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

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**Abstract—Heterozygous mutations in CIC-2 have been associated in rare cases with increased susceptibility to generalized, idiopathic epilepsy. Initially, it was hypothesized that mutations in CIC-2 may be associated with epilepsy due to a direct role for CIC-2 in the modification of hippocampal neuronal excitability. However, the absence of an overt seizure-susceptibility phenotype in young CIC-2 knockout (KO) mice rendered this hypothesis- implausible. A recent study of older CIC-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 CIC-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 CIC-2 expression plays a subtle neuroprotective role in the aging hippocampus.** 

**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 $\gamma$2subunit (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

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*Abbreviations:* ECoG, electrocorticogram; GFAP, glial fibrillary acidic protein; H&E, Hematoxylin and Eosin; Hi, hippocampus; MGPY, Methyl Green Pyronine Y; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP nick end labeling.

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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 $\mu$m) 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 nuclear 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: $\alpha$1 (1:500, Upstate Biotechnology) $\beta$2/3 (1:1000, Upstate Biotechnology) and $\gamma$2 (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, $\alpha$ 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-