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***Hlf* is a genetic modifier of epilepsy caused by voltage-gated sodium channel mutations**

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Abstract

Mutations in voltage-gated sodium channel genes cause several types of human epilepsies. Often, individuals with the same sodium channel mutation exhibit diverse phenotypes. This suggests that factors beyond the primary mutation influence disease severity, including genetic modifiers. Mouse epilepsy models with voltage-gated sodium channel mutations exhibit strain-dependent phenotype variability, supporting a contribution of genetic modifiers in epilepsy. The *Scn2a*^{Q54} (Q54) mouse model has a strain-dependent epilepsy phenotype. Q54 mice on the C57BL/6J strain exhibit delayed seizure onset and improved survival compared to [B6xSJL/J]F1.Q54 mice. We previously mapped two dominant modifier loci that influence Q54 seizure susceptibility and identified *Hlf* (hepatic leukemia factor) as a candidate modifier gene at one locus. *Hlf* and other PAR bZIP transcription factors had previously been associated with spontaneous seizures in mice thought to be caused by down-regulation of the pyridoxine pathway. An *Hlf* targeted knockout mouse model was used to evaluate the effect of *Hlf* deletion on Q54 phenotype severity. *Hlf*^{KO/KO};Q54 double mutant mice exhibited elevated frequency and reduced survival compared to Q54 controls. To determine if direct modulation of the pyridoxine pathway could alter the Q54 phenotype, mice were maintained on a pyridoxine-deficient diet for 6 weeks. Dietary pyridoxine deficiency resulted in elevated seizure frequency and decreased survival in Q54 mice compared to control diet. To determine if *Hlf* could modify other epilepsies, *Hlf*^{KO/+} mice were crossed with the *Scn1a*^{KO/+} Dravet syndrome mouse model to examine the effect on premature lethality. *Hlf*^{KO/+}; *Scn1a*^{KO/+} offspring exhibited decreased survival compared to *Scn1a*^{KO/+} controls. Together these results demonstrate that *Hlf* is a genetic modifier of epilepsy caused by voltage-gated sodium channel mutations and that modulation of the pyridoxine pathway can also influence phenotype severity.

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1. INTRODUCTION

Epilepsy is a common neurological disorder affecting approximately 50 million people worldwide (WHO, 2012). Over 1000 mutations identified in voltage-gated sodium channel genes result in several human epilepsy syndromes (Meisler et al., 2010). Often, individuals with the same mutation can exhibit strikingly different clinical severity. This suggests that the effect of the primary mutation is influenced by other factors, which may include genetic modifiers.

Several mouse models have been generated in order to study genetic epilepsies. Frequently, strain background alters the disease phenotype, supporting a contribution of genetic modifiers in epilepsy. The *Scn2a*^{Q54} (Q54) mouse model has a gain-of-function mutation that results in persistent sodium current and epilepsy (Kearney et al., 2001). On the C57BL/6J (B6) strain, Q54 mice have infrequent, adult-onset focal motor seizures and a 75% survival rate beyond six months of age (Bergren et al., 2005). When crossed with the SJL/J (SJL) strain, resulting F1.Q54 mice experience high seizure frequency with juvenile onset and less than 25% survival at six months of age (Bergren et al., 2005). We mapped two dominant loci that modify seizure susceptibility of Q54, designated *Moe1* (Modifier of Epilepsy) on chromosome 11 and *Moe2* on chromosome 19 (Bergren et al., 2005). In contrast to the overall effect, at the *Moe1* locus B6 alleles confer increased seizure risk (Hawkins and Kearney, 2012). Fine-mapping and candidate gene analysis by RNA-seq suggested hepatic leukemia factor (*Hlf*) as a candidate modifier at the *Moe1*. B6 mice express lower levels of *Hlf* transcript compared to SJL (Hawkins and Kearney, 2012), leading us to hypothesize that deletion of *Hlf* would increase seizure susceptibility.

Hlf is a member of the PAR bZIP transcription factor family, which includes *Hlf*, *Dbp* and *Tef*. Deletion of this family in a knock-out mouse model resulted in spontaneous generalized tonic-clonic and absence seizures, and premature lethality (Gachon et al., 2004). Two-fold reduction in expression of pyridoxal kinase (PDXK) was implicated as a likely contributor to the epilepsy phenotype. PDXK is essential for conversion of pyridoxine to pyridoxal 5' phosphate (PLP), a key coenzyme involved in amino acid and neurotransmitter metabolism, including GABA and glutamate (John, 1995). Pyridoxine deficiency in humans results in epilepsy that is successfully treated by PLP administration (Plecko and Stockler, 2009). Based on its prior association with seizures and the pyridoxine pathway, we hypothesized that genetic variation in *Hlf* could modify the Q54 epilepsy phenotype (Hawkins and Kearney, 2012).

To determine if *Hlf* could modify the Q54 phenotype, we evaluated the effect of *Hlf* deletion on seizures and survival in Q54 mice. Additionally, we tested whether manipulation of the pyridoxine pathway could modify the Q54 phenotype. Finally, to determine whether *Hlf* would modify epilepsy in another model, we evaluated the effect of *Hlf* deletion on phenotype of the *Scn1a*^{+/-} Dravet syndrome model.

2. MATERIAL AND METHODS

2.1 Mice

Hlf^{tm1Schb} (*Hlf*^{KO/+}) knockout embryos congenic on C57BL/6 were obtained from the European Mouse Mutant Archive (www.emmanet.org) (Gachon et al., 2004). Q54 transgenic mice [Tg(Eno2-Scn2a1*)Q54Mm] congenic on C57BL/6J were previously described (Bergren et al., 2005; Kearney et al., 2001). *Scn1a*^{tm1Kea} (*Scn1a*^{KO/+}) heterozygous knockout mice congenic on 129S6/SvEvTac (129) were previously described (Miller et al., 2014). Genotyping was performed by PCR of DNA isolated from postnatal day 14 (P14) tail biopsies. Mice were group-housed with access to food and water *ad libitum*. All studies were approved by the local Animal Care and Use Committees.

Hlf;Q54 double mutant mice were generated by crossing *Hlf*^{KO/+} females with Q54 males to generate *Hlf*^{KO/+};Q54 offspring, which were then crossed with *Hlf*^{KO/+}. Resulting offspring included *Hlf*^{KO/KO};Q54 and *Hlf*^{KO/+};Q54 test mice, and single mutant Q54, *Hlf*^{KO/+}, and *Hlf*^{KO/KO} control littermates. *Hlf*; *Scn1a*^{KO/+} double mutant mice were generated by crossing B6. *Hlf*^{KO/+} and 129.*Scn1a*^{KO/+} mice. Resulting F1 offspring included double mutant *Hlf*^{KO/+}; *Scn1a*^{KO/+} test mice, and *Scn1a*^{KO/+}, and *Hlf*^{KO/+} control littermates.

2.2 Phenotyping

Mice were phenotyped as previously described (Hawkins and Kearney, 2012). Briefly, littermates underwent 30 minute video-taping at three and six weeks of age. Behavioral seizures were scored offline by a blinded observer using ObserverXT software (Noldus). Prior extensive video-electroencephalogram (EEG) monitoring of Q54 mice demonstrated strong correlation between behavioral and EEG seizures ($\kappa=0.988$) (Anderson et al., 2014; Kearney et al., 2001). The number of focal motor seizures with forelimb clonus and repetitive movements lasting 1–5 seconds, and generalized tonic-clonic seizures (GTCS) with rearing and falling lasting approximately 1 min were counted. Seizure counts from the three and six week observations were combined to obtain the total number of focal motor seizures and GTCS in 60 minutes for each animal.

2.2 Video-EEG Monitoring

Hlf^{KO/KO};Q54 (n=4) and *Hlf*^{KO/+};Q54 (n=2) mice were implanted with headmounts for video-EEG monitoring (Pinnacle Technology, Inc.) as previously described (Hawkins et al., 2011). After 24 hours of recovery, EEG was recorded from freely moving mice. *Hlf*^{KO/KO};Q54 and *Hlf*^{KO/+};Q54 mice were monitored for a total of 151 and 52 hours, respectively. Acquisition and analysis was performed with Sirenia software along with contemporaneous video recordings (Pinnacle Technology, Inc.). Epileptiform activity was scored manually.

2.3 Pyridoxine Deficient Diet

Diets pellets were formulated by Research Diets, Inc. to contain a standard dietary amount of pyridoxine HCl (AIN-93G/5.8 ppm) or with no added pyridoxine (AIN-93G/no pyridoxine HCl). Wildtype and Q54 littermates were randomly assigned to pyridoxine

deficient or control diet at 3 weeks of age and maintained on the diet for 6 weeks. At nine weeks of age, surviving mice underwent 30 minute video-taping for seizures, as described above.

3. RESULTS AND DISCUSSION

3.1 Loss of *Hlf* Exacerbates Q54 Phenotype

To determine whether *Hlf* could act as a modifier of the Q54 epilepsy phenotype, we evaluated the effect of *Hlf* deletion on Q54 seizure severity using an *Hlf* knockout model. The number and type of seizures at 3 and 6 weeks of age were compared between *Hlf*;Q54 double mutant mice and littermate controls. There was no difference in the number of mice exhibiting focal motor seizures (Figure 1a) or in the average number of focal motor seizures observed (Figure 1b). GTCS were only observed in *Hlf*^{KO/KO};Q54 mice (Figure 1a & 1b). This more severe seizure type has never been observed in Q54 mice of this age in our previous studies (Bergren et al., 2009; Hawkins and Kearney, 2012; Jorge et al., 2011; Kearney et al., 2001). Video-EEG monitoring performed on additional *Hlf*^{KO/KO};Q54 mice confirmed the generalized electrographic seizure activity of *Hlf*^{KO/KO};Q54 mice (Figure 1d).

Hlf^{KO/KO};Q54 mice exhibited a significant reduction in survival compared to Q54 (Figure 1c). By six weeks of age, 25% of *Hlf*^{KO/KO};Q54 died, compared to only 3% of Q54 mice. Consistent with previous reports, *Hlf*^{KO/+} and *Hlf*^{KO/KO} mice had 100% survival throughout the study (data not shown) (Gachon et al., 2004). Together, our seizure and survival data demonstrate that complete loss of *Hlf* exacerbated the Q54 epilepsy phenotype.

3.2 Effect of Pyridoxine Deficient Diet on Q54

We hypothesized that B6.Q54 mice might be sensitized to pyridoxine deficiency based on previous data that showed reduced *Hlf* brain transcript in B6 mice (Hawkins and Kearney, 2012), and involvement of *Hlf* in the transcriptional regulation of PDXK, a key enzyme in the pyridoxine pathway (Gachon et al., 2004). To determine if direct modulation of the pyridoxine pathway would modify their phenotype, Q54 mice were maintained on either a pyridoxine deficient or control diet for six weeks, beginning at 3 weeks of age. Survival was monitored during this time, and seizure frequency was evaluated in a 30 minute session at nine weeks of age. Q54 mice maintained on a pyridoxine deficient diet had significantly more focal motor seizures compared to those on control diet (Figure 2a). No GTCS were observed in the pyridoxine deficient or control diet group. Q54 mice maintained on the pyridoxine deficient diet had a significant reduction in lifespan compared to controls (Figure 2b). Only 69% of Q54 mice on the deficient diet survived, compared to 94% on control diet. Thus, pyridoxine deficiency exacerbated the Q54 epilepsy phenotype, although this was not as dramatic as *Hlf* deletion since we did not observe GTCS. This suggests that *Hlf* deletion may have additional effects. Nonetheless, these results suggest that maintaining adequate pyridoxine levels is important in the context of epilepsy caused by a sodium-channel mutation.

3.3 Loss of *Hlf* exacerbates phenotype of *Scn1a*^{KO/+} Dravet mice

To determine if the *Hlf* modifier effect would translate to other epilepsy models, we assessed survival of *Hlf*^{KO/+};*Scn1a*^{KO/+} double mutant mice. We observed a significant reduction in lifespan between *Hlf*^{KO/+};*Scn1a*^{KO/+} and *Scn1a*^{KO/+} mice (Figure 3). By four weeks of age, only 45% of *Hlf*^{KO/+};*Scn1a*^{KO/+} mice survived, compared to 71% of *Scn1a*^{KO/+} mice. *Scn1a*^{KO/+} mice recapitulate many features of Dravet syndrome, including development of early onset epilepsy, spontaneous seizures and premature death (Kalume et al., 2013; Mistry et al., 2014; Yu et al., 2006). It is hypothesized that reduced GABAergic inhibitory function responsible for the *Scn1a*^{KO/+} seizure phenotype (Yu et al., 2006). This mechanism differs from Q54 mice, where seizures result from hyperexcitability of principal excitatory neurons (Kearney et al., 2001). Exacerbation of the epilepsy phenotype in a second model with a different underlying mechanism suggests that loss of *Hlf* alters excitability in a common pathway, perhaps by altering neurotransmitter synthesis.

3.4 Conclusions

We demonstrated that loss of *Hlf* modifies phenotypes in two genetic epilepsy models, Q54 and *Scn1a*^{KO/+} mice. The mechanism whereby *Hlf* exerts its effect remains to be determined in future studies. Elucidation of the underlying mechanism may suggest novel therapeutic strategies for improved treatment of epilepsy.

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HIGHLIGHTS

- Genetic deletion of Hepatic leukemia factor (*Hlf*) exacerbated epilepsy in *Scn2a^{Q54}* mice.
- Pyridoxine deficiency exacerbated the epilepsy phenotype of *Scn2a^{Q54}* mice.
- Genetic deletion of *Hlf* worsened survival in the *Scn1a^{+/-}* model of Dravet syndrome.
- *Hlf* acts as a genetic modifier of epilepsy, possibly by pyridoxine pathway modulation.

Figure 1. Loss of *Hlf* exacerbates the *Scn2a*^{Q54} phenotype

A) Mice were observed for focal motor seizures (FMS) and generalized tonic clonic seizures (GTCS) in 30 minute sessions at 3 and 6 weeks of age. The percentages of mice exhibiting FMS or GTCS during the observation period are shown (n=30–32 per group). Significant differences between groups were determined by Fisher's exact test. *p < 0.0134 compared to Q54 littermate controls. **B)** The average number of FMS (±S.E.M) and total number of GTCS observed is shown (n=30–32 per group). **C)** Kaplan-Meier plot of survival to 6 weeks of age. *Hlf*^{KO/KO};Q54 had a significant decrease in survival compared to Q54 mice (p=0.0215, LogRank Mantel-Cox). **D)** Representative EEG recording from a *Hlf*^{KO/KO};Q54 during a seizure. Time span of plot is 3 minutes. Top trace is right posterior to left posterior; bottom trace is right anterior to left posterior.

Figure 2. Pyridoxine deficiency exacerbates the *Scn2a*^{Q54} phenotype

A) Average FMS frequency of Q54 mice maintained on pyridoxine deficient or control diets from 3 to 9 weeks of age. The number of FMS was counted in a 30 minute session at 9 weeks of age. Significant differences were determined by Student's T-test (* $p < 0.03$). Data are represented as mean $\pm$ SEM. **B)** Survival of Q54 mice maintained on pyridoxine deficient or control diets was monitored until nine weeks of age. Kaplan Meier survival plot is shown. Q54 mice on the deficient diet had a significant decrease in survival compared to control diet ($p < 0.002$, LogRank Mantel-Cox).

Figure 3. Loss of *Hlf* exacerbates premature lethality phenotype of *Scn1a*^{KO/-} Dravet mice
 Survival of $Hlf^{KO/+}$, $Scn1a^{KO/+}$ and $Hlf^{KO/+};Scn1a^{KO/+}$ mice was monitored until nine weeks of age. Kaplan Meier survival plot is shown. $Hlf^{KO/+};Scn1a^{KO/+}$ mice had reduced survival compared to $Scn1a^{KO/+}$ mice ($p < 0.006$, LogRank Mantel-Cox).