Mechanisms of increased hippocampal excitability in the Mashl+/− mouse model of Na+/K+-ATPase dysfunction

Arsen S. Hunanyan1 | Ashley R. Helseth1 | Elie Abdelnour1 | Bassil Kherallah1 | Monisha Sachdev1 | Leeyup Chung2 | Melanie Masoud1 | Jordan Richardson1 | Qiang Li3,4,5 | J. Victor Nadler6 | Scott D. Moore3,4,5 | Mohamad A. Mikati1,2

1Division of Pediatric Neurology, Department of Pediatrics, Duke University Medical Center, Durham, NC, USA
2Division of Neurobiology, Duke University School of Medicine, Durham, NC, USA
3Durham Veterans Affairs Medical Center, Durham, NC, USA
4Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Durham, NC, USA
5Veterans Affairs Mid-Atlantic Region Mental Illness Research, Education, and Clinical Center, Durham, NC, USA
6Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC, USA

Summary

Objective: Na+/K+-ATPase dysfunction, primary (mutation) or secondary (energy crisis, neurodegenerative disease) increases neuronal excitability in the brain. To evaluate the mechanisms underlying such increased excitability we studied mice carrying the D801N mutation, the most common mutation causing human disease, specifically alternating hemiplegia of childhood (AHC) including epilepsy. Because the gene is expressed in all neurons, particularly γ-aminobutyric acid (GABA)ergic interneurons, we hypothesized that the pathophysiology would involve both pyramidal cells and interneurons and that fast-spiking interneurons, which have increased firing rates, would be most vulnerable.

Methods: We performed extracellular recordings, as well as whole-cell patch clamp recordings from pyramidal cells and interneurons, in the CA1 region on hippocampal slices. We also performed immunohistochemistry from hippocampal sections to count CA1 pyramidal cells as well as parvalbumin-positive interneurons. In addition, we performed video—electroencephalography (EEG) recordings from the dorsal hippocampal CA1 region.

Results: We observed that juvenile knock-in mice carrying the above mutation reproduce the human phenotype of AHC. We then demonstrated in the CA1 region of these mice the following findings as compared to wild type: (1) Increased number of spikes evoked by electrical stimulation of Schaffer collaterals; (2) equalization by bicuculline of the number of spikes induced by Schaffer collateral stimulation; (3) reduced miniature, spontaneous, and evoked inhibitory postsynaptic currents, but no change in excitatory postsynaptic currents; (4) robust action potential frequency adaptation in response to depolarizing current injection in CA1 fast-spiking interneurons; and (5) no change in the number of pyramidal cells, but reduced number of parvalbumin positive interneurons.

Significance: Our data indicate that, in our genetic model of Atp1a3 mutation, there is increased excitability and marked dysfunction in GABAergic inhibition. This supports the performance of further investigations to determine if selective expression of the mutation in GABAergic and or glutamatergic neurons is necessary and sufficient to result in the behavioral phenotype.
1 | INTRODUCTION

Major functions of the Na⁺/K⁺-ATPase pump are to regulate membrane excitability, Na⁺/K⁺ ionic gradients, synaptic transmission, neurotransmitter reuptake, and cell volume. Dysfunction of this pump is involved in many neurologic disorders including Alzheimer’s disease, mood disorders, rapid-onset dystonia-parkinsonism, spongiform encephalopathy, α-synucleinopathies, schizophrenia, epilepsy in animal models, epilepsy in humans, and alternating hemiplegia of childhood (AHC).

The ATP1A3 gene encodes for the Na⁺/K⁺ pump α₃ subunit, which is found exclusively in neurons, most prominently in γ-aminobutyric acid (GABA)ergic interneurons. One role of the α₃ isoform is rapid reversal of the large transient increase of [Na⁺]ᵢ that occurs during sustained action potentials of cells. Thus, it is likely that dysfunction of the α₃ subunit affects primarily the function of rapidly firing GABA interneurons.

Autosomal dominant mutations in the α₃ isoform are associated with a number of neurologic disorders, including AHC. AHC manifests episodes of hemiplegia, dystonia, epileptic seizures, and sudden unexpected death in epilepsy. Epilepsy usually starts in childhood, and is often focal and drug resistant. The most common ATP1A3 mutation that causes AHC in about one-third of patients is the D801N mutation. Despite the development of excellent mouse models of AHC, the mechanisms leading to excitation/inhibition imbalance at the cellular level due to ATP1A3 dysfunction are still not fully understood. Recently, we created a knock-in mouse model carrying the D801N mutation and demonstrated that adult heterozygous mice (Mashl+/-/C0) strikingly manifest a phenotype recapitulating essentially all of the human AHC-related symptoms including epilepsy.

Because epilepsy in AHC is first observed in childhood, our aim in this study was to investigate the neural mechanisms of increased excitability in juvenile Mashl+/− mice. Our hypothesis was that hippocampal slices will manifest increased excitability with reduced GABAergic inhibition. We addressed this question by performing electrophysiology experiments in CA1 hippocampal neurons in P21-30 Mashl+/− mice.

2 | METHODS

All procedures were approved by Duke University Institutional Animal Care and Use Committee.

Key points
- D801N knock-in mutation of Na/K pump induced spontaneous seizures in juvenile mice
- Mashl+/− manifest increased excitability of CA1 pyramidal cells
- Mashl+/− mice manifest reduced spontaneous inhibitory postsynaptic currents (sIPSCs), miniature inhibitory postsynaptic currents (mIPSCs), and evoked inhibitory postsynaptic currents (eIPSCs) but not excitatory postsynaptic currents (EPSCs) to CA1 pyramidal neurons compared to wild-type littersmates
- Mashl+/− mice CA1 PV+ interneurons show action potential frequency adaptation compared to wild-type littersmates
- Mashl+/− mice have reduced number of PV+ interneurons in the CA1 region compared to wild-type littersmates

2.1 | Mice

Generation of Mashl+/− mutant mice, as described previously, is detailed in the Supporting Information.

2.2 | Video-EEG recording

Video-electroencephalography (EEG) recordings were performed in Mashl+/− and wild-type (WT) age- and sex-matched littersmates from both sexes at P30 (see Data S1 for detailed description of recordings). No differences were found in these or the other experiments described below between the 2 sexes, so the data were combined.

2.3 | Slice electrophysiology

Recordings were performed from P21-P30 mice. Preparation of hippocampal slices were done as described previously with some modifications. Mice were deeply preanesthetized with 3% isoflurane in an induction chamber and kept in a face mask with 2-4% isoflurane. After transcranial perfusion the brains were removed and hippocampal sagittal slices were cut and used for both extracellular and whole cell patch clamp recordings (see Supplementary Material for detailed description of procedures). We recorded from CA1 pyramidal neurons located in a deep layer, for example, on the border of the stratum pyramidale...
and considered statistically significant if $P < .05$. Results are presented as mean ± standard error of the mean (SEM) and considered statistically significant if $P < .05$.

3 | RESULTS

3.1 | In vivo model shows spontaneous recurrent epileptic seizures, risk of sudden unexpected death of epilepsy, and other AHC manifestations

In our previous study, we demonstrated that adult Mash1+/− mice manifested the clinical features of AHC. In this study we documented the occurrence of the same phenotype in juvenile Mash1+/− mice including spontaneous recurrent seizures, dystonias, hemiplegias, and myoclonus. These occurred spontaneously or upon stimulation. Inducing stimuli included exposure to room temperature water, vestibular stimulation, or even simple manipulations (see Video S1 for spontaneous seizure and Video S2 for hemiplegia). Upon observation of an average time of 38 hours, 7 of 38 Mash1+/− mice (15 female, 23 male) mice had seizures with an average duration of 1.04 minutes (range 5 seconds to 4 minutes) and estimated seizure frequency was 0.42 seizures/animal/day. In addition, we observed that we could precipitate seizures in any mutant mouse if we applied triggering stimuli such as manipulation with vestibular stimulation or exposure to water. Of 10 consecutive observed seizures precipitated by manipulation in 10 different mice, 4 mice died during the seizures. Of 6 mutants age P30 and older recorded with depth electrodes and video-monitoring, 4 had electrographic seizures, most of which (54/60) had epileptic behavioral correlates. None of the 5 wild-type had any seizures. Forty-one of 60 observed seizures (mean duration 288.9 ± 140 s/seizure, documented during total of 222 hours), started either exclusively or concurrently from the hippocampus (Figure S1, Video S3). Eleven of those started exclusively from the hippocampus and 10 others started exclusively from frontal cortex. Finally, we demonstrated that the mortality rate in these mutant mice increased significantly as compared to wild-type ($P < .05$) (Figure S2).

3.2 | Increased excitability of the CA1 subregion of the hippocampus and effect of bicuculline in Mash1+/− as compared to wild-type

We found increased number of population spikes recorded from CA1 stratum pyramidale to 1 Hz single-pulse (wild-type: $1.5 ± 0.18, n = 3$ mice/8 slices vs Mash1+/−: $2.5 ± 0.24, n = 3$ mice/9 slices, $P = .004$, Figure 1A,E) as well as paired-pulse stimulus (100 msec interval, at first stimulation, wild-type: $1.87 ± 0.22, n = 3$ mice/8 slices vs Mash1+/−: $3.33 ± 0.33, n = 3$ mice/9 slices, $P = .003$, at second stimulation, wild-type: $2.5 ± 0.32$ vs Mash1+/−: $3.77 ± 0.14$, responses analyzed at 30th pulse, $P = .002$, Figure 1C,F), but not to low frequency 0.1 Hz (data not shown) as compared to wild-type. Because GABA<sub>A</sub> receptor activation has been shown to play a major role in the regulation of network excitability, we then measured the above responses in the presence of the GABA<sub>A</sub> receptor blocker bicuculline (10 µmol/L) and found that the number of spikes was equalized in both wild-type and Mash1+/− genotypes in the presence of bicuculline in both stimulation paradigm (for single pulse, $P = .133$, Figure 1B,E, for paired-pulse, $P = .148$, Figure 1D,F).

3.3 | No change in fiber volley amplitude and in the input-output curves between genotypes

Increased excitability in Mash1+/− vs wild-type mice hippocampal slices could be because of the difference of the fiber volley amplitude or field excitatory postsynaptic potential (fEPSP) slope in a given stimulus intensity. However, there were no significant differences in the slopes of the fiber volley vs stimulus intensity curves between genotypes nor were there any significant differences in the input-output curves between the 2 genotypes (Figure S3A-D).

3.4 | Pyramidal cells show some abnormalities in membrane properties in Mash1+/− mice

To determine whether increased excitability in the hippocampus of Mash1+/− mice could be due to abnormal...
membrane properties of pyramidal cells, we performed whole-cell current clamp recordings. CA1 pyramidal cells were identified by their pyramidal shape as well as by their action potential phenotype during increasing depolarization-induced current pulses. We found that there were some electrophysiologic differences between the 2 genotypes (Table S1). In Mashl<sup>+/−</sup> mice, pyramidal cells, resting membrane potential was depolarized as compared to wild-
Mash1+/− mice was significantly smaller than that in wild-type mice (P = .005; Figure 2A inset, 2B). It is important to note that Mash1+/− pyramidal cells showed double action potentials in 11 cells of 50 (6 of 16 mice, Figure 2D1), whereas in wild-type mice no such cells were observed in 53 cells (16 mice, P = < .003 Chi-square test, Figure 2C). Moreover, in contrast to wild-type mice 4 cells in mutants showed bursting action potential (P = .037, Fisher’s exact test). Representative examples of rheobase current induced normal in wild-type, double and bursting action potentials in Mash1+/− mice pyramidal cells, are shown in Figure 2C, D1, and D2, respectively.

3.5 Reduced threshold for action potential in CA1 pyramidal neurons in response to Schaffer collateral stimulation in Mash1+/− as compared to wild-type

Because population spikes can be affected by the properties of pyramidal cells and the amplitude of the EPSP, in the next experiments, we performed current clump recording from pyramidal cells during stimulation of Schaffer collaterals. Of interest, the threshold of stimulus intensity to induce action potential was significantly less in Mash1+/− mice (wild-type: 375 ± 22.9 μA, n = 4 mice/8 cells vs Mash1+/−: 289.1 ± 32.2 μA, n = 4 mice/8 cells, P = .047, Figure 2E,F).

3.6 Fast-spiking CA1 interneurons show reduced excitability due to marked action potential frequency adaptation in Mash1+/− mice

CA1 interneurons are known to be important in the regulation of hippocampal excitability, and dysfunction of these interneurons has been involved in facilitation of seizures in temporal lobe epilepsy. GABAergic interneurons in the CA1 area are highly diverse and they differ in their firing patterns.22 Because Atp1a3 is expressed highly in interneurons, we next performed whole cell recordings from CA1 SP-SO-alveus interneurons in hippocampal slices from wild-type littermate and Mash1+/− mice. Because our goal was to study physiologically defined types of interneurons that were classified after patching based on their firing characteristics,21–24 we studied 146 interneurons (73 mutants and 73 in wild-type). Of these, 16 in mutants and 27 in wild-type were non-fast-spiking nonaccommodating, 39 in mutants and 26 in wild-type were non–fast-spiking accommodating, and 18 in mutants and 20 in wild-type were fast-spiking. We found decreased excitability due to marked accommodation in only the fast-spiking interneurons in mutants as compared to wild-type (Figures 3 and 4). Because we found differences in the fast-spiking interneurons we then sought to determine if those are PV positive (PV+) or not. So we injected biocytin and then stained for PV in a subgroup of slices and found that 9/9 (4 mice) mutant and 11/11 (5 mice) of wild-type fast-spiking interneurons were PV+. None of the non–fast-spiking interneurons stained with PV. This is consistent with a previously reported study that found that PV+ interneurons are fast-spiking.25 To further validate our findings, we recorded from CA1 PV interneurons specifically expressing tdTomato as reporter in mice expressing tdTomato and the Mash1+/− mutations. Consistent with above results we found adaptation of fast-spiking PVtdTomato interneurons in Mash1+/− mice hippocampal slices but not in wild-type tdTomato interneurons (Figure 4, WT n = 3 mice/5 cells/4 slices, Mash1+/− n = 2 mice/4 cells/4 slices). We also recorded non–fast-spiking SO interneurons (wild-type: n = 30 mice/53 cells; Mash1+/−: n = 26 mice/55 cells, Figure 3), and post hoc immunohistochemistry using fast-spiking marker PV did not show any co-staining with biocytin. There were no significant differences in the number of action potentials during rising stimulus current in those non-PV+ interneurons between genotypes (Figure 3, Table S1). However, in non–fast-spiking accommodating interneurons the mean amplitude of action potential, measured at rheobase current, in Mash1+/− mice (52.6 ± 2 mV) was significantly smaller compared with wild-type (60.2 ± 2 mV, P = .015). We did not use other interneuron markers for identification of the subtypes of the non-PV+ interneurons because in the hippocampal CA1 area there are at least 21 types of interneurons and because doing so would constitute a separate study by itself.22–26

Taken together, these results demonstrate that in Mash1+/− mice excitability of fast-spiking PV+ and non–fast-spiking accommodating interneurons is dramatically decreased.

3.7 Excitatory postsynaptic inputs to CA1 pyramidal neurons are not increased in Mash1+/− mice

Increased excitability of the CA1 hippocampal region could be due to increased excitatory synaptic inputs on to the CA1 pyramidal neurons. To assess this hypothesis, we recorded spontaneous excitatory postsynaptic currents (sEPSC) and miniature excitatory postsynaptic currents (mEPSC) from the hippocampal CA1 pyramidal neurons in a voltage clamp mode. We found that neither amplitude
nor frequency of sEPSCs (amplitude, wild-type: 14.9 ± 0.9 pA, n = 6 mice/15 cells/15 slices vs Mashl+/−: 13.6 ± 0.7 pA, n = 5 mice/10 cells/10 slices, P = .949; frequency, wild-type: 0.72 ± 0.09 Hz vs Mashl+/−: 0.73 ± 0.17 Hz, P = .964) and mEPSCs (amplitude, wild-type: 11 ± 0.6 pA, n = 6 mice/15 cells/15 slices vs Mashl+/−: 11.2 ± 0.6 pA, n = 5 mice/10 cells/10 slices, P = .845; frequency, wild-type: 0.35 ± 0.05 Hz vs Mashl+/−: 0.32 ± 0.1 Hz, P = .767) were increased in Mashl+/− mice as compared to wild-type littermates (Figure 5A-F). These results demonstrate that basal glutamatergic neurotransmission to CA1 pyramidal cells are not augmented in mutant mice.

3.8 | Inhibitory postsynaptic currents in CA1 pyramidal neurons are markedly reduced in Mashl+/− mice

To investigate if observed increases in excitability of area CA1 could be due to decrease in GABA synaptic...
transmission, we recorded inhibitory postsynaptic currents (IPSCs) from CA1 pyramidal cells. The Kolmogorov-Smirnov test revealed a significant difference in peak current of events (Figure 5G-I; \( P = .001 \)). The mean of spontaneous inhibitory postsynaptic current (sIPSC) amplitude (\( \text{Mashl}^{+/-}/C_0 \): 38.8 ± 3.9 pA, \( n = 12 \) mice/22 cells/22 slices vs wild-type: 53.6 ± 4.3 pA, \( n = 10 \) mice/19 cells/19 slices; \( P = .016 \); Tukey test, Figure 5G-I) was significantly smaller than in wild-type mice, but the frequency of events was not statistically different (\( \text{Mashl}^{+/-}/C_0 \): 4.7 ± 0.3 Hz vs WT: 5.5 ± 0.3 Hz, \( P = .135 \), Figure 5I). There was no statistically significant difference in sIPSCs rise time (10-90%) and decay time between genotypes (wild-type: rise time 3.3 ± 0.6 msec, \( \text{Mashl}^{+/-}/C_0 \): rise time 3.4 ± 0.4 msec, \( P = .83 \); wild-type: decay time 24 ± 1.8 msec, \( \text{Mashl}^{+/-}/C_0 \): decay time 22 ± 2.1 msec, \( P = .34 \)). Next, we recorded miniature inhibitory postsynaptic currents (mIPSCs) and found that in \( \text{Mashl}^{+/-}/C_0 \) mice hippocampal slices, the mean of mIPSC amplitude (\( \text{Mashl}^{+/-}/C_0 \): 25.2 ± 2.4 pA, \( n = 7 \) mice/13 cells/13 slices vs wild-type: 40.8 ± 4.5 pA, \( n = 5 \) mice/13 cells/13 slices; \( P = .006 \); Figure 5J-L) and the frequency of events (\( \text{Mashl}^{+/-}/C_0 \): 3.9 ± 0.3 Hz vs WT: 4.7 ± 0.2 Hz, \( P = .048 \)) were significantly smaller than in wild-type mice. To ensure that mIPSCs were induced by activation of GABA\(_A\) receptors, bicuculline (10 \( \mu \)mol/L) was added to the recording chamber. Bicuculline blocked all recorded events (data not presented).

We further examined the disinhibition of CA1 pyramidal neurons by electrically eliciting GABA input onto the CA1 pyramidal neurons. The stimulation electrode was positioned in the stratum oriens 100 \( \mu \)m away from the recorded CA1 cell and monosynaptic GABA receptor mediated IPSCs were isolated in the presence of CNQX (20 \( \mu \)mol/L) and AP5 (50 \( \mu \)mol/L). Stimulation of stratum oriens induced evoked
inhibitory postsynaptic currents (eIPSCs) with threshold currents that were usually 20-30 μA and were not different between genotypes (n = 6 mice for each genotype, P > .05). The mean amplitude of eIPSCs in Mashl−/− mice was depressed starting from the 30th pulse of 1 Hz stimulation (Figure 5M,N). These results show decreased GABA transmission from SO interneurons to CA1 pyramidal neurons during repetitive stimulation, and that there is abnormal short-term plasticity mediated by inhibitory synaptic transmission in the hippocampus.

3.9 | Immunohistochemistry

Immunohistochemistry was performed on dorsal hippocampal slices from Mashl−/− and WT littermates to count the number of CA1 pyramidal cells using neurogranin (NG) as a marker. The number of NG+ cells was not different between genotypes (wild-type: 20937 ± 1537, n = 5 mice vs Mashl−/−: 24122 ± 1633, n = 5 mice, P = .22, Figure 6A,C). We also quantitated the number of these PV+ interneurons in area CA1. A majority of PV+ cells were

FIGURE 4 Comparison of voltage responses to current steps of CA1 parvalbumin positive (PV+) fast-spiking interneurons in wild-type littermate (WT) and Mashl−/− mice hippocampal slices using whole-cell current-clamp recordings. A, Representative traces showing action potential firing pattern in a PV+ fast-spiking interneuron in WT (n = 14 mice/20 cells, blue traces) and (B) Mashl−/− mice (n = 15 mice/18 cells, red traces) in response to incremental steps to current. Presented responses were evoked by depolarization (100 pA, 200 pA, 300 pA and 450 pA, 1 s duration) injected currents. Note that in contrast to WT, fast-spiking interneurons in Mashl−/− mice show a strong firing frequency adaptation with depolarizing current injection. Inset shows superimposed action potentials recorded from WT (blue traces) and from Mashl−/− mice (red traces). C, The mean number of action potentials in response to each current step. D, Confocal image shows double immunostaining with biocytin (red) and parvalbumin (PV, green) after electrophysiologic recording. Scale bar, 20 μm. E, Comparison of voltage responses to current steps of CA1 parvalbumin-tdTomato interneurons in wild-type (WT: n = 3 mice/5 cells/4 slices) and Mashl−/− mice (n = 2 mice/4 cells/4 slices) hippocampal slices (F). Note that, in contrast to WT, PVtdTomato mouse Mashl−/−, PVtdTomato mouse interneuron shows strong adaptation of action potential frequency. Inset shows example of fluorescence image of expression of tdTomato in CA1 PV interneurons taken from WT mouse. SO - stratum oriens, SP - stratum pyramidale, SR-stratum radiatum. Data presented as mean ± SEM, ** P < .01, *** P < .001
located in the stratum oriens and stratum pyramidale in hippocampal slices prepared from both mutant mice and wild-type littermates. Stereological quantification revealed reduced number of PV+ cells in Mash1+/− compared to wild-type littermates (Mash1+/−: 3826 ± 347, n = 6 mice; wild-type: 6231 ± 285; n = 6 mice, P < .001; Figure 6B,D). These results suggest that the Atp1a3 mutation also caused a reduction in PV+ interneurons in the dorsal CA1 area.

4 | DISCUSSION

In this study, we found that the D801N mutation results in spontaneous recurrent seizures, increased excitability in CA1 hippocampal slices, marked impairment in GABA inhibition, PV+ cell loss, and action potential frequency adaptation in fast-spiking PV+ interneurons.

4.1 | Increased excitability in extracellular recordings and role of GABA

Our findings of increased excitability in juvenile Mash1+/− mice extend our prior findings in Mash1+/− adult mice and the findings of increased excitability in other AHC models. As in adults, we found in this study on juveniles increased spiking as a result of fast, 1 Hz, but not slow, 0.1 Hz stimulation. In the present study, we also investigated the effects of GABA receptor blockade and found a similar degree of spiking in juvenile mutants and wild-type mice in the presence of the GABAA receptor antagonist bicuculline. This is consistent with the hypothesis that GABA dysfunction is a major cause of increased excitability in our model. Of note is that in mice carrying the I810N mutation, valproic acid reduces the severity of seizures, and in those with the D801Y mutation, clonazepam corrects some of the behavioral changes.

4.2 | Increased excitability in the hippocampal CA1 pyramidal cells

Depolarization of resting membrane potential as well as doublets and bursting action potentials in some pyramidal cells can significantly contribute to overall increased excitability in Mash1+/− mice hippocampal slices. These observed abnormalities are most probably not due to a direct effect of α3 mutation on pyramidal cells because expression of the α3 subunit is lower in pyramidal neurons than in GABAergic interneurons where we did not find similar abnormalities. Given our findings of reduced inhibition, some of the observed changes in pyramidal cell properties, thus, may be secondary to changes in GABAergic tonic inhibition onto these neurons. Other explanations of the changes in pyramidal cells could be due to changes in [K+]o or K+ conductance. In contrast to our mouse model, Holm et al. found that in D801Y mice, which have a less severe phenotype of AHC than our model, there are only minor changes in CA1 pyramidal cell electrophysiology.

No change in EPSP amplitude and reduction in IPSPs suggest that GABAergic inhibition to pyramidal neurons is particularly compromised in the mutants. CA1 pyramidal cells receive massive GABAergic inputs from fast-spiking PV+ interneurons, which control the output of local networks by inhibiting action potential generation in principal cells.

4.3 | Markedly reduced GABAergic inhibition from fast-spiking interneurons

To our surprise, in Mash1+/− mice, fast-spiking CA1 interneurons exhibited marked action potential frequency adaptation in response to depolarizing stimuli, indicating reduced excitability of these interneurons. CA1 interneurons are activated by recurrent collaterals of pyramidal cells and therefore have a significant role in controlling excitability in area CA1. Because fast-spiking interneurons fire action potentials with higher frequency, these cells would require rapid restoration of Na+ and K+ ion gradients across the plasma membrane, which is one of the major roles of Atp1α3. Dysfunction of the Na/K pump due to mutation and/or due to secondary causes, such as ATP depletion resulting from ongoing seizure activity, may lead to the loss of ionic gradient across the membrane, and can ultimately lead to failure of action potential generation. Indeed, the Na+/K⁺-ATPase current density (or its activity) is known to be 3-to 7-fold larger in fast-spiking interneurons than in cortical pyramidal neurons. In addition, increases in K+ concentration due to seizure activity could further impair mutated pump function resulting depolarization block of fast-spiking interneurons. An additional potential could involve upregulation of calcium-dependent potassium currents in these interneurons.

Our recordings also revealed marked reductions in IPSC frequency and amplitude. This suggests both pre- and postsynaptic changes in GABA synapses. The Na+ gradient generated by Na+/K⁺-ATPase is important as a driving force for neurotransmitter uptake. The Atp1a3 mutation could increase GABA concentration in the synaptic cleft by reducing uptake. This sustained increase of GABA could potentially lead to internalization or desensitization of postsynaptic GABA A receptors. Consistent with our findings of abnormal firing in GABAergic neurons is that 2 other models of Atp1a3 mutations manifest abnormalities in cerebellar GABAergic neuronal firing. Whereas the types of observed abnormalities differ among the 3 models,
this is likely to be due to differences in the type of cells studied in the ages investigated, and to the likely different effects of different mutations. For example, the D801N mutation shows a dominant negative effect when expressed in *Xenopus laevis* oocytes, whereas knocking out the gene is not expected to do so.

We also found that the eIPSC amplitudes were depressed during repetitive stimulation at SO and only after...
the 30th pulse. This could be because in the resting state there is a not much change in the balance of excitatory and inhibitory synaptic responses from SO, whereas during repetitive stimulation there is robust excitation of SO interneurons, which leads to depression of action potential firing and ultimately an increased excitability of pyramidal cells. This is also consistent with the function of the 
Atp1a3 subunit being important under conditions of increased firing. Other histological subtypes of interneurons not investigated in this study may be involved too and should be the subject of future investigations.

We found reduction of PV+ cell numbers in our mice. The cause may be prior seizure activity, as loss of PV+ cells has been reported in chronic epilepsy including in the hippocampus. Alternatively, the reduction may be related to other effects of the Atp1a3 mutation. Dysfunction of the Na/K pump can lead to secondary calcium overload through reversed Na+/Ca2+ exchange. Interneurons that normally highly express the 
Atp1a3 subunit and that are fast-spiking, such as PV+ interneurons, would then be the most vulnerable to potential Ca2+-mediated toxicity. In addition, Na+/K+-ATPase is also a signal transducer that modulates cell growth, adhesion, and apoptotic threshold. Consequently, dysfunction in this molecule can potentially result in cell death and impaired neuronal migration.

Our findings of reduced GABAergic inhibition in a genetic model of epilepsy are similar to findings in some other epilepsy models. Dravet syndrome epileptic mice exhibit action potential frequency adaptation in PV+ interneurons, impaired GABAergic neurotransmission, and reduction in IPSC frequency, but also have increased AP threshold and rheobase not manifested in our mice. Finally, PV+ interneurons are important in other types of induced epilepsy models.

4.4 | Potential implications of our findings

Our findings raise the possibility that neurophysiologic dysfunction in other areas of the brain, similar to what we found in the hippocampus, may be responsible for the other AHC manifestations. Although spreading depression, the presumed mechanism of hemiplegias in AHC, has been traditionally related to glutamatergic mechanisms; there is also strong evidence that loss of GABAergic inhibition is important too. Our findings are also in line with emerging evidence to support the “interneuron energy hypothesis.” Taking into consideration the complexity of neuronal circuits and especially the great heterogeneity of GABAergic interneurons, experiments using conditional mice allowing the introduction of the Atp1a3 mutation in specific types of neurons should be a useful approach to dissect further the AHC- and Atp1a3-related epilepsy pathophysiology.

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DISCLOSURE

None of authors has any conflict of interest to disclose. 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.

ORCID

Mohamad A. Mikati http://orcid.org/0000-0003-0363-8715

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.