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Increased seizure susceptibility in a mouse model of neurofibromatosis type 1

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ABSTRACT

Neurofibromatosis type 1 (NF1) is a neurocutaneous disorder linked to higher rates of epilepsy as compared with the general population. Although some epilepsy cases in NF1 are related to intracranial lesions, epileptogenic lesions are not always identified. It is unknown whether the genetic mutation itself, which leads to lower levels of the tumor suppressor protein neurofibromin, alters seizure susceptibility. The purpose of this research was to determine whether *Nf1*$^{+/-}$ mice have altered seizure susceptibility to the chemical convulsants kainic acid and pilocarpine. Young adult *Nf1*$^{+/-}$ or WT control (*Nf1*$^{+/+}$) mice were injected with either 20 mg/kg kainic acid or scopolamine 1 mg/kg and pilocarpine 300 mg/kg and assessed for various behavioral seizure parameters. Another subset of mice were implanted with intracranial electrodes and injected with 10 mg/kg kainic acid for electrographic seizure testing. Histological analyses were performed one week after kainic acid challenge to assess hippocampal damage. A higher proportion of *Nf1*$^{+/-}$ mice had behavioral seizures after kainic acid or pilocarpine challenge, with shorter seizure latency, longer seizure duration, and higher Racine scores compared to WT mice. *Nf1*$^{+/-}$ and WT mice with severe behavioral seizures demonstrated similar levels of hippocampal damage. EEG recordings confirmed decreased seizure latency and longer seizure duration in response to KA in the *Nf1*$^{+/-}$ group. These data demonstrate increased seizure susceptibility in a mouse model of NF1 and support the use of the *Nf1*$^{+/-}$ mouse for further investigations into the mechanistic link between NF1 and seizures.

1. Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant condition caused by a mutation in the NF1 gene encoding the tumor suppressor protein neurofibromin. It affects approximately 1 in 2,600-3,000 people, featuring various cutaneous manifestations (Anderson and Gutmann, 2015). Many patients also have neurological symptoms such as neurocognitive deficits or epilepsy. Reported rates of epilepsy in NF1 are as high as 14–20% (Syrbe et al., 2007; Hirabaru and Matsuo, 2018; Perek-Polnik et al., 2006) versus 1% in the general population. While intracranial lesions are epileptogenic in a number of NF1 patients, some series have found up to 47% of NF1 patients with epilepsy are non-lesional on imaging (Santoro et al., 2018) and some epilepsies appear to be generalized as opposed to focal in onset (Pecoraro et al., 2017; Hsieh et al., 2011). This raises the question of whether the genetic mutation itself predisposes neural circuitries to hyperexcitability. To date there are no reports of seizure susceptibility in animal models of NF1 and molecular mechanisms are unknown. The goal of this study was to determine whether the *Nf1*$^{+/-}$ mouse demonstrates increased seizure susceptibility to chemical convulsants.

2. Methods

2.1. Mice

This study was conducted in strict accordance with the policies and guidelines of the Canadian Council on Animal Care. Studies employed *Nf1*$^{+/-}$ mice on a C57BL/6 J background (Jackson Labs, Bar Harbor, ME; strain 008,192). *Nf1*$^{+/+}$ littermates were used as WT controls. Mice were housed under a 12 -h light/dark cycle with *ad libitum* access

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Table 1
Modified Racine Score. Scoring system used for kainic acid and pilocarpine-induced behavioral seizures.

Racine Stage	Behavioural Activity
Stage 0	No behavioral seizure activity
Stage 1	Immobility/rigidity
Stage 2	Head nodding, tail and/or forelimb extension
Stage 3	Forelimb clonus, mild full body convulsions
Stage 4	Rearing with forelimb clonus
Stage 5	Continuous rearing and falling
Stage 6	Severe tonic-clonic seizures

to food and water.

2.2. Kainic acid-induced behavioral seizure susceptibility

Young adult male and female mice were injected with kainic acid (20 mg/kg i.p.; Abcam Inc., Cambridge, MA). Seizure activity was scored according to a modified Racine Scale (Racine, 1972; Table 1) for every 10-minute period over the following 180 min. Other parameters included proportion of mice with seizures, latency to seizure onset, duration of seizure activity, average and maximum Racine score over 180 min, and mortality.

2.3. Pilocarpine-induced behavioral seizure susceptibility

Male and female young adult mice were pretreated with scopolamine (1 mg/kg s.c.) 30 min prior to pilocarpine administration (300 mg/kg i.p.; Sigma-Aldrich, Milwaukee, WI). Behavioral seizures were scored as described above.

2.4. EEG recordings

A separate group of mice were implanted with polyamide-insulated twisted stainless steel wire electrodes (outer diameter 200 um; Plastics One, Roanoke, VA) in CA3 of the hippocampus (bregma: 2.5 mm posterior, 3.0 mm lateral, 3.0 mm depth) and contralateral sensorimotor neocortex (2.0 mm posterior, 2.0 mm lateral, 1.5 mm depth) with a reference electrode positioned at a frontal area (1.0 mm anterior, 1.0 mm lateral, 0.5 mm depth). Mice were allowed to recover for one week before injection of 10 mg/kg KA ip. A lower dose was used due to reports of increased mortality with KA in mice with intracranial electrodes (Balzekas et al., 2016). EEG signals were recorded for 180 min using a two-channel microelectrode AC amplifier (model 1800, A–M Systems; Carlsborg, WA) with an input frequency band of 0.1–1,000 Hz and amplification gain of 1000 ×. Output signals were digitized at 5000 Hz (Digidata 1400A, Molecular Devices; Sunnyvale, CA). Data acquisition, storage, and analyses were done using PClamp software (Version 10; Molecular Devices). EEG was analyzed for latency to electrographic seizure activity and total seizure duration. Electrographic seizure activity was defined as repetitive spike and waves with an amplitude greater than four times the baseline and a duration of at least five seconds.

2.5. Histology

WT controls and *Nf1*$^{+/-}$ mice injected with 20 mg/kg KA with a minimum Racine 5 seizure were transcardially perfused seven days later with PBS followed by 4% paraformaldehyde. A separate group of WT and *Nf1*$^{+/-}$ mice were injected with saline as histological controls. 40 um brain sections were stained with cresyl violet (Sigma) and neuronal loss in pyramidal layers of hippocampal subfields was scored as follows: 0, no lesion; 1, minimal lesion localized to CA1/2 or CA3; 2, cellular loss in CA1/2 or CA3 with some preservation of cellular architecture and some normal cellular components; 3, complete disruption of normal cellular architecture and components (Noh et al., 2003). Average scores were compared for WT and *Nf1*$^{+/-}$ saline controls, and KA-treated WT and *Nf1*$^{+/-}$ groups.

2.6. Statistical analysis

Behavioral seizure scores were analyzed using a repeated-measures two-way ANOVA followed by Sidak's post-hoc test for multiple comparisons. Other behavioral and EEG seizure parameters were analyzed using two-tailed unpaired Student's t-tests. Mortality and seizure rates were compared using a Chi-square test. P-values less than 0.05 were considered statistically significant. Aside from proportional data, all data are expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. KA-induced behavioral seizures

Forty one mice were used for KA behavioral seizure testing (WT: 10 male, 8 female; *Nf1*$^{+/-}$: 13 male, 10 female). No differences were found between sexes (not shown), therefore data were combined within WT and *Nf1*$^{+/-}$ groups. 95.65% of *Nf1*$^{+/-}$ mice reached at minimum Racine stage 4 seizure activity (rearing with forelimb clonus) compared to 55.55% of WTs (Fig. 1A, $p < 0.05$). Mortality rate was 47.83% in *Nf1*$^{+/-}$ versus 16.67% in WTs (Fig. 1B, $p < 0.05$). Repeated-measures two-way ANOVA demonstrated a significant effect of time ($p < 0.0001$), genotype ($p < 0.005$), and a significant interaction of time and genotype ($p = 0.0003$). Sidak's multiple comparisons test demonstrated *Nf1*$^{+/-}$ mice had a higher average Racine score than WT mice from 20 to 100 min post-KA (Fig. 1C, $p < 0.025$). Average latency to Racine stage 2 was significantly shorter in *Nf1*$^{+/-}$ (Fig. 1E, $p < 0.02$). Average duration of minimum Racine stage 2 seizure activity and average maximum Racine score were both significantly higher in *Nf1*$^{+/-}$ (Fig. 1F,G, $p < 0.01$).

3.2. Pilocarpine-induced behavioral seizures

A total of 25 mice (11 WT, 14 *Nf1*$^{+/-}$) were used for pilocarpine testing. All *Nf1*$^{+/-}$ mice reached minimum Racine stage 4 versus 45.45% of WTs (Fig. 1A, $p < 0.05$) with similar mortality rates in both groups (Fig. 1B). Repeated-measures two-way ANOVA demonstrated a significant effect of time ($p < 0.0001$), genotype ($p < 0.0001$), and a significant interaction of time and genotype ($p < 0.0001$). Sidak's multiple comparisons test demonstrated *Nf1*$^{+/-}$ mice had significantly higher Racine scores from 10 min through 180 min (Fig. 1D, $p < 0.01$). Latency to Racine stage 2 was significantly lower in *Nf1*$^{+/-}$ (Fig. 1E, $p < 0.0001$). Average duration of minimum Racine stage 2 seizure activity and average maximum Racine score were both significantly higher in *Nf1*$^{+/-}$ (Fig. 1F, G, $p < 0.01$).

3.3. KA-induced electrographic seizures

Fourteen mice (7 WT, 7 *Nf1*$^{+/-}$) were monitored with EEG after injection of 10 mg/kg KA (Fig. 2A). There was no difference between the proportion of mice demonstrating electrographic seizures (85.7% WT, 100% *Nf1*$^{+/-}$). Seizure activity was first detected at the hippocampal electrode before spread to the neocortical electrode. Latency to electrographic seizure onset was significantly shorter in *Nf1*$^{+/-}$ ($p < 0.05$, Fig. 2B) and electrographic seizure duration was significantly longer ($p < 0.01$, Fig. 2C).

3.4. Histology

Semi-quantitative analysis of hippocampal cell death showed similar damage in both KA-treated WT and *Nf1*$^{+/-}$ groups versus saline controls, but did not reveal any differences in degree of hippocampal

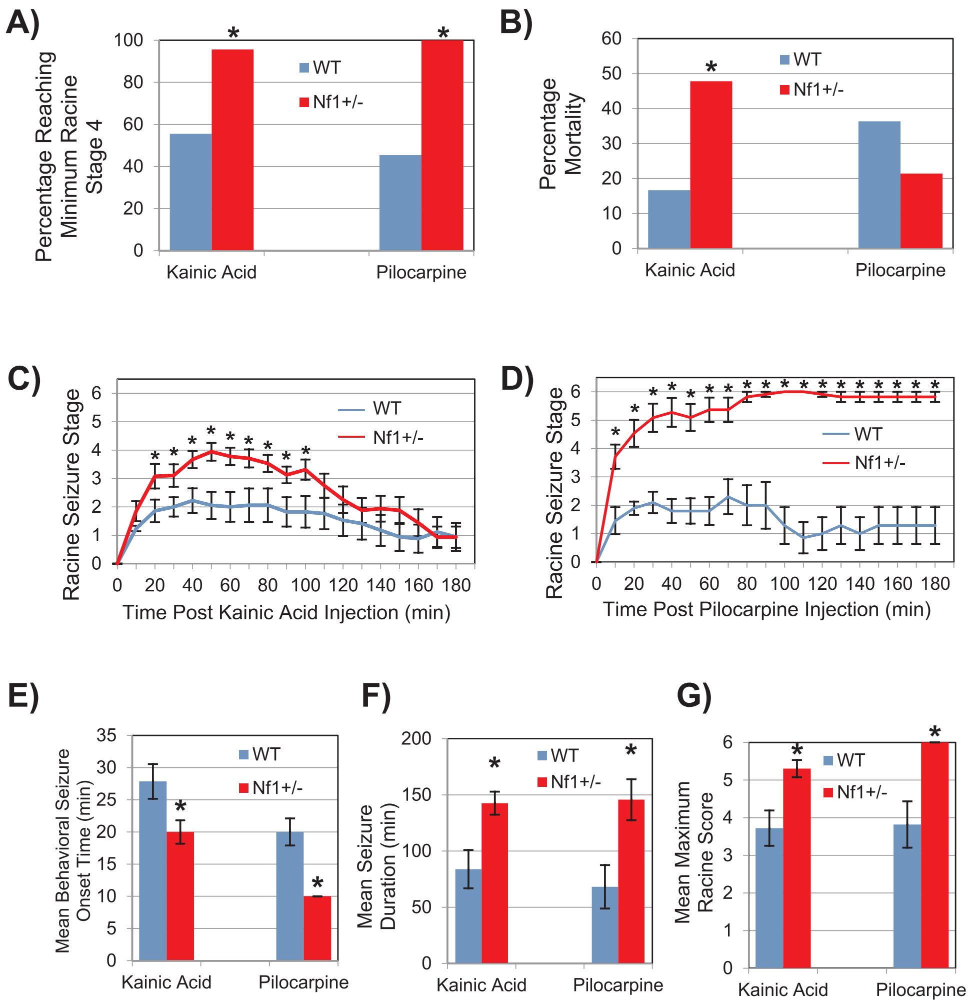

Fig. 1. A) A significantly higher percentage of mice in the *Nf1*$^{+/-}$ group reached a minimum Racine stage 4 seizure after systemic administration of either kainic acid or pilocarpine as compared to the wild-type (WT) group (for kainic acid n = 18 for WT and n = 23 for *Nf1*$^{+/-}$, for pilocarpine n = 11 for WT and n = 14 for *Nf1*$^{+/-}$; *p < 0.05). **B)** A significantly higher percentage of *Nf1*$^{+/-}$ mice died after kainic acid injection compared to WT mice (*p < 0.05). There was no significant difference in mortality between groups after pilocarpine administration. **C)** Racine seizure stage (mean +/− SEM) was higher in the *Nf1*$^{+/-}$ group (red line) between 20 and 100 min post-kainic acid injection compared to the WT group (blue line) (*p < 0.05). There was no difference between groups for the remainder of the 180 min monitoring period post injection. **D)** Racine seizure stage (mean +/− SEM) was higher in the *Nf1*$^{+/-}$ group between 10 and 180 min post-pilocarpine injection compared to the WT group (*p < 0.05). **E)** Behavioral seizure onset time (mean +/− SEM) was significantly lower in *Nf1*$^{+/-}$ mice after either kainic acid or pilocarpine administration compared to the WT group (*p < 0.05). **F)** Behavioral seizure duration (mean +/− SEM) was significantly longer in the *Nf1*$^{+/-}$ group compared to the WT group after either kainic acid or pilocarpine injection (*p < 0.05). **G)** Maximum Racine score (mean +/− SEM) reached by mice in the *Nf1*$^{+/-}$ group was significantly higher than that reached by mice in the WT group after either kainic acid or pilocarpine administration (*p < 0.05).

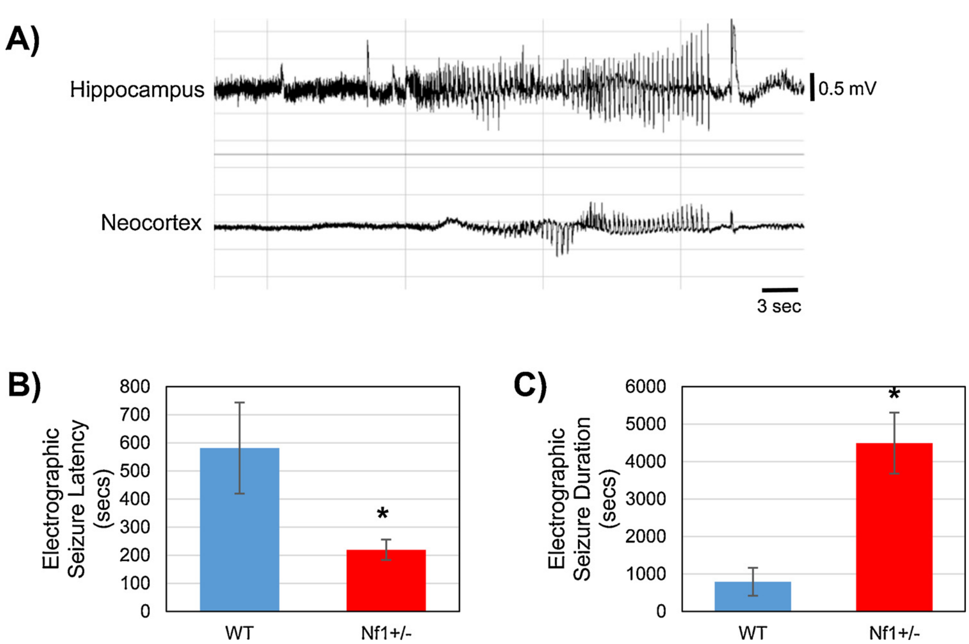

Fig. 2. A, B) Representative 60 s trace of EEG recording during a kainic acid-induced seizure in a wild-type (WT) **(A)** and an *Nf1*$^{+/-}$ **(B)** mouse. Upper tracing is from hippocampus, lower tracing from contralateral sensorimotor cortex. Electrographic seizure onset (arrow) was seen first in the hippocampus before spread to the neocortex. **C)** Electrographic seizure onset time was significantly shorter in the *Nf1*$^{+/-}$ group compared with the WT group (*p < 0.05). **D)** Electrographic seizure duration was significantly longer in the *Nf1*$^{+/-}$ group versus the WT group (*p < 0.05).

damage between KA-treated WT and *Nf1*$^{+/-}$ mice (Suppl Fig. 1).

4. Discussion

Nf1$^{+/-}$ mice have an increased susceptibility to the chemical convulsants pilocarpine and KA. A higher proportion of *Nf1*$^{+/-}$ mice have behavioral seizures after injection of either drug as compared to WT, with shorter seizure latency, longer seizure duration, and higher Racine scores. EEG recordings confirm decreased seizure latency and longer seizure duration in response to KA. Mice with this *Nf1* mutation are known to not have intracranial lesions, supporting the genetic mutation itself is responsible for increased seizure susceptibility.

Various mechanisms may contribute to seizures and epilepsy in NF1. Although structural changes remain the most likely cause in many patients, the genetic mutation may also contribute to altered synaptic plasticity, ion channel alterations, and neurotransmitter abnormalities (Stafstrom et al., 2017). Loss of neurofibromin leads to increased Ras activity and enhanced activation of Ras-PI3K, MAPK, and mTOR (Anderson and Gutmann, 2015). *Nf1*$^{+/-}$ mice have cognitive deficits related to increased GABAergic signaling (Cui et al., 2008), which can be reversed via Ras-ERK inhibitors (Li et al, 2005). Although somewhat counter-intuitive, increased GABAergic signaling may play a role in ictogenesis (see de Curtis et al., 2019). *Nf1*$^{+/-}$ mice also have increased calcium currents in hippocampal neurons (Wang et al., 2010a) in addition to alterations in peripheral sodium channels (Wang et al., 2010b), suggesting a possible role for ion channel alterations in seizures in NF1. Our demonstration of increased seizure susceptibility in *Nf1*$^{+/-}$ mice supports use of this model to further investigate mechanistic links.

Similar differences between WT and *Nf1*$^{+/-}$ seizure susceptibility were found with both behavioral and electrographic seizure monitoring. Relative differences in seizure onset time and duration as assessed by behavioral versus electrographic monitoring can be accounted for by the fact that electrographic changes precedes behavioral changes, a different scoring system utilizing 10 min bins for seizure behavior versus exact time of onset of electrographic seizure activity, and the different doses of KA used. Despite these differences, the same result of shorter seizure latency and longer seizure duration in the *Nf1*$^{+/-}$ group was found using both methods. However, it is not known whether these mice demonstrate spontaneous seizures. Future studies are needed to explore rates of epilepsy with long-term video-EEG monitoring.

We did not find any difference in hippocampal damage after KA-induced Stage 5 seizures in *Nf1*$^{+/-}$ and WT mice. This could be because we only analyzed mice reaching Stage 5 seizures in order to avoid differences in hippocampal damage due to different seizure severities. While a greater proportion of *Nf1*$^{+/-}$ mice had Stage 5 seizures than WT mice, the effect of the seizure on the brain seems similar.

5. Conclusions

The *Nf1*$^{+/-}$ mouse demonstrates increased susceptibility to seizures induced by kainic acid or pilocarpine. These data support the use of the *Nf1*$^{+/-}$ mouse model for further investigations into the mechanistic link between NF1 and seizures.

Declaration of Competing Interest

None of the authors have 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.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.eplepsyres.2019.106190.

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