus.

mined for young adult mice (1–3 months of age) and older mice (6–10 months of age). As shown in Fig. 1,i (A–H), in the younger age group, there was no obvious difference in brain morphology nor in neuronal number between Clcn2 knockout mice and their wild type littermates. On the other hand, as shown in the Fig. 1,ii, there is a clear defect in the CA3 region of the hippocampus of the older Clcn2−/− mice in comparison with their wild type siblings. Neuronal number was reduced in the CA3a/b but not CA1 or granule cell layer of the dentate gyrus as determined by NeuN immunoreactivity (Fig. 1,ii, A–E). Quantitation of cell num-

ber in H&E-stained sections confirmed that this reduction was not due to epitope masking (Fig. 1,ii, F–H). To assess viability of remaining cells, sections were processed for MGPY, fluorojade B, and TUNEL. A significant increase in degenerating morphology was detected in the CA3a/b of MGPY-stained (Fig. 1I–K) and fluorojade-stained sections (Fig. 1L–N). Cells were not TUNEL+ (Fig. 1N, inset).

ClC-2 knockout mice do not exhibit spontaneous seizures but they do exhibit an abnormal neurophysiological phenotype with interical spikes

ClC-2 knockout mice do not exhibit spontaneous, overt myoclonic seizure activity regardless of their age (1–10 months). Since profound neurodegeneration in the CA3 region of the hippocampus was detected in older mice (Fig. 1,ii), we sought to determine whether these older mice exhibited a more subtle neurological phenotype. In contrast to young adult mice (1–3 months of age), the ECoG recordings made form monopolar differential recordings in older ClC-2 knockout mice (6–10 months) exhibited an abnormal ECoG, revealing an interictal spike pattern (Fig. 2). Although occasional interictal spikes were recorded from wild type controls, the interictal spike frequency was significantly greater in the knockout animals than the wild type controls (Fig. 2, bar graph). This observation suggested that disruption of ClC-2 expression leads to a progressive neurological pathology associated with enhanced excitability.

In order to identify the brain region most affected by the disruption of ClC-2 expression, we compared the ECoG's detected when electrode pairs were inserted into the bilateral frontal and parietal cortices and other subcortical such as the thalamus and hippocampus (Fig. 3A, B). Only those pairs which included an electrode inserted in the hippocampus led to recapitulation of the interictal spike pattern observed when the electrodes were implanted over the frontal and parietal simultaneously. These findings suggest that the abnormal ECoG detected in the ClC-2 knockout mice originated from a specific perturbation in hippocampal circuitry.

Localization of the electrophysiological defect to the hippocampus is consistent with previous reports of ClC-2 expression in this region of the brain. We confirmed these previous experiments and compared the hippocampal expression of ClC-2 relative to the other regions of mouse brain. Tissue lysates obtained from older Wt mice were analyzed by SDS-PAGE and immunoblotting conducted using an anti-ClC-2 antibody. In Fig. 3, panel C, it is clear that in the mouse and rat, ClC-2 protein is expressed in the hippocampus, thalamus and cortex. These findings suggest that the altered ECoG activity and enhanced neurodegeneration observed in the hippocampus of ClC-2 KO mice, may reflect a specific functional role for ClC-2 in this tissue. As previous studies reported an increase in astrocyte activation in the cerebellum of older ClC-2 KO mice, we were prompted to determine if astrocyte activation was associated with the hippocampal phenotype discovered in the current project. As shown in Fig. 3D, expression of the glial fibrillary acidic protein (GFAP, an astrocyte specific
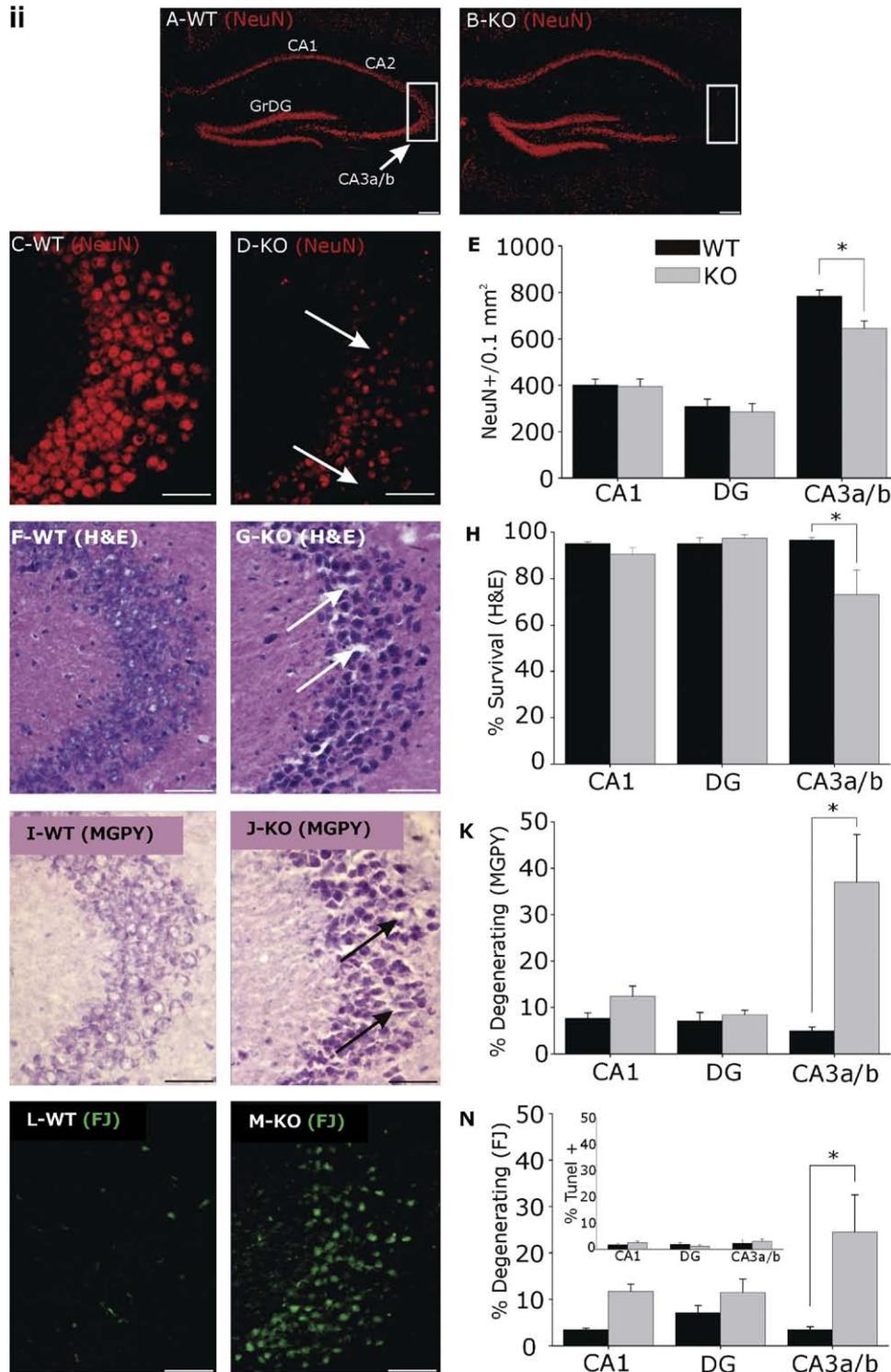
Fig. 1. (*Continued.*) (ii) Evidence of neuronal degeneration in ClC-2 KO mice. (A, B) NeuN+ neuronal number is reduced in the CA3a/b of adult ClC-2KO mice. (C, D) Higher magnification of the outlined CA3a/b area. White arrows point to areas within the CA3a/b pyramidal cell layer devoid of neurons. (E) NeuN+ cell number is significantly reduced in the CA3a/b but not the CA1 or GrDG by ClC2 null-mutation. (F ,G) H&E assessment of cell number. Arrows point regions of cell loss. (H) Cell survival is significantly reduced in the CA3a/b but not CA1 or GrDG of ClC2 KO mice. (I, J) Remaining neurons in KO mice are compromised as assessed by MGPY staining. Arrows point to representative cells with degenerating morphology as described in Experimental Procedures. (K) Degeneration of remaining cells is significantly increased in the CA3a/b of ClC-2 KO mice. (L, M) Fluorojade B+ labelling of terminally damaged neurons is detected in the CA3a/b of ClC-2 KO mice. (N) A significant increase in fluorojade B labelling is observed in the CA3a/b but not TUNEL labelling of apoptotic-like death (inset). Scale bars, 100 μm (A, B); 50 μm (C, D, F, G, I, J, L, M). * indicates statistical significance between Wt and ClC2 KO groups; $P<0.05$.
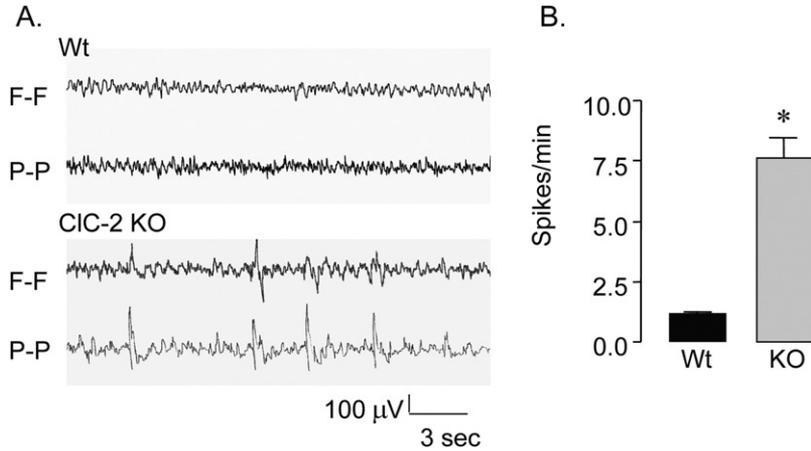
Fig. 2. Neurodegeneration associated with abnormal electrocorticography (ECoG) in older ClC-2 KO mice. (A) Unlike their wild type (Wt) siblings (top panel), the baseline ECoG in older (>6 mon) ClC-2 KO mice exhibited spontaneous slow spike and wave discharges. Recording electrodes were placed in the left and right frontal (F–F) or parietal (P–P) lobes. Time/voltage scale indicates 100 μV/3 s. Sensitivity=20 μV/mm; HFF=100 Hz; LFF=1 Hz; 60 Hz Filter ON; paper speed 15 mm/s; F–F (left frontal–right frontal), P–P (left parietal–right parietal). (B) Bars (mean±SEM) indicate the number of bursting spikes per minute in Wt (black, n=7) or KO siblings (>grey, n=7). * indicates statistically significant difference between Wt and KO groups (Student's t-test, P<0.05).

marker) was upregulated in the whole brain (WB) and hippocampus (Hi) of ClC-2 KO mice relative to their Wt controls. These findings point to an association between

inflammatory status, neuronal degeneration and abnormal activity as revealed in ECoG of ClC-2 KO mice. While interictal spikes can have functional consequences since
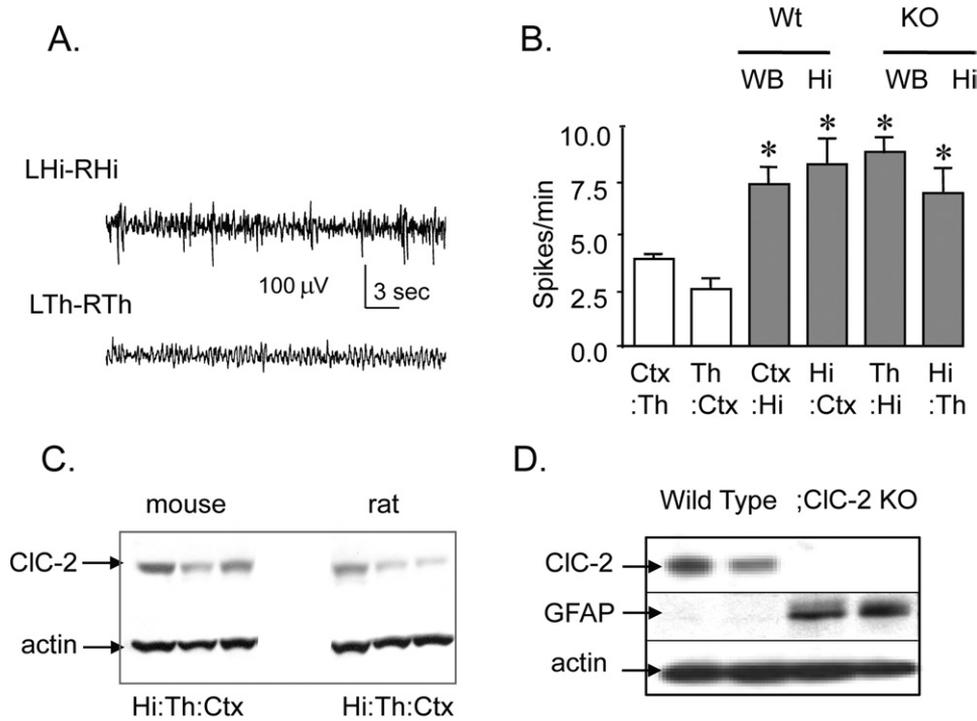

Fig. 3. Abnormal ECoG localized to hippocampus of ClC-2 KO mice. (A) Representative ECoG traces when electrode pairs in left and right hippocampus (LHi–RHi) or left and right thalamus (LTh–RTh). (B) Bars (mean±SEM) indicate the number of bursting spikes per minute in older KO mice (>6 mon, n=7). Electrode pairs (left and right): Ctx–Th (cortex and thalamus), Ctx–Hi (cortex and hippocampus), Th–Hi (thalamus and hippocampus). The numbers of bursting spikes per minute is significantly greater in electrode pairs recording from the hippocampus (grey bars, two way ANOVA, with post-hoc Bonneferroni analysis: * P<0.05). (C) Immunoblots showing relative expression levels of ClC-2 protein in hippocampus (Hi), thalamus (Th) and cortex (Ctx) in normal mice or rats. Actin expression was probed to control for loading. (D) Immunoblot showing GFAP (glial fibrillary acidic protein) a specific marker of astrocyte activation, exhibits enhanced expression in whole brain (WB) and hippocampal (Hi) homogenates obtained from KO mice (3, >6 mon) relative to Wt controls. The same blots were stripped and reprobed with an anti-ClC-2 antibody to confirm the genotype of these tissues and with an anti-actin antibody to confirm equal sample loading in each lane.

they have the potential to affect learning and memory, there is no evidence that interictal spikes result in neuronal injury (Holmes and Lenck-Santini, 2006; Lee et al., 2001; Leung, 2009).

Expression of other hippocampal chloride channels/transporters unaffected in ClC-2 KO mice

Disruption of the expression of ClC-3 (another member of the ClC family of channels and transporters) has also been associated with neuronal degeneration in the mouse hippocampus (Dickerson et al., 2002; Stobrawa et al., 2001; Yoshikawa et al., 2002). Further, disruption of the expression of the $GABA_A$ receptor subunit $\gamma 2$ has been linked to neurodegeneration (Karle et al., 2001). Similarly, KCC3 KO mice, lacking expression of this potassium: chloride co-transporter exhibited hippocampal degeneration and reduced seizure threshold upon exposure to flurothyl (Boettger et al., 2003). Therefore, in order to determine if reduction in the expression of these chloride transporters (secondary to the disruption of ClC-2 expression) could account for the hippocampal degeneration observed in ClC-2 knockout mice, we compared the expression of these proteins in four knockout mice ($\sim$6 months of age) with appropriate wild type sibling controls. The immunoblots of whole brain lysates in Fig. 4, confirm that ClC-2 protein expression is completely disrupted in the ClC-2 knockout mice studied. Importantly, there was no significant change in the expression of ClC-3, KCC3 or any of the $GABA_A$ subunits. These findings support the conclusion that the hippocampal neurodegeneration and abnormal ECoG documented in the older ClC-2 KO mice (Figs. 1, 2 and 3), is caused by the specific loss of ClC-2 expression and not due to secondary effects on the expression of other chloride channels/transporter implicated in hippocampal neurotransmission.

Older mice exhibit overt seizure activity in response to $GABA_A$ inhibitors: bicuculline and picrotoxin

Initially, it was suggested that ClC-2 may function to regulate neuronal excitability by facilitating $GABA_A$ mediated

Fig. 4. Expression of other hippocampal chloride channels/transporters unaffected in ClC-2 KO mice. Whole brain tissues obtained from four ClC-2 KO and Wt siblings (>6 mon) analyzed by SDS-PAGE and immunoblotting, probing for expression of chloride channels (ClC-2 and $GABA_A$ subunits commonly expressed in hippocampus) and transporters(ClC-3 and KCC3). Disruption of ClC-2 expression in ClC-2 KO does not appear to affect expression of other chloride channels/transporters implicated as important in hippocampal health and function.

neurotransmission (Staley et al., 1996). Further, loss of ClC-2 expression may convert GABA mediated neurotransmission from inhibitory to excitatory. If the abnormal ECoG behaviour exhibited in older ClC-2 KO mice is related to GABA mediated excitatory neurotransmission, one would predict that treatment with the $GABA_A$ inhibitors, bicuculline and picrotoxin would reverse this phenotype. In Fig. 5 we show that, in contrast to this prediction, these $GABA_A$ inhibitors caused enhanced seizure activity in the ClC-2 KO mice. The latency of seizure onset was significantly reduced and the frequency of seizures significantly increased in ClC-2 KO animals relatively to their Wt siblings. These findings argue that ClC-2 expression does not directly regulate $GABA_A$ mediated neurotransmission. Rather, seizure susceptibility may be secondary to inflammation and neurodegeneration in the CA3 region of the hippocampus and the disruption of neural networks.

DISCUSSION

In the current study, we showed that disruption of ClC-2 expression is associated with hippocampal neurodegeneration in aging mice and an abnormal ECoG with the occurrence of spontaneous interictal spikes. Together with evidence for enhanced astrocyte activation in the hippocampus of ClC-2 KO mice and specific neurodegeneration in the CA3 region of the hippocampus, a region which is particularly susceptible to age-related neuroinflammation (Rizzi et al., 2003), we suggest that ClC-2 function may normally modulate astrocyte: neuron interactions in the hippocampus.

Neurodegeneration observed in ClC-2 KO mice is different from that observed in ClC-3 and KCC transporter KO mice

ClC-3 KO mice also exhibit neurodegeneration, however the time course and the lesion location differs from that observed in ClC-2 KO mice. In the case of ClC-3 KO mice, neurodegeneration has an early onset, starting at 2 weeks after birth and results in the complete loss of the hippocampus by 3 months (Stobrawa et al., 2001). A lesion first appears in the CA1 region as early as postnatal day 21 with progression to CA3 and other regions of the hippocampus at later times (Stobrawa et al., 2001). Similarly, photoreceptor degeneration in the retina is apparent at P12. The early onset of such a severe phenotype points to a primary role of ClC-3 in neuronal function. Although the cellular mechanism underlying this severe phenotype remains unclear, it has been speculated that it is related to glutamate toxicity due to abnormal glutamate uptake by synaptic vesicles. In contrast to ClC-3, ClC-2 protein is not expressed on synaptic vesicles, but rather in the endosomes and plasma membrane of neurons (Sik et al., 2000), and hence is not expected to contribute directly to glutamate uptake and release from synaptic vesicles. As for the ClC-3 knockout mice, the neurodegeneration and enhanced seizure susceptibility induced by disrupted expression of the potassium: chloride cotransporters, KCC3 and KCC2 is also apparent in young mice (less than a
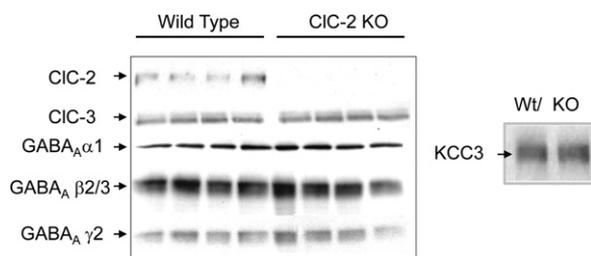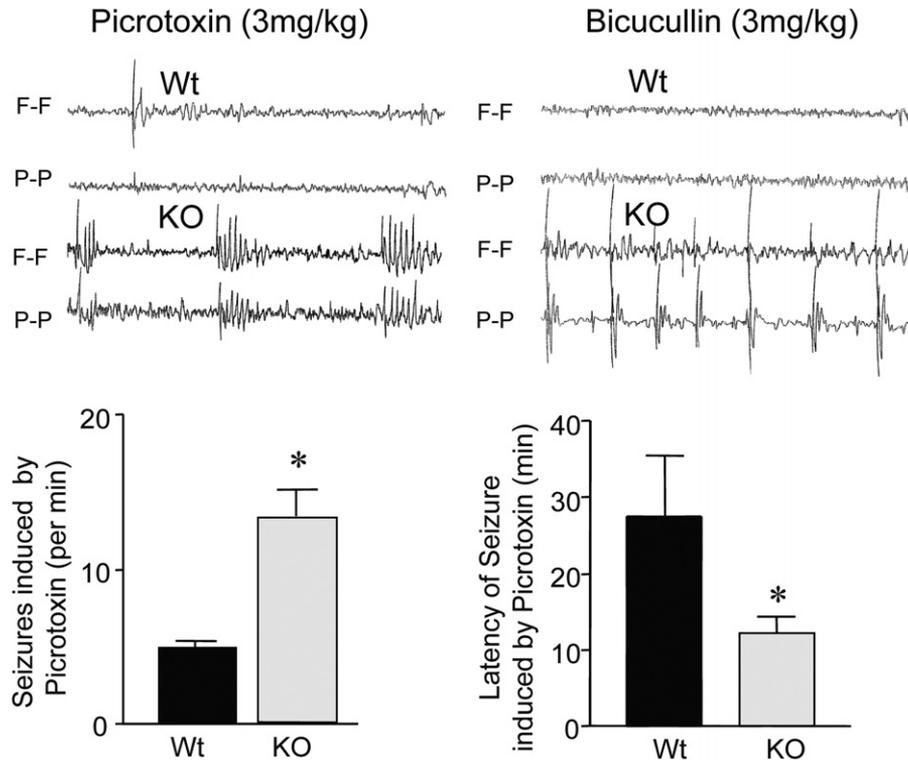
Fig. 5. Inhibition of GABA$_A$ function induces seizures. Age matched (>6 mon) Wt (*n*=7) and ClC-2 KO (*n*=7) mice were treated with the GABA$_A$ receptor inhibitors, picrotoxin or bicucullin. ECoG were recorded with (left-right) electrode pairs: F–F (frontal–frontal), P–P (parietal–parietal). The number of seizures associated with exacerbation of spike and wave discharges on the ECoG were recorded. The bar graphs show seizure frequency and the latencies prior until seizures for Wt and ClC-2 KO animals after picrotoxin treatment, mean±SEM. * indicates statistically significant difference between Wt and KO groups using the Students paired "*t*" test ($P<0.05$).

month of age) (Boettger et al., 2003; Hubner et al., 2001). The acute and early onset of neuronal degeneration in KCC2 and KCC3 knockout mice is thought to reflect their fundamental role in regulating neuronal chloride concentration and inhibitory neurotransmission (Boettger et al., 2003; Hubner et al., 2001). A similar mechanism is unlikely to account for the slowly progressive hippocampal neurodegeneration observed in ClC-2 knockout mice.

Astrocyte activation and neurodegeneration caused by disruption of ClC-2 expression

Within the hippocampus, ClC-2 is localized to neuronal somata, dendrites and axon initial segments associated with active zones of symmetrical (presumed to be GABAergic) synapses (Sik et al., 2000). Interestingly, ClC-2 is also highly concentrated on astrocytic end feet that come into close contact with neighboring astrocytes, pyramidal neurons and small blood vessels (Sik et al., 2000). The distinctive expression pattern of ClC-2 lead to the hypothesis that ClC-2 has a functional role in modifying astrocyte: neuron interactions (Sik et al., 2000). The observed hippocampal astrocyte activation and neurodegeneration caused by the loss of ClC-2 supports this hypothesis and a role for ClC-2 in the maintenance of neuronal health (Rossi and Volterra, 2009). Although the molecular basis for the functional role of ClC-2 in astrocyte: neuron communication is largely unknown, it may relate to a role in maintain-

ing extracellular ion homeostasis (Sik et al., 2000). For example, ClC-2 is thought to contribute to male germ cell development in mice by maintaining the ionic and pH environment that is critical to the functional interactions between male germ cells and Sertoli cells (Bosl et al., 2001).

Activation of astrocytes has been implicated in multiple neurodegenerative diseases including acute traumatic brain injury, Alzheimer's disease and inflammatory demyelinating diseases (Nair et al., 2008; Rossi and Volterra, 2009). Recently, Gavilan et al. (2007) showed (in the aging rodent brain) that neuroinflammation-related degeneration of somatostatin-expressing GABAergic neurons was highly concentrated in the hilus of the dentate gyrus and the CA3 region (Gavilan et al., 2007). Interestingly, both of these regions are distinctive with respect to the relatively large extent of their vascularization (Cavaglia et al., 2001). Loss of somatostatin-expressing GABAergic neurons has also been reported to contribute to a reduced seizure threshold in an experimental model of temporal lobe epilepsy, a process which may also involve neuroinflammation (Cossart et al., 2001). This model of temporal lobe epilepsy bears remarkable similarities with the morphological and electrophysiological phenotype described in the present study of the older ClC-2 KO mice. Although speculative, it is possible that the observed neurodegeneration due to loss of ClC-2 is related to neuroinflammation and the loss

of GABAergic neurons in the CA3 region, thereby accounting for the enhanced basal excitability and seizure susceptibility. Future studies which reveal the basis for enhanced neuroinflammation and confirm the identity of specific neurons lost in the CA3 region of the ClC-2 KO mouse will be critical to understanding the neuroprotective role that ClC-2 plays in the aging hippocampus.

Abnormal electrical activity recorded in older ClC-2 KO mice suggests a propensity for temporal lobe epilepsy

The current studies of ClC-2 KO mice do not support a primary role for ClC-2 in the etiology of generalized epilepsy, as the morphological and electrophysiological phenotypes do not appear until the mice are relatively old. Rather, our findings suggest that the impact of additional genetic and/or environmental factors conspire with the consequences of disrupting ClC-2 expression to promote a proinflammatory state in the hippocampus which leads in turn to neuroinflammation and neurodegeneration. Further, our data suggest that the search for linkage of CLCN2 mutations to modification of seizure susceptibility should extend beyond idiopathic generalized epilepsies to include localization related epilepsies such as the temporal lobe epilepsies.

REFERENCES

Al-Hazzaa AA, Bowen ID (1998) Improved cytochemical methods for demonstrating cell death using LR White as an embedding medium. Histochem J 30(12):897–902.

Bennett SA, Stevenson B, Staines WA, Roberts DC (1995) Periodic acid-Schiff (PAS)-positive deposits in brain following kainic acid-induced seizures: relationships to fos induction, neuronal necrosis, reactive gliosis, and blood-brain barrier breakdown. Acta Neuropathol 89(2):126–138.

Bennett SA, Tenniswood M, Chen JH, Davidson CM, Keyes MT, Fortin T, Pappas BA (1998) Chronic cerebral hypoperfusion elicits neuronal apoptosis and behavioral impairment. Neuroreport 9(1):161–166.

Blanz J, Schweizer M, Auberson M, Maier H, Muenscher A, Hübner CA, Jentsch TJ (2007) Leukoencephalopathy upon disruption of the chloride channel ClC-2. J Neurosci 27(24):6581–6589.

Boettger T, Rust MB, Maier H, Seidenbecher T, Schweizer M, Keating DJ, Faulhaber J, Ehmke H, Pfeffer C, Scheel O, Lemcke B, Horst J, Leuwer R, Pape HC, Völkl H, Hübner CA, Jentsch TJ (2003) Loss of K-Cl co-transporter KCC3 causes deafness, neurodegeneration and reduced seizure threshold. EMBO J 22(20):5422–5434.

Bösl MR, Stein V, Hübner C, Zdebik AA, Jordt SE, Mukhopadhyay AK, Davidoff MS, Holstein AF, Jentsch TJ (2001) Male germ cells and photoreceptors, both dependent on close cell-cell interactions, degenerate upon ClC-2 Cl(−) channel disruption. EMBO J 20(6):1289–1299.

Cavaglia M, Dombrowski SM, Drazba J, Vasanji A, Bokesch PM, Janigro D (2001) Regional variation in brain capillary density and vascular response to ischemia. Brain Res 910(1–2):81–93.

Combi R, Grioni D, Contri M, Redaelli S, Redaelli F, Bassi MT, Barisani D, Lavitrano ML, Tredici G, Tenchini ML, Bertolini M, Dalprà L (2009) Clinical and genetic familial study of a large cohort of Italian children with idiopathic epilepsy. Brain Res Bull 79(2):89–96.

Cossart R, Dinocourt C, Hirsch JC, Merchan-Perez A, De Felipe J, Ben-Ari Y, Esclapez M, Bernard C (2001) Dendritic but not somatic GABAergic inhibition is decreased in experimental epilepsy. Nat Neurosci 4(1):52–62.

Dhani SU, Mohammad-Panah R, Ahmed N, Ackerley C, Ramjeesingh M, Bear CE (2003) Evidence for a functional interaction between the ClC-2 chloride channel and the retrograde motor dynein complex. J Biol Chem 278(18):16262–16270.

Dickerson LW, Bonthius DJ, Schutte BC, Yang B, Barna TJ, Bailey MC, Nehrke K, Williamson RA, Lamb FS (2002) Altered GABAergic function accompanies hippocampal degeneration in mice lacking ClC-3 voltage-gated chloride channels. Brain Res 958(2):227–250.

Gavilán MP, Revilla E, Pintado C, Castaño A, Vizuete ML, Moreno-González I, Baglietto-Vargas D, Sánchez-Varo R, Vitorica J, Gutiérrez A, Ruano D (2007) Molecular and cellular characterization of the age-related neuroinflammatory processes occurring in normal rat hippocampus: potential relation with the loss of somatostatin GABAergic neurons. J Neurochem 103(3):984–996.

Holmes GL, Lenck-Santini PP (2006) Role of interictal epileptiform abnormalities in cognitive impairment. Epilepsy Behav 8(3):504–515.

Hübner CA, Stein V, Hermans-Borgmeyer I, Meyer T, Ballanyi K, Jentsch TJ (2001) Disruption of KCC2 reveals an essential role of K–Cl cotransport already in early synaptic inhibition. Neuron 30(2):515–524.

Kang JQ, Shen W, Macdonald RL (2009) The GABRG2 mutation, Q351X, associated with generalized epilepsy with febrile seizures plus, has both loss of function and dominant-negative suppression. J Neurosci 29(9):2845–2856.

Karle J, Woldbye DP, Diemer NH (2001) GABAA receptor antisense epilepsy: histological changes following infusion of antisense oligodeoxynucleotide to GABAA receptor gamma 2 subunit into rat hippocampus. Neurol Res 23(1):39–46.

Lee CL, Hannay J, Hrachovy R, Rashid S, Antalffy B, Swann JW (2001) Spatial learning deficits without hippocampal neuronal loss in a model of early-onset epilepsy. Neuroscience 107(1):71–84.

Leung LS (2009) Kindling, long-term potentiation and spatial memory performance. Can J Neurol Sci 36 (Suppl 2):S36–S38.

Löscher W, Fiedler M (1996) The role of technical, biological and pharmacological factors in the laboratory evaluation of anticonvulsant drugs. VI. Seasonal influences on maximal electroshock and pentylenetetrazol seizure thresholds. Epilepsy Res 25(1):3–10.

Lu Y, Wang X (2009) Genes associated with idiopathic epilepsies: a current overview. Neurol Res 31(2):135–143.

Macdonald RL, Kang JQ (2009) Molecular pathology of genetic epilepsies associated with GABA(A) receptor subunit mutations. Epilepsy Curr 9(1):18–23.

Moffitt P (1994) A methyl green-pyronin technique for demonstrating cell death in the murine tumour S180. Cell Biol Int 18(6):677–679.

Nair A, Frederick TJ, Miller SD (2008) Astrocytes in multiple sclerosis: a product of their environment. Cell Mol Life Sci 65(17):2702–2720.

Papale LA, Beyer B, Jones JM, Sharkey LM, Tufik S, Epstein M, Letts VA, Meisler MH, Frankel WN, Escayg A (2009) Heterozygous mutations of the voltage-gated sodium channel SCN8A are associated with spike-wave discharges and absence epilepsy in mice. Hum Mol Genet 18(9):1633–1641.

Rizzi M, Perego C, Aliprandi M, Richichi C, Ravizza T, Colella D, Velísková J, Moshé SL, De Simoni MG, Vezzani A (2003) Glia activation and cytokine increase in rat hippocampus by kainic acid-induced status epilepticus during postnatal development. Neurobiol Dis 14(3):494–503.

Rossi D, Volterra A (2009) Astrocytic dysfunction: insights on the role in neurodegeneration. Brain Res Bull 80(4-5):224–232.

Saint-Martin C, Gauvain G, Teodorescu G, Gourfinkel-An I, Fedirko E, Weber YG, Maljevic S, Ernst JP, Garcia-Olivares J, Fahlke C, Nabbout R, LeGuern E, Lerche H, Poncer JC, Depienne C (2009) Two novel CLCN2 mutations accelerating chloride channel deactivation are associated with idiopathic generalized epilepsy. Hum Mutat 30(3):397–405.

Sík A, Smith RL, Freund TF (2000) Distribution of chloride channel-2-immunoreactive neuronal and astrocytic processes in the hippocampus. Neuroscience 101(1):51–65.

Staley K, Smith R, Schaack J, Wilcox C, Jentsch TJ (1996) Alteration of GABAA receptor function following gene transfer of the CLC-2 chloride channel. Neuron 17(3):543–551.

Stewart LS, Bercovici E, Shukla R, Serbanescu I, Persad V, Mistry N, Cortez MA, Snead OC 3rd (2006) Daily rhythms of seizure activity and behavior in a model of atypical absence epilepsy. Epilepsy Behav 9(4):564–572.

Stobrawa SM, Breiderhoff T, Takamori S, Engel D, Schweizer M, Zdebik AA, Bösl MR, Ruether K, Jahn H, Draguhn A, Jahn R, Jentsch TJ (2001) Disruption of ClC-3, a chloride channel expressed on synaptic vesicles, leads to a loss of the hippocampus. Neuron 29(1):185–196.

Yoshikawa M, Uchida S, Ezaki J, Rai T, Hayama A, Kobayashi K, Kida Y, Noda M, Koike M, Uchiyama Y, Marumo F, Kominami E, Sasaki S (2002) CLC-3 deficiency leads to phenotypes similar to human neuronal ceroid lipofuscinosis. Genes Cells 7(6):597–605.

(Accepted 21 January 2010)
(Available online 29 January 2010)