Targeted inhibition of upregulated sodium-calcium exchanger in rat inferior colliculus suppresses alcohol withdrawal seizures

Luli R Akinfiresoye
Howard University College of Medicine
Jamila Newton
Howard University College of Medicine
Shubhankar Suman
Georgetown Lombardi Comprehensive Cancer Center
Kamal Datta
Georgetown Lombardi Comprehensive Cancer Center
Prosper N'Gouemo (prosper.ngouemo@howard.edu)
Howard University College of Medicine https://orcid.org/0000-0002-7829-7292

Research Article

Keywords: calcium signaling, generalized tonic-clonic seizures, protein expression, SN-6, KB-R7943

Posted Date: April 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1517756/v1

Abstract

The inferior colliculus (IC) is critical in initiating acoustically evoked alcohol withdrawal-induced seizures (AWSs). Recently, we reported that systemic inhibition of Ca^{2+} entry via the reverse mode activity of the Na^{+}/Ca^{2+} exchanger (NCX_{rev}) suppressed AWSs, suggesting remodeling of NCX expression and function, at least in the IC the site of AWS initiation. Here, we probe putative changes in protein expression in the IC of NCX isoforms, including NCX type 1 (NCX1), 2 (NCX2), and 3 (NCX3). We also evaluated the efficacy of targeted inhibition of $NCX1_{rev}$ and $NCX3_{rev}$ activity in the IC on the occurrence and severity of AWSs using SN-6 and KB-R943, respectively. We used our well-characterized alcohol intoxication/withdrawal model associated with enhanced AWS susceptibility. IC tissues from the alcohol-treated were collected 3 hours (before the onset of AWS susceptibility), 24 hours (when AWS susceptibility is maximal), and 48 hours (when AWS susceptibility is resolved) following alcohol withdrawal; in comparison, IC tissues from the control-treated group were collected at 24 hours after the last gavage. Analysis shows that NCX1 protein levels were markedly higher 3 and 24 hours following alcohol withdrawal. However, NCX3 protein levels were only higher 3 hours following alcohol withdrawal. The analysis also reveals that intra-IC microinjection of SN-6 (but not KB-R7943) markedly suppressed the occurrence and severity of AWSs. Together, these findings indicate that NCX1 is a novel molecular target that may play an essential role in the pathogenesis and pathophysiology of AWSs.

Introduction

Seizures are one of the most common features of alcohol withdrawal syndrome [1–3]. These alcohol withdrawal-induced seizures (AWSs) are usually generalized tonic-clonic seizures (GTCSs) of subcortical origin and can be resistant to modern antiseizure medications [4–10]. Interestingly, brainstem auditory evoked potentials (BAEP) are altered in alcoholic epilepsy suggesting changes in neuronal excitability of the inferior colliculus (IC), one of the generators of BAEP wave V [11, 12]. In rodents, the IC is critically involved in initiating acoustically evoked AWSs [13–18]. Therefore, alcohol withdrawal-induced hyperexcitability of IC neurons can serve as the functional correlates underlying increased susceptibility to AWSs. Accordingly, electrophysiological studies in awake and behaving rats revealed increased firing of IC neurons before and during acoustically evoked AWSs [19, 20]. Similarly, *in vitro* studies and studies in anesthetized rats showed increased IC neuronal firing during alcohol withdrawal associated with enhanced susceptibility to AWSs [21–23]. We have previously reported that Ca^{2+} influx through the L-type of voltage-gated Ca^{2+} (Ca_V) channel plays a role in the occurrence—but not necessarily in the initiation—of AWSs, suggesting that other Ca^{2+} entry mechanisms play more critical roles in the pathogenesis of AWSs [24–26]. Of interest is the Na^{+}/ Ca^{2+} exchanger (NCX), a membrane transporter, when operating in its reverse mode (NCXrev), is also an essential pathway for Ca^{2+} entry into neurons and may play a role in the pathophysiology of seizures, including of AWSs [27–31]. Accordingly, blocking the reverse mode activity of NCX (NCX_{rev}) suppressed acoustically evoked AWSs [32]. Three NCX isoforms, including type 1 (NCX), type 2 (NCX2), and type 3 (NCX3), are expressed in the brain, and each isoform has a distinct molecular expression pattern and pharmacological sensitivity [33–38]. Here, we probe the putative role of

NCX in the pathogenesis and pathophysiology of AWSs by i) quantifying protein expression associated with NCX1, NCX2, and NCX3 in IC neurons at various time points during alcohol withdrawal associated with seizure susceptibility and ii) evaluating the efficacy of intra-IC inhibition of $NCX1_{rev}$ and $NCX3_{rev}$ activity on the occurrence and severity of acoustically evoked AWSs.

Material And Methods

Animals

We used 70 male Sprague-Dawley (SD) rats (250-300 g; Taconic, Germantown, New York, Unites States) for the experiments. These animals were housed in standard polycarbonate cages with chow and water *ad libitum* and maintained in a temperature- and humidity-controlled room on a 12/12-hour light/dark cycle. Efforts were made to minimize the number of animals used in these experiments and their discomfort. All experimental procedures were approved by the Institution Animal Care and Use Committee (Protocol MED-20-03) and performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals [39].

Pharmacological probes

To probe the role of NCX in the AWS pathophysiology, we used SN-6 (2-[[4-[(4-nitrophenyl)methoxy]phenyl]methyl]-4-thiazoli dinecarboxylic acid ethyl ester, R&D Systems, Minneapolis, Minnesota, United States) and KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl] isothioureamethanesulfonate, R&D Systems). SN-6 preferentially blocks $NCX1_{rev}$ activity, while KB-7943 inhibits $NCX3_{rev}$ more potently than its forward mode [40-42]. SN-6 and KB-R7943 were dissolved in dimethyl sulfonic acid (0.1%) and phosphate-buffered saline (pH 7.4) using sonication (80 kHz, 100% power). The solutions containing SN-6 or KB-R7943 were filtered before intra-IC microinjections at 5 µg/hemisphere; this dose was chosen based on our published reports [24,43].

Cannula guide implantation

We used ketamine/xylazine (85/3 mg/kg, IP) to anesthetize the animals. Guide cannulae (21-gauge, Plastics One, Roanoke, VA, USA) were implanted bilaterally over the IC (9.15 mm posterior to bregma, 1.5 mm lateral to bregma), and the injection cannula was subsequently inserted vertically to 4.5 mm below the surface of the brain [44]. A stylet was placed in each guide cannula to prevent clogging when not in use.

Ethanol administration procedure

Ethanol intoxication and withdrawal were performed as previously described [26,25,45-47]. Briefly, ethanol solution (30%, v/v, from a 95% stock solution, U.S.P., The Warner-Gram Company, Cockeysville, Maryland, Unites States) in Isomil (Abbotts's lab, Chicago, Illinois, United States) was administered by intragastric intubation three times per day (at 8-hours intervals) for four days. The first dose of ethanol was 5 g/kg

body weight, and subsequent doses were reduced and adjusted for each animal to achieve a moderate degree of intoxication determined based on a well-described intoxication scale [48,49]. The control-treated animals were maintained under similar conditions but received thrice daily the Isomil alone (without ethanol).

In our model, blood ethanol concentrations were elevated 3-hours after the last dose of ethanol when no acoustically evoked seizure susceptibility was observed but returned to control levels 24- and 48-hours later when the seizure susceptibility peaked and resolved, respectively [24-26,43,45,48].

Acoustically evoked seizure testing

Acoustically evoked seizure testing following alcohol withdrawal was performed as previously described [24,43]. Briefly, a**nimals** were placed in an acoustic chamber. An auditory stimulus consisting of pure tones (100-105 decibels sound pressure level; Med Associated, St Albans, VT) was first presented until seizure activity was elicited or 60 seconds passed with no seizure activity. Animals that did not respond to tones were tested again 1-hour later using mixed sound at 110 decibels produced by an electrical bell. Acoustically evoked seizures following ethanol withdrawal consisted of wild running seizures (WRSs) that evolved into bouncing generalized tonic-clonic seizures (i.e., tonic-clonic seizures while the animal is lying on its belly, GTCSs) [24,50].

Western blot procedure

For Western blot analysis, animals subjected to ethanol withdrawal were not subjected to seizure testing because various degrees of seizure severity and duration can alter NCX protein expression [48]. Control-treated SD rats (n=8) and ethanol-treated SD rats (n=8 per group) subjected to withdrawal (3-hour group, 24-hour group, and 48-hour group) were deeply anesthetized with Nembutal (100 mg/kg; i.p.), colliculi were surgically dissected and stored at –80°C until use. Tissue homogenates from each animal were lysed in 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 1% IGEPAL (Sigma-Aldrich, St. Louis, MO), 10% glycerol, 1 mM EDTA, and 1 mM Na3VO4 as described previously [25,42]. Briefly, nitrocellulose membranes (Bio-Rad, Hercules, CA) were incubated overnight at 4°C with primary rabbit antibodies (Alpha Diagnostic International, San Antonio, TX) against NCX1 (1:1000, Cat. #NCX12-A), NCX2 (1:1000; Cat. #NCX21-A), or NCX3 (1:1000, Cat. #NCX31-A). In addition, the membranes were incubated overnight with anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH) antibody (1:10,000; Thermo Fisher Scientific, Waltham, Massachusetts, United States) at 4°C as an internal control. The membranes were probed with goat anti-rat IRDye800 (1:10,000; LI-COR Biosciences) and goat anti-rabbit IR-Dye680 (1:10,000; LI-COR Biosciences) for 1 hour at room temperature, then scanned using an Odyssey Fc imager (LI-COR Biosciences). All experiments were duplicated.

Four SD rats from the cohort subjected to ethanol withdrawal were tested at 3, 24, and 48 hours following ethanol withdrawal to monitor acoustically evoked AWS susceptibility. Accordingly, 100% and 75% of tested SD rats (n=4) exhibited WRSs and GTCSs 24 hours following ethanol withdrawal; no AWS

susceptibility was observed at 3 and 48 hours following ethanol withdrawal, consistent with earlier reports [24,25,43,45,48].

Focal microinjections

Only animals exhibiting acoustically evoked AWSs (AWS-sensitive, n=24 out of 30 rats) were used for focal pharmacological studies to evaluate the antiseizure effects of NCX_{rev} inhibitors. For microinjection procedures, stylets were removed from the implanted cannula guide tubes, and the vehicle, SN-6, or KB-R7943 was infused through an injection cannula (26 gauge, Plastics One) at a rate of 1 µl/min for 2.5 minutes; the injection cannula remained in place for additional 2.5 minutes. Acoustically evoked AWS-sensitive rats were closely monitored following intra-IC administration of vehicle (2.5 µl/hemisphere, n=8), SN-6 (5 µg/2.5 µl/hemisphere, n=8) or KB-R7943 (5 µg/2.5 µl/hemisphere, n=8) and tested for AWS susceptibility at 0.5, 1, 2, and 4 hours post-microinjections. Animals that do not show acoustically evoked AWSs (AWS-resistant) were used to assess the potential proconvulsant effects of SN-6 (5 µg/2.5 µl/hemisphere, n=3) or KB-R7943 (5 µg/2.5 µl/hemisphere, n=3). In another set of experiments, naive SD rats received intra-IC microinjections of SN- 6 (5 µg/2.5 µl/hemisphere, n=4) or KB-R7943 (5 µg/2.5 µl/hemisphere, n=4) and were subjected to 24 hours monitoring for potential abnormal behaviors. At the end of the pharmacological experiments, the animals received bilateral infusions of Fast green (0.25 µl/hemisphere, Electron Microscopy Science, Hatfield, PA, USA) at the microinjection sites. Animals were then euthanized with Nembutal or Euthasol (100 mg/kg i.p.), and coronal sections of the IC were obtained to verify the locations of microinjections microscopically.

Data analysis

The investigators were blinded to group allocation during experiments and data analysis. Origin 2022 software (Origin Northampton, Massachusetts, United States) was used for statistical analyses and graphs. For Western blot, we used densitometry to measure protein levels of NCX1, NCX2, and NCX3 in ethanol-treated samples relative to control-treated samples using LI-COR Image Studio Software; data from each sample was normalized to GAPDH. One-way ANOVA followed by a Bonferroni post hoc correction was performed to evaluate differences in protein expression levels. Data were first subjected to a normality test (Kolmogorov–Smirnov) and tests for homogeneity of variance (Levene's test and Brown–Forsythe's test) before ANOVA. For in vivo pharmacological studies and seizure testing, animals that did not display seizures, following intra-IC microinjection of SN-6 or KB-R7943, within the 60-s acoustic stimulation period were protected from seizure activity. For each group, the incidences of WRS and GTCSs were recorded and analyzed using Fisher's Exact test. The seizure latency and seizure duration were analyzed using two-way ANOVA followed by Bonferroni post hoc correction. Finally, the seizure severity score was analyzed using the Kruskal-Wallis test. The summary data are presented as fold-change±S.E.M. for protein expression, mean±S.E.M. for seizure latency and seizure duration, median seizure score±mean average deviation for seizure severity, and the percentage (%) for the incidence of WRSs and GTCSs. Differences between groups were considered significant at $P<0.05$.

Results

Ethanol withdrawal upregulates NCX1 and NCX3 at the protein level.

To determine which of the three NCX isoforms most contributes to the pathogenesis and pathophysiology of AWS susceptibility, we examined whether changes in NCX protein expression in the IC correlated with the time course of acoustically evoked AWS susceptibility. Figure 1 summarizes the protein levels of NCX1 (panel A), NCX2 (panel B), and NCX3 (panel C) in the IC. Analysis showed that ethanol withdrawal significantly ($F(3,28) = 11.93$, $P = 0.00003$) altered the overall protein levels of NCX1 in the IC. Detailed analysis using the Bonferroni correction revealed that protein levels of NCX1 in the IC were significantly higher 3 hours ($t = 2.84$, $P = 0.049$) and 24 hours ($t = 5.95$, $P = 0.00001$) after ethanol withdrawal compared to the control-treated group (Fig. 1A). The protein levels of NCX1 in the IC were also significantly higher at 24 hours than 3 hours ($t = 3.11$, *$P = 0.026$*, Fig. 2A) or 48 hours ($t = 3.52$, $P = 0.009$, Fig. 1A) time points during ethanol withdrawal. Quantification showed that ethanol withdrawal also significantly altered the overall protein levels of NCX3 ($F(3,28) = 6.5$, $P = 0.002$) in the IC. Detailed quantification using the Bonferroni correction revealed that protein levels of NCX3 in the IC were higher at 3 hours following ethanol withdrawal compared to the control-treated group ($t = 3.23$, $P = 0.019$, Fig. 1C). Furthermore, protein levels of NCX3 in the IC were higher at 3 hours than 24 hours ($t = 3.49$, $P = 0.009$), or 48 hours ($t = 3.95$, $P = 0.003$) following ethanol withdrawal (Figure IC). In contrast to NCX1 and NCX3, the protein expression levels of NCX2 in the IC were not notably altered by ethanol withdrawal (Fig. 1B).

We also quantified the protein expression ratios of NCX1, NCX2, and NCX3 in the IC (Table 1). Analysis revealed that the protein expression of NCX1 in the IC was favored throughout the first 48 hours and the last 24 hours of ethanol withdrawal over NCX2 and NCX3, respectively. In addition, when comparing expression ratios of NCX2 and NCX3 in the IC, we observed a shift in which NCX3 was favored before the onset of AWS susceptibility, whereas NCX2 became dominant when AWS susceptibility was maximal and resolved.

Inhibition of reverse mode NCX1 activity within the inferior colliculus suppresses acoustically evoked alcohol withdrawal seizures.

Histological analysis showed that the microinjection sites of SN-6 and KB-R7943 were within the central nuclei of the IC (Supplementary Information). Twenty-four hours of monitoring revealed that intra-IC microinjections of SN-6 or KB-R7943 in naive SD rats or AWS-resistant rats did not alter their gross behaviors. No Straub tail, sedation, lethargy, ataxia, change in body temperature, and spontaneous seizures were observed following intra-IC microinjection of SN-6 and KB-7943 in a set of naive SD rats (n = 4 per group).

We evaluated the effects of intra-IC microinjections of SN-6 (5 µg/2.5 µl/hemisphere, n = 8) or KB-R7943 (5 µg/2.5 µl/hemisphere, n = 8) on the incidences of WRSs. Fisher Exact test revealed a significantly ($P = 0.026$) lower incidence of WRSs at 2 hours ($P = 0.026$) and 4 hours ($P = 0.026$) post-treatment following intra-IC microinjections of SN-6 compared with the vehicle-treated group; no notable changes were seen

0.5 and 1 hour post-treatment (Fig. 2A). However, intra-IC microinjections of KB-R7943 did not considerably alter the incidences WRSs at all tested post-microinjection time points compared with the vehicle-treated group (Fig. 2A). The analysis also showed lower incidences of GTCSs at all tested time points following intra-IC microinjections of SN-6 compared with the vehicle-treated group ($P = 0.007$; Fig. 2B). The incidences of GTCSs were also lower at 1-, 2-, and 4- hours following intra-IC microinjections of KB-R7943 compared with the vehicle-treated group ($P = 0.026$; Fig. 2B).

Analysis using the Kruskal-Wallis test revealed that bilateral intra-IC microinjections of SN-6 significantly reduced or suppressed the seizure severity at 1hour ($z = 2.799$; $P = 0.015$), 2 hours ($z = 3.052$, $P = 0.007$), and 3 hours ($z = 2.699$, $P = 0.020$) post-microinjection compared to the vehicle-treated group; no notable effect was seen at 0.5 hour ($z = 2.342$, $P = 0.058$) post-treatment (Fig. 3A). However, intra-IC microinjections of KB-R7943 did not notably alter the seizure severity at all tested post-microinjection time points compared with the vehicle-treated group (Fig. 3A).

We also evaluated the effects of intra-IC microinjections of SN-6 (5 µg/2.5 µl/hemisphere, n = 8) or KB-R7943 (5 µg/2.5 µl/hemisphere, n = 8) on the seizure latency (Fig. 3B) and duration (Fig. 3C). Two-way ANOVA revealed significant effects of both time ($F(3,95) = 3.300$, $P = 0.024$) and treatment ($F(2,95) = 15.494$; $P = 0.0000001$). Detailed analysis using the Bonferroni showed significantly longer seizure latencies at 2 hours ($t = 3.793$, $P = 0.018$) and 4 hours ($t = 3.903$, $P = 0.013$) following intra-IC microinjection of SN-6 (Fig. 3B); no effect was seen at 0.5 and 1hour post-intra-IC microinjections (Fig. 3B). However, intra-IC-microinjection of KB-R7943 did not notably delay the seizure latency at all tested post-microinjection time points compared with the control testing conditions (Fig. 3B). Two-way ANOVA also revealed a significant effect of treatment with NCX inhibitors on the seizure duration ($F(2,95) = 24.611$; $P = 0.0000001$) on seizure latency. Detailed analysis using the Bonferroni correction revealed a significantly shorter seizure duration at 2 hours ($t = 3.861$, $P = 0.15$) and 4 hours ($t = 3.758$, $P = 0.021$) following intra-IC microinjection of SN-6 compared with the vehicle-treated group (Fig. 3C). However, intra-IC-microinjection of KB-R7943 did not notably alter the seizure duration at all tested post-microinjection time points compared with the vehicle-treated group (Fig. 3C).

Discussion

We have previously reported that systemic administration of NCX_{rev} activity inhibitors suppressed AWSs in adult male rats; these findings suggest NCX remodeling, at least in the IC, the site of AWS initiation [32]. Here, we explored a potentially novel mechanism underlying AWSs—the upregulation of NCX1 in the IC. We found that the expression of NCX isoforms differed from one another following alcohol withdrawal. Specifically, protein expression levels of NCX1 were significantly higher 3 and 24 hours after alcohol withdrawal, before the onset of AWS susceptibility, and when AWS susceptibility peaked, respectively, compared to the control conditions. Furthermore, the NCX1 to NCX2 and NCX3 revealed that NCX1 protein expression was dominant before AWS onset and when AWS peaked compared to NCX2, while NCX1 protein expression was dominant when AWS peaked to NCX3. In addition, intra-IC inhibition of $NCX1_{rev}$ markedly suppressed the occurrence of AWSs and AWS severity suggesting a role of NCX1 in the

mechanism underlying seizure initiation. Intra-IC inhibition of $NCX1_{rev}$ also delayed the seizure onset and reduced the seizure duration, suggesting roles of NCX1 in the propagation of seizure activity from the initiation site, the IC, to brain sites implicated in GTCS generation and seizure termination, respectively. Together, these findings suggest that upregulation of NCX1 plays the most significant role in the pathogenesis and pathophysiology of AWSs.

Though NCX3 protein expression was also significantly higher before the onset of AWSs, intra-IC inhibition of $NCX3_{rev}$ activity failed to notably alter AWSs, suggesting that NCX3 may play a role in AWS initiation, but not in AWS propagation and termination. Our comparison analysis between NCX2 versus NCX3 protein expression revealed that NCX3 was more highly expressed before the onset of acoustically evoked AWSs, whereas NCX2 became dominant when AWS susceptibility was maximal and resolved. This shift could represent a switch in the specific type of cells that were altered at time points during alcohol withdrawal; upstream neurons may favor the expression of NCX3, whereas downstream neurons could favor NCX2. Higher and lower NCX3 protein levels were found in mice's hippocampus and dentate gyrus after 30 days and 60 days after chronic ethanol exposure, respectively [52]. Whether these changes in NCX3 were associated with enhanced GTCS susceptibility remains unknown. The upregulation of NCX1 and NCX3 before the onset of AWSs may also represent a compensatory mechanism to massive Na^+ influx in IC neurons during alcohol intoxication. The upregulation of NCX1 and NCX3 proteins is consistent with the reports that chronic alcohol exposure causes an increase in NCX proteins in brain synaptic membranes and NCX activity in synaptosomes; this increased NCX activity and NCX protein expression returned to baseline within one week of alcohol withdrawal [53, 54]. Thus, we can speculate that alcohol withdrawal may also increase $NCX1_{rev}$ and $NCX3_{rev}$ activities in IC neurons.

The upregulation of NCX1 could contribute to AWSs if the exchanger is operating in its reverse mode. Higher intracellular Ca^{2+} and altered Ca^{2+} homeostasis have been implicated in the pathogenesis and pathophysiology of seizures [55–59]. We previously reported higher current density carried by L-type $Ca_V1.x$ and P/Q-type $Ca_V2.1$ channels in IC neurons during alcohol withdrawal [26]. Although Ca_V channels are the main routes of Ca^{2+} influx in neurons, evidence indicates that NCX_{rev} activity is also an important pathway for Ca^{2+} entry into neurons [27, 28, 30]. Thus, massive Ca^{2+} entry into the neuron following activation of NCX_{rev} can disturb Ca^{2+} homeostasis, resulting in neuronal hyperexcitability that can lead to seizures. Our findings posit that alcohol withdrawal upregulates NCX1 in IC neurons and likely drives the NCX1 activity into its reverse mode resulting in Ca^{2+} entry. We have previously reported higher and modest protein and mRNA expression of L-type $Ca_V1.3$ channels in IC neurons before AWS onset [25]. However, higher L-type $Ca_V1.2$ protein expression was found when the incidence of AWSs was maximal, but not before the onset of AWS susceptibility [25]. We also reported higher P/Q-type $Ca_V2.1$ mRNA expression was before the onset of AWS and when the incidence of AWS was maximal, but no change in protein expression [45]. Nevertheless, Intra-IC inhibition of L-type $Ca_V1.x$ but not P/Q-type $Ca_V2.1$ channels suppressed AWSs suggesting a potential role of phosphorylation of Ca_V channels in the increased current density in IC neurons following alcohol withdrawal [24, 40]. In the present study, only

higher mRNA (Supplemental data) and protein expressions of NCX1 occurred before the onset of AWS susceptibility and when AWS incidence was maximal. Together, these findings indicate that the remodeling of NCX1 in the IC is essential for AWS susceptibility. At the same time, NCX3 and L-type $Ca_V2.1$ may play minor roles in AWS initiation, and $Ca_V1.2$ and $Ca_V1.3$ channels may play minor roles in the seizure severity.

Acoustically evoked seizures GTCSs during alcohol withdrawal resemble GTCSs observed in the moderated seizure severity substrain of the genetically epilepsy-prone (GEPR-3) rat [50, 60]. In the GEPR-3s, systemically blocking $NCX1_{rev}$ activity also prevents the occurrence of GTCSs, suggesting a molecular remodeling of NCX1, at least, in the IC [61]. Thus, upregulation of NCX1 in IC neurons represents a plasticity mechanism that may predispose the animals to develop AWSs (acquired GTCSs) and inherited GTCSs. Furthermore, inhibition of $NCX1_{rev}$ and $NCX3_{rev}$ activities preferentially suppressed the occurrence of the GTCS component of pentylenetetrazole and pilocarpine-induced seizures but not the limbic component of pilocarpine-induced seizures [29, 30]. These findings suggest a role of NCX_{1rev} and NCX3rev activities in the pathophysiology of GTCS initiated in the limbic system. In the present study, NCX3 protein expression is increased before the onset of AWS susceptibility and inhibiting $NCX3_{rev}$ activity in the IC failed to suppress AWSs. Whether a limited role of NCX3 in seizure pathogenesis is also present in GEPR-3s remains unknown. Nevertheless, in the Mongolian gerbil model of GTCSs, an upregulation of NCX3 protein expression levels is reported in pyramidal neurons of hippocampal CA1-3 regions following episodes of spontaneous GTCSs in seizure-prone animals [62]. However, elevated expression of NCX3 proteins in the hippocampus and cortex has been linked to low susceptibility to GTCSs in seizure-resistant gerbils and a model of hyperthermia-induced seizures [62, 63]. It is, therefore, tempting to speculate that the upregulation of NCX3 in IC neurons following alcohol withdrawal may serve as a compensatory mechanism for the alcohol withdrawal-induced hyperexcitability of IC neurons that leads to increased AWS susceptibility.

In conclusion, alcohol withdrawal differentially affects the expression of NCX isoforms in the rat IC neurons. Alcohol withdrawal triggered higher NCX1 expression in IC neurons and targeted inhibition of NCXrev activity in this brain site, which may attenuate the elevated intraneuronal Ca^{2+}, suppressed AWSs. These findings indicate that NCX1 in IC neurons is an essential molecular target that plays a critical role in the pathogenesis and pathophysiology of AWS.

Declarations

Acknowledgments

This work was supported by the National Institute of Alcoholism and Alcohol Abuse (R01 AA027660 to PN) at the National Institutes of Health. The National Institute of Alcoholism and Alcohol Abuse had no other role in the design of the study, collection and data analysis, and decision to publish.

Funding

This work was supported by the National Institute of Alcoholism and Alcohol Abuse (R01 AA027660 to PN) at the National Institutes of Health.

Competing Interests

The authors declare no conflicts of interest.

Author Contributions

LRA and JN performed mRNA and protein extraction, Western blotting experiments, and data analysis; SS and KD contributed to qPCR experiments and data analysis; PN designed the study, performed in vivo experiments and data analysis, and wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Data Availability

All data are available upon request.

Ethical Approval

All experimental procedures were approved by the Institution Animal Care and Use Committee (Protocol MED-20-03) and were performed per the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council US, 2011).

Consent to Participate

Not applicable

Consent to Publish

Not applicable

References

1. Hillbom M, Pieninkeroinen I, Leone M (2003) Seizures in alcohol-dependent patients. CNS Drugs 17:1013-1030. doi: 10.2165/00023210-200317140-00002.
2. Ripley JB (1990) A 39-year-old man with fatal alcohol withdrawal seizures. J Emerg Nurs 16:67-69.
3. Victor M, Brausch C (1967) The role of abstinence in the genesis of alcohol epilepsy. Epilepsy 8:1-20. doi: 10.1111/j.1528-1157.1967.tb03815.x.
4. Jesse S, Bråthen G, Ferrara M, Keindl M, Ben-Menachem E, Tanasescu R, Brodtkorb E, Hillbom M, Leone MA, Ludolph AC (2017) Alcohol withdrawal syndrome: mechanisms, manifestations, and management. Acta Neurol Scand 135:4-16. doi: 10.1111/ane.12671.

5. Long D, Long B, Koyfman A (2017) The emergency medicine management of severe alcohol withdrawal. Am J Emerg Med 35:1005-1011. doi: 10.1016/j.ajem.2017.02.002.
6. McMicken D, Liss JL (2011). Alcohol-related seizures. Emerg Med Clin North Am 29:117-124. doi: 10.1016/j.emc.2010.08.010.
7. Schmidt KJ, Doshi MR, Holzhausen JM (2016) Treatment of severe alcohol withdrawal. Ann Pharmacol Ther 50:389-401. doi: 10.1177/1060028016629161.
8. Sand T, Brathen G, Michler R, Brodtkorb E, Helde G, Bovim G (2002) Clinical utility of EEG in alcohol-related seizures. Acta Neurol Scand 105:18-42. doi: 10.1034/j.1600-0404.2002.00058.x.
9. Neiman J, Noldy NE, El-Nesr B, McDonough M, Carlen PL (1991) Late auditory evoked potentials in alcoholics: identifying those with a history of epileptic seizures during withdrawal. Ann NY Acad Sci 620:73-81. doi: 10.1111/j.1749-6632.1991.tb51575.x.
10. Touchon J, Besset A, Baldy-Moulinier M, Billiard M, Uziel A, Passouant P (1981) Electrophysiological aspect of alcoholic epilepsy. Rev Electroencephal Neurophysiol Clin 11:514-519. doi: 10.1016/s0370-4475(81)80094-9.
11. Hughes JR, Fino JJ (1985) A review of generators of the brainstem auditory evoked potentials: contribution of an experimental study. J Clin Neurophysiol 2:355-381.
12. Touchon J, Rondouin G, DeLustrac C, Billard M, Baldy-Moulinier M, Cadilhac M (1984) Brainstem auditory evoked potentials in alcoholic epilepsy. Rev Electroencephal Neurophysiol Clin 14:133-137. doi: 10.1016/s0370-4475(84)80018-0.
13. McCown TJ, Breese GR (1993) A potential contribution to ethanol withdrawal kindling: reduced GABA function in inferior collicular cortex. Alcoholism: Clin Exp Res 17:1290-1294. doi: 10.1111/j.1530-0277.1993.tb05243.x.
14. McCown TJ, Breese GR (1990) Multiple withdrawals from chronic ethanol “kindles” inferior collicular seizure activity: evidence for kindling seizures associated with alcoholism. Alcoholism: Clin Exp Res 14:394-399. doi: 10.1111/j.1530-0277.1990.tb00492.x.
15. Eckardt MJ, Campbell GA, Marietta CA, Majchrowicz E, Wixon HN, Weight FF (1986) Cerebral 2-deoxyglucose uptake in rats during ethanol withdrawal and post-withdrawal. Brain Res 366:1-9. doi: 10.1016/0006-8993(86)91276-x.
16. Frye GD, McCown TJ, Breese GR (1983) Characterization of susceptibility to audiogenic seizures in ethanol-dependent rats after microinjection of gamma-aminobutyric acid (GABA) agonists into the inferior colliculus, substantia nigra or medial septum. J Pharmacol Exp Ther 227:663-670.
17. Maxson SC, Sze PY (1976) Electroencephalographic correlates of audiogenic seizures during ethanol withdrawal in mice. Psychopharmacology 47:17-20. doi: 10.1007/BF00428695.
18. Hunter BE, Boast CA, Walker DW, Zornetzer SF (1973) Alcohol withdrawal syndrome in rats: neural and behavioral correlates. Pharmacol Biochem Behav 1:719-725. doi: 10.1016/0091-3057(73)90036-1.
19. Chakravarty DN, Faingold CL (1998) Comparison of neuronal response patterns in the external and central nuclei of inferior colliculus during ethanol administration and ethanol withdrawal. Brain Res

783:102-108. doi: 10.1016/s0006-8993(97)01193-1.

20. Faingold CL, Riaz A (1995) Ethanol withdrawal induces increased firing in the inferior colliculus neurons associated with audiogenic seizure susceptibility. Exp Neurol 132:91-98. 22. doi: 10.1016/0014-4886(95)90062-4.
21. Evans MS, Li Y, Faingold CL (2000) Inferior colliculus intracellular response abnormalities in vitro associated with susceptibility to ethanol withdrawal seizures. Alcoholism: Clin Exp Res 24:1180-1186.
22. Faingold CL, Li Y, Evans MS (2000) Decreased GABA and increased glutamate receptor-mediated activity on inferior colliculus neurons in vitro are associated with susceptibility to ethanol withdrawal seizures. Brain Res 868:287-295. doi: 10.1016/s0006-8993(00)02342-8.
23. N'Gouemo P, Caspary DM, Faingold CL (1996) Decreased GABA effectiveness in the inferior colliculus neurons during ethanol withdrawal in rat susceptible to audiogenic seizures. Brain Res 724:200-204. doi: 10.1016/0006-8993(96)00304-6.
24. N'Gouemo P (2015) Altered voltage-gated calcium channels in rat inferior colliculus neurons contribute to alcohol withdrawal seizures. Eur Neuropsychopharmacol 25:1342-1352. doi: 10.1016/j.euroneuro.2015.04.008.
25. N'Gouemo P, Akinfiresoye LR, Allard, JS, Lovinger DM (2015) Alcohol withdrawal—induced seizure susceptibility is associated with an upregulation of CaV1.3 channels in the rat inferior colliculus. Int J Neuropsychopharmacol 18:pyu123. doi: 10.1093/ijnp/pyu123.
26. N'Gouemo P, Morad M (2003) Ethanol withdrawal seizure susceptibility is associated with upregulation of L- and P-type Ca2+ channels currents in rat inferior colliculus neurons. Neuropharmacology 45:429-437. doi: 10.1016/s0028-3908(03)00191-6.
27. Annunziato L, Pignataro G, Di Renzo GF (2004) Pharmacology of brain Na+/Ca2+ exchanger: from molecular biology to therapeutic perspectives. Pharmacol Rev 56:633-654. doi: 10.1124/pr.56.4.5.
28. Blaustein MP, Lederer WJ (1999) Sodium/calcium exchange: its physiological implications. Physiol Rev 79:763-854.
29. N'Gouemo P (2013) Probing the role of the sodium/calcium exchanger in pentylenetetrazole-induced generalized seizures in rats. Brain Res Bull 90:52-57. doi: 10.1016/j.brainresbull.2012.09.007
30. Martinez Y, N'Gouemo P (2010) Blockade of the sodium calcium exchanger exhibits anticonvulsant activity in a pilocarpine model of acute seizures in rats. Brain Res 1366:211-216. doi: 10.1016/j.brainres.2010.09.100.
31. Saito R, Kaneko E, Tanaka Y, Honda K, Matsuda T, Baba A, Komuro I, Kita S, Iwamoto T, Takano Y (2009) Involvement of Na+/Ca2+ exchanger in pentylenetetrazol-induced convulsion by use of Na+/Ca2+ exchanger knockout mice. Biol Pharm Bull 32:1928-1930.
32. Newton J, Akinfiresoye LR, N'Gouemo P (2021) Inhibition of the sodium calcium exchanger suppresses alcohol withdrawal-induced seizure susceptibility. Brain Sci 11: 279. doi: 10.3390/brainsci11020279.

33. Annunziato L, Pignataro G, Boscia F, Sirabella R, Formisano L, Saggese M, Cuomo O, Gala R, Secondo A, Viggiano D, Molinaro P, Valsecchi V, Tortiglione A, Adornetto A, Scorziello A, Cataldi M, Di Renzo GF (2007) ncx1, ncx2, and ncx3 gene product expression and function in neuronal anoxia and brain ischemia. Ann NY Acad Sci 1099:413-426. doi: 10.1196/annals.1387.050.
34. Iwamoto T (2004) Forefront of Na+/Ca2+ exchanger studies: molecular pharmacology of Na+/Ca2+ exchange inhibitors. J Pharmacol Sci 96:27-32. doi: 10.1254/jphs.fmj04002x6.
35. Thurneysen T, Nicoll DA, Philipson KD, Porzig H (2002) Sodium/calcium exchanger subtypes NCX1, NCX2 and NCX3 show cell-specific expression in rat hippocampal cultures. Mol Brain Res 197:145-156. doi: 10.1016/s0169-328x(02)00461-8.
36. Papa M, Canitano A, Boscia F, Castaldo P, Sellitti S, Porzig H, Taglialatela M, Annunziato L (2003) Differential expression of the Na+-Ca2+ exchanger transcripts and proteins in rat brain regions. J Comp Neurol 461:31-48. doi: 10.1002/cne.10665.
37. He S, Ruknudin A, Bambrick LL, Lederer WJ, Schulze DH (1998) Isoform-specific regulation of the Na+/Ca2+ exchanger in rat astrocytes and neurons by PKA. J Neurosci 18:4833-4841. doi: 10.1523/JNEUROSCI.18-13-04833.1998.
38. Yu L, Colvin RA (1997) Regional differences in expression of transcripts for Na+/Ca2+ exchanger isoforms in rat brain. Brain Res Mol Brain Res 50:285-292. doi: 10.1016/s0169-328x(97)00202-7.
39. National Research Council (U.S.), Institute for Laboratory Animal Research (U.S.), National Academies Press (U.S.). Guide for the Care and Use of Laboratory Animal, 8th edn. Washington, DC: National Academies Press; 2011.
40. Hu HJ, Wang SS, Wang YX, Liu Y, Feng XM, Shen Y, Zhu L, Chen HZ, Song M (2019) Blockade of the forward Na(+)/Ca(2+) exchanger suppresses the growth of glioblastoma cells through Ca(2+)-mediated cell death. Br J Pharmacol 176:2691–2707. doi: 10.1111/bph.14692.
41. Watanabe Y, Koide Y, Kimura J (2006) Topics on the Na+/Ca2+ exchanger: Pharmacological characterization of Na+/Ca2+ exchanger inhibitors. J Pharmacol Sci 102:7–16.
42. Pignataro G, Gala R, Cuomo O, Tortiglione A, Giaccio L, Castaldo P, Sirabella R, Matrone C, Canitano A, Amoroso S, et al (2004) Two sodium/calcium exchanger gene products, NCX1 and NCX3, play a major role in the development of permanent focal cerebral ischemia. Stroke 35:2566–2570. doi: 10.1016/j.nbd.2011.10.007.
43. Akinfiresoye LR, Miranda C, Lovinger DM, N'Gouemo P (2016). Alcohol withdrawal increases protein kinase A activity in the rat inferior colliculus. Alcohol Clin Exp Res 40:2359-2367. doi: 10.1111/acer.13223.
44. Paxinos G, Watson C (1998). The rat brain in stereotaxic coordinates. Academic Press, San Diego.
45. Newton J, Suman S, Akinfiresoye LR, Datta K, Lovinger DM, N'Gouemo P (2018) Alcohol withdrawal upregulates mRNA encoding for CaV2.1-a1 subunit in the rat inferior colliculus. Alcohol 66:21-26. doi: 10.1016/j.alcohol.2017.07.007.
46. N'Gouemo P, Morad M (2014) Alcohol withdrawal is associated with a downregulation of large-conductance Ca²⁺-activated K⁺ channels in rat inferior colliculus neurons. Psychopharmacology

231:2009-2018. doi: 10.1007/s00213-013-3346-8.

47. N'Gouemo P, Yasuda RP, Morad M (2006). Ethanol withdrawal is accompanied by downregulation of calcium channel alpha 1B subunit in rat inferior colliculus neurons. Brain Res 1108:216-220. doi: 10.1016/j.brainres.2006.06.028.
48. Faingold CL (2008) The Majchrowicz binge alcohol protocol: an intubation technique to study alcohol dependence in rats. Curr Protoc Neurosci 44:9.28.1-9.28.12. doi: 10.1002/0471142301.ns0928s44.
49. Majchrowicz E (1975) Induction of physical dependence on alcohol and the associated metabolic and behavioral changes in rats. Psychopharmacologia 43:245-254. doi: 10.1007/BF00429258.
50. Faingold CL, N'Gouemo P, Riaz A (1998) Ethanol and neurotransmitter interaction-from molecular to integration effects. Prog Neurobiol 55:509-535. doi: 10.1016/s0301-0082(98)00027-6.
51. Ueda Y, Tusru N (1995) Simultaneous monitoring of the seizure related change in extracellular glutamate and gamma-aminobutyric acid concentration in bilateral hippocampi following development of amygdaloid kindling. Epilepsy Res 20:213-219. doi: 10.1016/0920-1211(94)00081-7.
52. Wang C, Wang X, Li Y, Xia Z, Liu Y, Yu H, Xu G, Wu X, Zhao R, Zhang G (2019) Chronic ethanol exposure reduces the expression of NCX3 in the hippocampus of male C57BL/6 mice. Neuroreport 30:397-403. doi: 10.1097/WNR.0000000000001214.
53. Chen X, Michaelis ML, Michaelis EK (1997) Effects of chronic ethanol treatment on the expression of calcium transport carriers and NMDA/glutamate receptor proteins in brain synaptic membranes. J Neurochem 69:1559-1569. doi: 10.1046/j.1471-4159.1997.69041559.x.
54. Michaelis ML, Michaelis EK, Nunley EW, Galton N (1987) Effects of chronic alcohol administration on synaptic membrane Na+-Ca2+ exchange activity. Brain Res 414:239-244. doi: 10.1016/0006-8993(87)90004-7.
55. Albowitz B, König P, Kuhnt U (1997) Spatiotemporal distribution of intracellular calcium transients during epileptiform activity in guinea pig hippocampal slices. J Neurophysiol 77:491-501. doi: 10.1152/jn.1997.77.1.491.
56. Davies G, Peterson DW (1989) Normal extracellular calcium levels block kindled seizures. Exp Neurol 106:99-101. doi: 10.1016/0014-4886(89)90150-7.
57. Stringer JL, Lothman EW (1988) In vitro effects of extracellular calcium concentrations on hippocampal pyramidal cell responses. Exp Neurol 101:132-46. doi: 10.1016/0014-4886(88)90070-2.
58. Somjen GG (1980) Stimulus-evoked and seizure-related responses of extracellular calcium activity in spinal cord compared to those in cerebral cortex. J Neurophysiol 44:617-632. doi: 10.1152/jn.1980.44.4.617.
59. Heinemann U, Lux H, Gutnick MJ (1977) Extracellular free calcium and potassium during paroxysmal activity in the cerebral cortex of the cat. Exp Brain Research 27:237-243. doi: 10.1007/BF00235500.
60. Faingold CL (1999) Neuronal networks in the genetically epilepsy-prone rat. Adv Neurol 79:311-321.

61. Quansha H, N'Gouemo P (2014) Amiloride and SN-6 suppress audiogenic seizure susceptibility in genetically epilepsy-prone rats. CNS Neurosci Ther 20:860-866. doi: 10.1111/cns.12296.
62. Park DK, Park KH, Ko JS, Kim DS (2011) Alteration in NCX-3 immunoreactivity within the gerbil hippocampus following spontaneous seizures. BMB Rep 44:306-311. doi: 10.5483/BMBRep.2011.44.5.306.
63. Sun D, Xiao JH, Bai Y, Chen MS, Hu JS, Wu GF, Mao B, Wu SH, Hu Y (2015) Na+/Ca2+exchanger 3 is downregulated in the Hippocampus and cerebrocortex of rats with hyperthermia-induced convulsion. Chin Med J (Engl) 128:3083-3087. doi: 10.4103/0366-6999.169103.
64. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method Methods 25:402-408. doi: 10.1006/meth.2001.1262.

Tables

Table 1. Effects of ethanol withdrawal on NCX protein ratios

Effects of ethanol withdrawal on the ratios of protein expression of NCX isoforms. The NCX1 protein expression over NCX2 or NCX3 was dominant throughout the ethanol withdrawal period or the last 24 hours. Analysis of the ratios NCX2 over NCX3 proteins revealed a shift such that NCX3 was prevalent at the 3-hour time point, before the onset of alcohol withdrawal seizure susceptibility, whereas NCX2 became dominant when the susceptibility to ethanol withdrawal seizures peaked and resolved. The NCX protein expression was measured and quantified as described in Methods.

Ethanol withdrawal	NCX1 vs. NCX2	NCX1 vs. NCX3	NCX2 vs. NCX3
3 hours	1.30 : 1.00	1.05 : 1.00	1.00 : 1.24
24 hours	1.37 : 1.00	1.81 : 1.00	1.32 : 1.00
48 hours	1.22 : 1.00	1.46 : 1.00	1.20 : 1.00

Figures

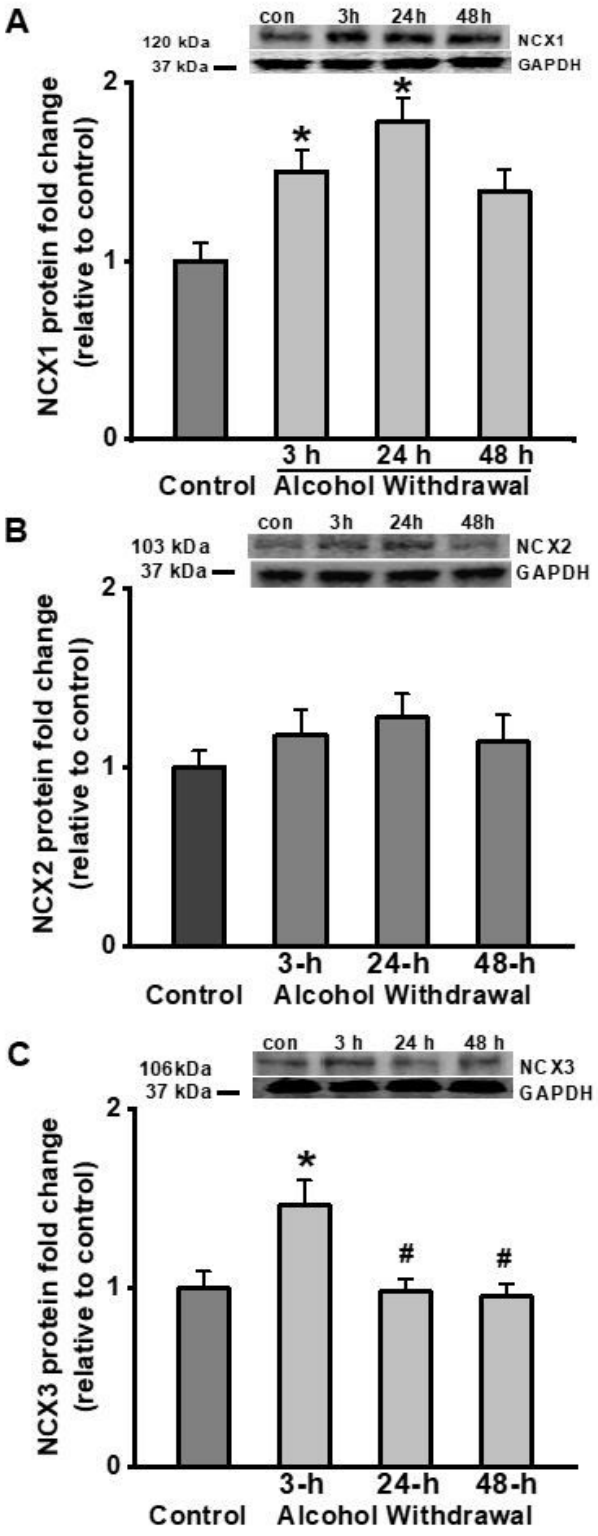

Figure 1

Ethanol withdrawal increases the protein levels of NCX1 and NCX3 in the IC before the onset of seizure susceptibility

Shown in insets are representative immunoblots of NCX1 (panel **A**), NCX2 $Ca_V1.2$ (panel **B**), and NCX3 (panel **C**) measured from the control-treated samples collected 24 hours after vehicle administration and

samples obtained at the indicated times after ethanol withdrawal. The bar graphs summarize the relative protein levels of NCX1, NCX2, and NCX3 in the IC after ethanol withdrawal, expressed as a percentage of the control-treated group. The density of the 120-kDa immunoreactive band (i.e., NCX1; panel **A**) increased significantly in the IC 3 and 24 hours after ethanol withdrawal compared to the control-treated group. The density of the 103-kDa immunoreactive bands (i.e., NCX2; panel **B**) did not change significantly after ethanol withdrawal compared to the control-treated group. The density of the 106-kDa immunoreactive band (i.e., NCX3; panel **C**) increased significantly in the IC 3 hours after ethanol withdrawal compared to the control-treated group, and the ethanol withdrawal 24-hour group and 48-hour group. The summary data are shown as the mean ± S.E.M. (8 rats per group). *$P < 0.05$ versus control (ANOVA followed by a Bonferroni correction).

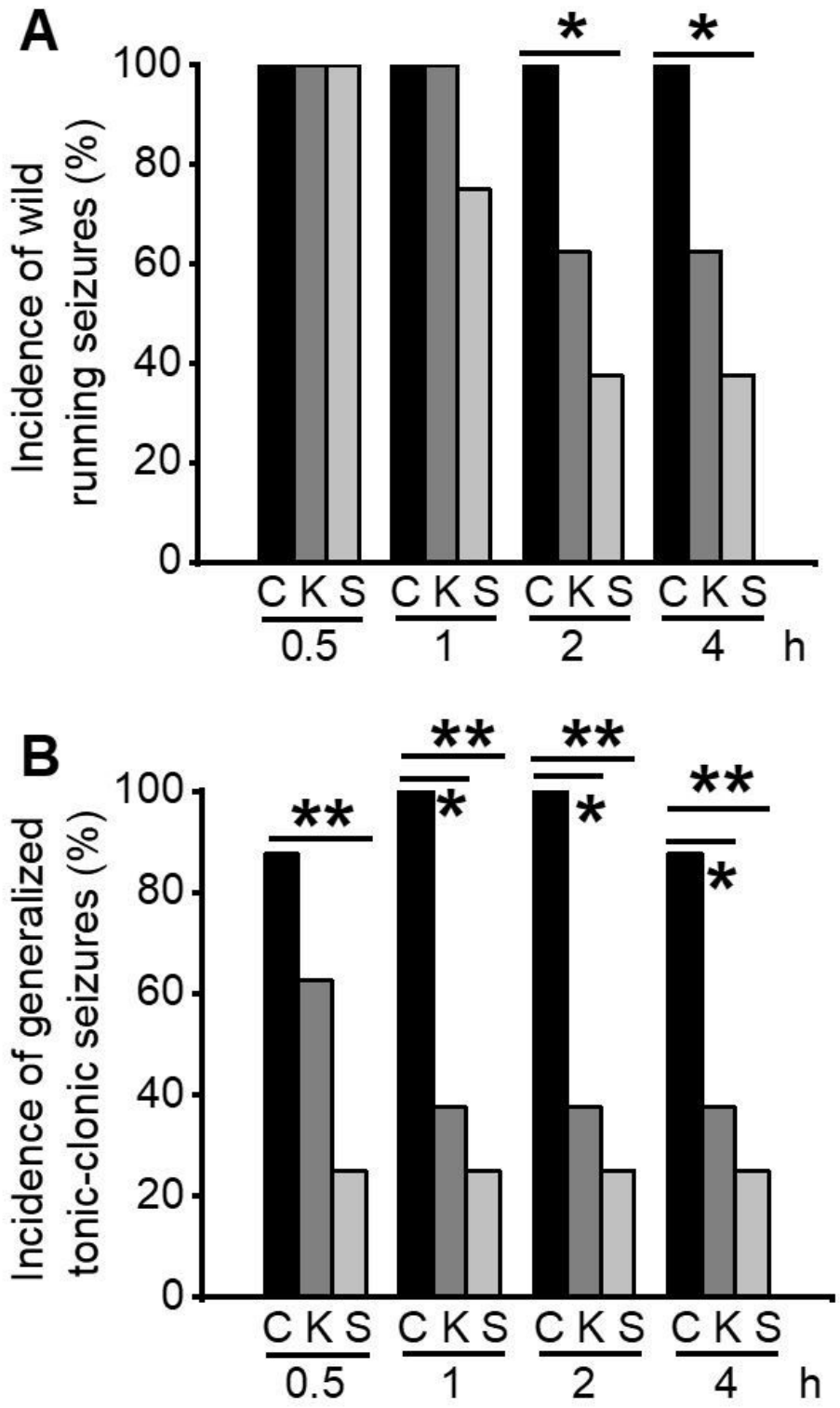

Figure 2

Effects of intra-IC microinjections of SN-6 and KB-R7943 on the incidence of alcohol withdrawal seizures

The effects of intra-IC microinjections of SN-6 (5µg/2.5µl/hemisphere) and KB-R7843 (5µg/2.5µl/hemisphere), inhibitors of NCX1rev and NCX3rev activity, respectively, were evaluated at various post-treatment time points in animals exhibiting AWS susceptibility. **A**. Lower incidence of WRSs

was observed a 2- and 4-hours post-treatment time points. No notable change in WRSs incidence was observed following intra-IC microinjections of KBR-7943. **B**. Intra-IC microinjection of SN-6 markedly reduced the incidence of GTCs at all tested posttreatment time points. KB-R7943 intra-IC microinjection also lowered the incidence of GTCS but at 1-, 2-, and 3-hours posttreatment time points. The summary data are shown as the incidence (%) of WRSs and GTCSs (8 rats per group). *$P < 0.05$, **$P < 0.01$ versus vehicle-treated group Fisher Exact test).

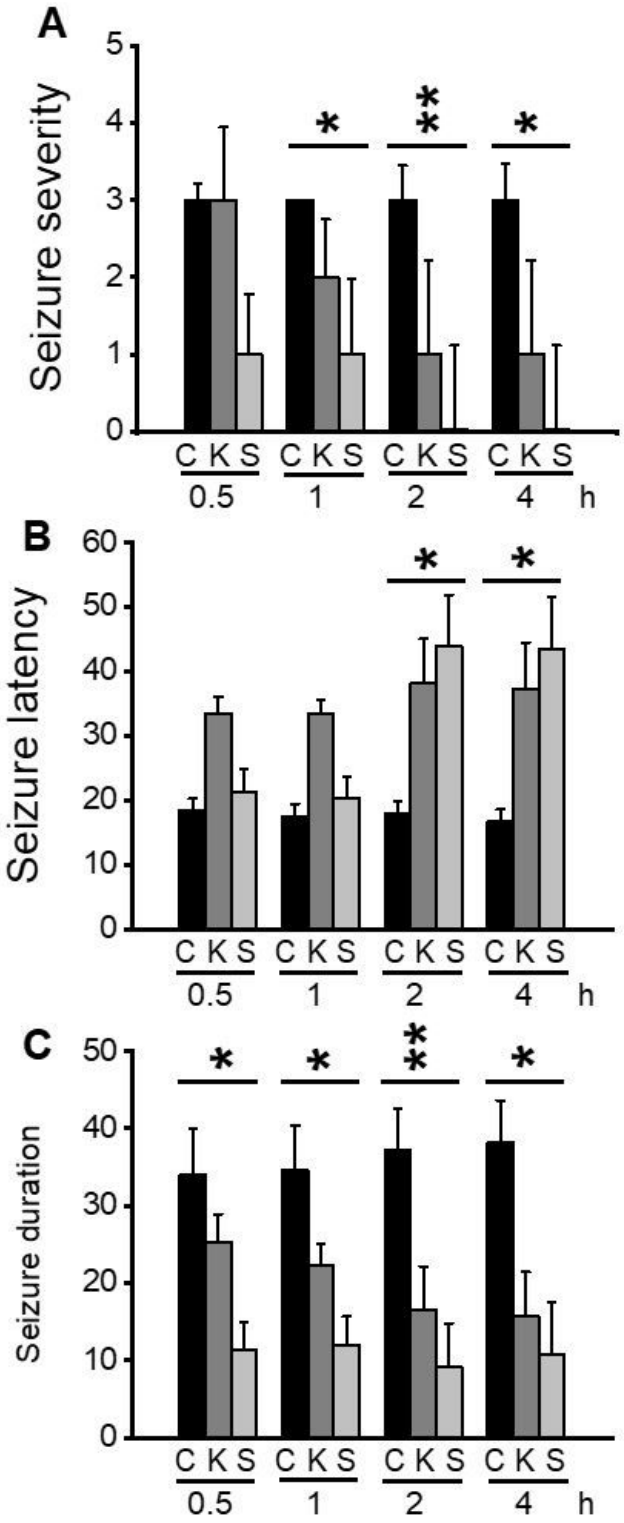

Figure 3

Effects of intra-IC microinjections of SN-6 and KB-R7943 on the seizure severity, seizure latency, and seizure duration.

The effects of intra-IC microinjections of SN-6 (5μg/2.5μl/hemisphere) and SN-6 (5μg/2.5μl/hemisphere), inhibitors of NCX1rev and NCX3rev activity, respectively, were evaluated at various post-treatment time points in animals exhibiting AWS susceptibility. **A**. Intra-IC SN-6 microinjections reduced and suppressed the seizure severity after the 0.5-hour post-treatment time point; SN-6 slightly reduced the seizure severity at 0.5-hour post-treatment time points. No notable reduction in seizure severity was observed following intra-IC microinjections of KBR-7943. **B**. Intra-IC microinjection of SN-6 significantly delayed the onset of seizures at 2- and 4-hours posttreatment time points; no notable effects were seen at 0.5- and 1-hour post-treatment time points. KB-R7943 intra-IC microinjection does not substantially alter the seizure onset. **C**. Intra-IC microinjection of SN-6 significantly reduced the seizure duration at all tested posttreatment time points. KB-R7943 intra-IC microinjection did not considerably change the seizure duration and lowered the incidence of GTCS but at 1-, 2-, and 3- hour posttreatment time points. The summary data are shown as the incidence (%) of WRSs and GTCSs (8 rats per group). *$P < 0.05$, **$P < 0.01$ versus vehicle-treated group Kruskal Wallis test for seizure severity, two-way ANOVA followed by Bonferroni correction for seizure latency and severity.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- MolecularNeurobiolManuscriptSupplementalInformation.pdf