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Focal Scn1a knockdown induces cognitive impairment without seizures

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Abstract

Cognitive impairment is a common comorbidity in pediatric epilepsy that can severely affect quality of life. In many cases, antiepileptic treatments fail to improve cognition. Therefore, a fundamental question is whether underlying brain abnormalities may contribute to cognitive impairment through mechanisms independent of seizures. Here, we examined the possible effects on cognition of $Na_v1.1$ down-regulation, a sodium channel principally involved in Dravet syndrome but also implicated in other cognitive disorders, including autism and Alzheimer's disease. Using an siRNA approach to knockdown $Na_v1.1$ selectively in the basal forebrain region, we were able to target a learning and memory network while avoiding the generation of spontaneous seizures. We show that reduction of $Na_v1.1$ expression in the medial septum and diagonal band of Broca leads to a dysregulation of hippocampal oscillations in association with a spatial memory deficit. We propose that the underlying etiology responsible for Dravet syndrome may directly contribute to cognitive impairment in a manner that is independent from seizures.

Keywords

Scn1a; Nav1.1; sodium channel; Dravet syndrome; Alzheimer's disease; oscillations; cognition; epilepsy; medial septum

INTRODUCTION

Understanding the mechanisms of cognitive impairment in epilepsy syndromes is essential to improving quality of life. Although emphasis has been placed on the role of seizures, etiology may contribute to cognitive dysfunction through mechanisms independent of seizures. Dravet syndrome (DS) is a life-long epilepsy syndrome with childhood onset that is associated with severe cognitive and quality-of-life impairments (Ohtsuka et al., 1991; Caraballo and Fejerman, 2006; Wolff et al., 2006; Akiyama et al., 2010; Ragona et al.,

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2010). Intellectual disability in DS begins early in development and is permanent; scores on the developmental quotient typically drop to 20-40% of normal within the first 6 years of age and, as adults, IQ scores are below 50 in the majority of cases (Ohtsuka et al., 1991; Wolff et al., 2006; Akiyama et al., 2010). Currently, emphasis has been placed on the impact of seizures, and treatments have targeted seizure control. However, amelioration of cognitive impairment with antiepileptic drugs has so far been elusive, suggesting that mechanisms other than seizures could importantly contribute to adverse outcomes.

In 85% of cases, DS is caused by mutations in the *SCN1A* gene, resulting in loss of function of the type I voltage-gated sodium channel ($Na_v1.1$). $Na_v1.1$ is one of four sodium channels expressed in the brain that are critical for initiating and propagating action potentials in neurons (Catterall, 2000), and deficits in $Na_v1.1$ have been linked to multiple neurological disorders associated with cognitive impairment. In addition to epilepsy, *SCN1A* mutations have also been identified in some sporadic and familial cases of autism (Weiss et al., 2003; O'Roak et al., 2011) and are now believed to be a potential risk factor for the disorder (O'Roak et al., 2012). Additionally, evidence of $Na_v1.1$ dysregulation has been found in Alzheimer's disease (AD) and has been linked to the Alzheimer's phenotype in AD mice (Kim et al., 2007; Kovacs et al., 2010; Verret et al., 2012).

Although loss of function of $Na_v1.1$ has been associated with multiple neurocognitive disorders, it is unclear whether $Na_v1.1$ deficits contribute to cognitive impairment at all. In the context of DS, two mechanisms for cognitive impairment have been considered: (1) the presence of seizures negatively impacts cognitive development and function (Catarino et al., 2011); or (2) loss-of-function of $Na_v1.1$ impairs the normal function of neural networks that are involved in information processing (Bender et al., 2012). It is important to acknowledge that these possibilities are not mutually exclusive and may in fact both occur simultaneously. So far, however, studies have focused primarily on the role of $Na_v1.1$ in seizure susceptibility rather than cognitive function (Weiss et al., 2003; Yu et al., 2006; Oakley et al., 2009; Kovacs et al., 2010; Huth et al., 2012). The finding that neuropathological abnormalities are absent in DS has further supported the former view, and it has therefore been suggested that effective control of seizures will prevent cognitive decline (Catarino et al., 2011). On the other hand, studies in mouse models have demonstrated an underlying neurophysiological deficit that is likely to impact information processing. Specifically, loss of function of $Na_v1.1$ impairs the ability of GABAergic neurons to maintain high frequency firing (Yu et al., 2006; Tang et al., 2009; Martin et al., 2010). Thus, it is entirely possible that $Na_v1.1$ deficits may also compromise the neural substrate supporting normal cognitive function.

To investigate this hypothesis, we used *in vivo* RNA interference (RNAi) to knockdown $Na_v1.1$ expression in a focal brain region that is important for regulating activity in learning and memory networks. This approach allowed us to induce focal down-regulation of gene expression in a specific target at a specified time. In this study, we have targeted the medial septum and diagonal band of Broca (MSDB) – a basal forebrain region dense with GABAergic neurons and critical for spatial cognition by regulating theta oscillations in the hippocampus (Winson, 1978; Kelsey and Vargas, 1993; Buzsaki, 2002; Colom, 2006; Hangya et al., 2009). This brain rhythm is dynamically regulated during cognitive tasks and is believed to provide a temporal framework for neural network activity. By taking this approach, we have also avoided known abnormalities caused by a global $Na_v1.1$ deficit that would otherwise confound cognitive-behavioral assessments, including cerebellar ataxia and spontaneous seizures (Kalume et al., 2007; Oakley et al., 2009). Finally, by targeting adult rats we have avoided the potential impact of developmental seizures. In this study, we aimed to determine whether cognitive impairment could arise from neural processing abnormalities

alone. We report that knockdown of $Na_v1.1$ in the MSDB caused a spatial memory impairment associated with a dysregulation of hippocampal theta frequency.

METHODS

Animals

All procedures involving animals were approved by the Dartmouth College Institutional Animal Care and Use Committee and were performed in accordance with the Institute for Laboratory Animal Research (ILAR) Guide for the Care and Use of Laboratory Animals. Male, adult (postnatal day 90-120), Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were housed in standard facilities under USDA- and AAALAC-approved conditions with a 12:12 hour light:dark cycle and *ad libitum* access to food and water.

Surgery

Rats were anesthetized with isoflurane (1-3% in oxygen) during surgery. A guide cannula with a 33 gauge internal dummy extending 1 mm beyond the tip of the cannula (Plastics One, Inc., Roanoke, VA) was stereotaxically implanted into the MSDB (Fig. 1A,B). In order to avoid damaging the superior sagittal sinus during surgery, the cannula was implanted by lowering at an angle of 21 degrees from a starting position of 2.6 mm lateral, +0.72 mm anterior from bregma. The tip of the cannula was lowered to a final position of: 0.0 mm lateral, +0.72 mm anterior from bregma, 6.8 mm ventral (Paxinos and Watson, 2005), as illustrated in the lower right inset of Fig. 1A. A custom EEG recording electrode (see *Electrophysiology*) was implanted into the hippocampus using the following coordinates (measured from the tip of the longest EEG wire): 3.0 mm lateral, −3.8 mm posterior from bregma, 3.0 mm ventral, as illustrated in the upper right inset of Fig. 1B. Three small screws were attached to the skull surface and the implants were fixed to the skull and screws with dental cement. All animals were allowed 3 days to recover before further procedures were performed.

Small-interfering RNA (siRNA)-mediated RNA interference

siRNA constructs were obtained from Invitrogen (Stealth RNAi, Life Technologies Co., Carlsbad, CA). A negative control siRNA construct (Stealth RNAi Negative Control #12935300, Invitrogen) was used for control groups, and Alexa555-conjugated *Scn1a* and control siRNA constructs were used for tracking siRNA delivery. Before administering siRNA constructs *in vivo*, three siRNA sequences were initially screened *in vitro* for efficient knockdown of *Scn1a* expression. B50 rat neuroblastoma cells (HPA Cultures #85042302), were generously donated by David Schubert (Salk Institute, San Diego, CA) and grown according to HPA recommendations. Cells were plated at a density of 40,000 cells/cm^2 and were transfected after 1 day in culture with 10 nM siRNA complexes prepared in Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. A transfection efficiency of between 90-95% was achieved under these conditions (M=94.1%, SEM=0.90 cells exhibiting Alexa555 fluorescence). Cells were then harvested 24 or 48 hours later for analysis of *Scn1a* expression by real-time PCR or western blot (WB). A minimum of 3 independent experiments were performed for each group. The sense strand sequence for the siRNA that was selected for *in vivo* use was: 5′-CCAGAGCGAUUAUGUGACAAGCAUU-3′.

siRNA complexes were delivered *in vivo* using a cationic liposomal preparation with JetSI 10 mM (Polyplus transfection, BIOPARC, France). JetSI 10mM has been optimized for delivery to brain and validated *in vivo* in multiple studies (Hassani et al., 2005; Guissouma et al., 2006; Kumar et al., 2006; Cakir et al., 2009). siRNA complexes were prepared according to manufacturer's instructions. Briefly, JetSI 10 mM reagent was mixed with 80

mM DOPE (dioleoylphosphatidylethanolamine, Sigma Aldrich, St. Louis, MO) and diluted in ethanol to a concentration of 5 mM JetSI/10 mM DOPE. Next, 1.5 ul of JetSI/DOPE solution was combined with siRNA complexes in a glucose solution to a total volume of 50 ul resulting in a final concentration of 6 μM siRNA complexes in a 5% glucose solution. Between 30 and 60 minutes after preparation of the final siRNA solution, 2.5 μl was infused into the MSDB at a rate of 0.2 μl/min (see Supplementary Methods for further description). The injection cannula (Plastics One, Roanoke, VA) was held in place for an additional 5 minutes before removing and replacing with the dummy. Each rat was injected once per day on four consecutive days (Days 2 through 5; Fig. 1C). The rats tolerated the series of siRNA injections well with no differences in weight, activity level or response to handling observed in either the controls or siRNA groups following the injections. Subsequent experimental assessments were performed 1 day after the last injection (Day 6).

RT-PCR & Western blot (WB)

RNA or protein lysate was collected from cells treated with siRNA (see *siRNA-mediated RNA interference*) for quantification of *Scn1a* expression by real-time PCR and Western blot. RNA was extracted using the RNeasy Plus Mini kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocol. Cells were lysed directly in the plate by addition of homogenization buffer and homogenized with the QIAshredder spin column prior to RNA extraction. All RNA samples were tested for quality by spectrophotometric analysis (agilent bioanalyzer and nanodrop) at the Dartmouth genomics core facility. Equal amounts of RNA were reverse transcribed to cDNA using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Life Technologies Co., Carlsbad, CA) according to manufacturer's protocol. Equal volumes of cDNA were subsequently used for real-time quantitative PCR (RT-PCR) with primers for *Scn1a* (sequence 5′-GCTTCACTGAAGGCTGTGTGCAGAG-3′) and *GAPDH* (sequence 5′-GAAACCCATCACCATCTTCCAGGAG-3′). RT-PCR was performed using the Applied Biosystems Taqman Gene Expression assays with FAM dye-labeled, TaqMan MGB probes and the Applied Biosystems 7500 Real-time PCR machine following the manufacturer's guidelines. *Scn1a* expression levels are reported as normalized to *GAPDH*.

For WB analysis, cells plated at equal density and treated with siRNA (see *siRNA-mediated RNA interference*) were collected in RIPA buffer (Pierce, Thermo Fischer Scientific Inc., Rockford, IL) following manufacturer's recommendations and mixed 1:1 with Laemmli Sample Buffer (Bio-Rad Laboratories Inc. Hercules, CA) containing βME. Equivalent amounts of total protein were electrophoresed on a 4-15% Tris-HCl gradient polyacrylamide gel and transferred to a PVDF membrane. Membranes were blotted with antibodies for $Na_v1.1$ (1:200, Chemicon/Millipore, Billerica, MA) and β-tubulin (1:500, Abcam, Cambridge, MA) followed by horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000, Chemicon/Millipore). Proteins were detected by enhanced chemiluminescence (SuperSignal West Femto, Pierce), and band intensities were quantified using the G:BOX imaging system (Syngene, Frederick, MD). $Na_v1.1$ expression is reported as normalized to β-tubulin loading control.

Immunohistochemistry

Immunofluorescence was performed for analysis of $Na_v1.1$ expression in the MSDB. Rats were anesthetized with isoflurane and perfused intracardially with phosphate buffered saline (PBS). The brains were then rapidly removed and frozen over dry ice/95% ethanol in Tissue Tek O.C.T. embedding compound (Andwin Scientific, Schaumberg, IL). 10 μm coronal sections were collected on slides using a cryostat and fixed in methanol (−20° C, 10 min). Sections were washed in PBS, blocked with 10% NGS for 1 hr, and incubated with anti-Nav1.1 antibody (1:50, Chemicon/Millipore) in blocking buffer overnight at 4° C. Sections

were washed in PBS for 30 min and incubated in Alexa488-conjugated goat anti-rabbit secondary (1:500, Invitrogen) in blocking buffer for 3 hr at room temperature. Negative controls included sections from postnatal day 2 brains, which express undetectable levels of $Na_v1.1$ (Supplementary Fig. 2). Sections were also incubated without primary antibody to control for specificity of the secondary antibody. Sections were washed in PBS for 60 min, counterstained and coverslipped with Vectashield DAPI mounting medium (Vector Laboratories, Burlingame, CA), and viewed on an Olympus BX61WI fluorescent microscope (Olympus, Center Valley, PA). For images used as examples, images were taken in greyscale, pseudocolored to reflect the correct fluorophore, and brightness and contrast were adjusted (Photoshop, Adobe, San Jose, CA).

For quantification, sections from control- and Scn1a-siRNA rats were processed together on the same slides and using the same solutions. The area transfected, indicated by Alexa555 fluorescence (approximately 0.5 mm radius from injection site), was located, and images were captured with a fluorescent filter for Alexa488 (for $Na_v1.1$ immunofluorescence) at 40X magnification using identical exposure and camera settings for all sections. Images were analyzed manually using ImageJ (NIH) by an experimenter blinded to the group assignments. The mean fluorescent intensity for $Na_v1.1$ immunoreactive cells was calculated and an area outside of the $Na_v1.1$ staining was selected for subtraction of background signal. The proportion of total $Na_v1.1$ immunoreactive cells out of all cells with DAPI labeling was also determined. An average of 164.89 +/− 26.63 (mean +/− standard error) $Na_v1.1$ immunoreactive cells were analyzed for each rat (also see Supplementary Methods).

Behavior

We used a reaction-to-novelty task – a behavioral assay that could be completed within 24 hours after the last siRNA injection (Fig. 1C). The arena was composed of a grey cylinder (75 cm diameter; 40 cm height) with a white cue card on the inner wall spanning a 100° arc of the cylinder. The arena was located within an outer insulated metal chamber illuminated by ceiling lights. Two LEDs were attached to the rat headstage to track its position via a CCD video camera connected to a frame grabber integrated within a Neuralynx electrophysiology recording system (see *Electrophysiology*).

Twelve rats were implanted with an injection cannula into the MSDB and EEG electrodes into the dorsal hippocampus CA1 region and allowed to recover from surgery (see *Surgery*). All rats were then habituated to the behavioral arena for 15 minutes (Day 0). The next day (Day 1) they were placed back in the same arena and exposed to a “baseline” configuration of 3 different objects spaced equidistant around the perimeter of the arena (Fig. 1C). They were allowed to voluntarily explore this baseline configuration for 10 minutes. Hippocampal EEG and position data were recorded during exploration (see *Electrophysiology*). They were then randomized to receive either control-siRNA or *Scn1a*-siRNA injections with a total of 6 rats per group. siRNA injections were then administered once per day for 4 days (Days 2 to 5). Twenty-four hours after the last siRNA injection (Day 6), each rat was placed back in the behavioral arena with the 3 objects in the same “baseline” configuration and allowed to explore again for 10 minutes. After re-exposure to the “baseline” configuration, one object (Object A) was moved to a new location (halfway between the other two objects) while the rat was held outside the arena (approximately 1 minute). The rat was then returned to the center of the arena and allowed to explore this novel spatial configuration for 10 min (reaction to spatial change). In the following session, a different object (Object C) was removed and replaced with a new object (Object D) in the same location while the rat was held outside the arena (approximately 1 min). The rat was then returned to the center of the arena and explored again for 10 min (reaction to object change). These three sessions on Day 6 were performed consecutively for each rat. Hippocampal EEG and position data were recorded during exploration in all sessions (see *Electrophysiology*).

The rat position data was analyzed using Matlab (MathWorks, Natick, MA). A pilot study was conducted in a separate group of control animals to determine *a priori* the most sensitive time period for detecting a response to the novel stimulus (Supplementary Fig. 3). It was determined that this response was most prominent within the first 2 minutes of the session, consistent with that reported by Chauvière et al., 2009. Therefore, the time spent exploring each object (defined as the time within a 10 cm radius from the object center) was assessed during the first 2 min of each session in this study.

Electrophysiology

Custom made electrodes were implanted into the CA1 region of the dorsal hippocampus (see *Surgery* & Fig. 1A), and were used to recorded EEG simultaneously with the animal position data in the reaction to novelty task (see *Behavior*). EEG electrodes consisted of four insulated 25 μm nichrome wires (California Fine Wire) bundled together and threaded through a 25 gauge stainless steel guide cannula (Small Parts Inc.). Each wire was connected to gold-plated pins that were inserted and held in a plastic array (Mill-Max Mfg. Co., Oyster Bay, NY). The tips of the wires were cut to span 1-1.5 mm between the shortest and longest wires. The metal guide cannula was lowered to the surface of the brain and served as a ground. Signal from the electrodes was preamplified directly on the rat's implant by operational amplifiers mounted as followers (gain = 1) and sent to a Neuralynx recording system (Neuralynx Inc., Bozeman, MT). Bipolar EEG recordings were performed between the two of the four wires that gave the largest theta amplitude on Day 1. EEG data was sampled at 10 kHz, recorded using Cheetah32 software (Neuralynx Inc.), and analyzed using Matlab (Mathworks) with multitaper time-frequency spectrum analysis (window size 2s, window step 1s; Chronux software, www.chronux.org). Only EEG signal corresponding to speeds of greater than 5 cm/s were analyzed. To compare between rats, analysis of theta power (5 to 12 Hz) was performed as normalized to the sum of the power spectrum from 0 to 120 Hz. Theta frequency was calculated as the frequency corresponding to the maximum theta power (between 5 to 12 Hz).

Continuous EEG monitoring

In order to determine whether siRNA treatment induced seizures, continuous EEG monitoring was performed in two additional groups of animals from those used for the behavior and theta analyses. In the first group (n=4), a wireless EEG monitoring device (Epoch, Ripple, Salt Lake City, UT) was used to record EEG from the cortical surface for a total duration of 48 hours before the first siRNA injection and 48 hours again after the last siRNA injection. The recording device included a battery-operated emitter cemented to the rat skull and a receiver that was placed under the rat cage. The emitter and two recording electrodes were connected to two bone screws inserted through the skull to the surface of the left and right parietal cortices in the brain. Standard surgical procedures were followed. The output of the receiver was connected to an Axoscope A/D converter and associated software (Molecular Devices Inc., Sunyvale, CA). EEG data was sampled at 200 Hz.

In the second group (n=4), custom EEG electrodes were used to record intracranial EEG simultaneously from the MSDB and both hippocampi for two 24 hour periods: one before the first siRNA injection (Day 1) and another after the last siRNA injection (Day 6). The hippocampal EEG electrodes were built and implanted as described above (*Electrophysiology* and *Surgery*). The MSDB electrodes consisted of two 0.23 mm insulated wires attached to opposite sides of the MSDB cannula (Plastics One, Inc., Roanoke, VA) and were thus implanted simultaneously with the cannula as described in *Surgery*. Signal from the electrodes was preamplified directly on the rat's implant by operational amplifiers and transmitted via a custom cable and rotating commutator (Dragonfly Research & Development, Inc., Ridgeley, WV) to an Axoscope A/D converter and associated software

(Molecular Devices Inc., Sunyvale, CA). EEG data was sampled at 1 kHz. All rats had *ad libitum* access to food and water during 24 hour monitoring.

To investigate if a pro-convulsant agent would generate seizure activity when injected into the MSDB, bicuculline was injected in one rat at a dose previously shown to induce seizures within 5 minutes when injected into the hippocampus (Bragin et al., 2009). Intracranial EEG was recorded in the MSDB and both hippocampi, as described above, for 10 minutes before and 90 minutes after injection of 2 μl of 100 μM bicuculline methiodide (Tocris Bioscience, Bristol, UK) dissolved in sterile saline.

Statistics

An unpaired student's t-test and one-way ANOVA were used to compare changes in expression of *Scn1a* and $Na_v1.1$ between control- and Scn1a-siRNA treated groups. Total exploration distance and the percent time spent in the center/periphery of the arena were assessed using a two-way repeated measures ANOVA with Bonferroni correction for multiple comparisons. A one-way ANOVA was also used for direct comparison of object exploration within groups.

For comparison of the behavioral performance and theta power/frequency between groups, we used generalized estimating equations (GEE). GEE was used because these data include multiple sessions for each animal and potential confounding variables also exist (e.g. speed). It is therefore not appropriate to consider each of these observations as being independent. GEE is a multivariable, repeated-measures, regression method which allows potential confounding variables to be tested and allows the assumption of the most appropriate distribution for the data. Individual comparisons between sessions and groups are made if a significant effect is found, but the primary comparison being tested is whether the pattern of responses over time differs between the two groups. For spatial recognition, the time spent near object A was evaluated across sessions with the time near objects B and C as covariates. For novel object recognition, the time near object C/D was assessed with the time near objects A and B as covariates. For analysis of theta power and frequency, speed was used as a covariate. Session, group and group-by-session effects were tested.

To evaluate the relationship between theta frequency and performance on the spatial and novel object sessions in each group, a linear regression was used with time near the moved/novel object as the dependent variable and frequency as the independent variable. To test if these relationships were significantly different between groups while also accounting for the potential effect of speed, a generalized linear model was used with percent time near the moved/novel object as the dependent variable and theta frequency and speed as covariates. Effects by group, frequency, speed, and group × frequency were tested. A p-value less than 0.05 was considered significant for all statistical tests. For clarity in the results, the t-statistic is reported for all t-tests, the F-statistic is reported for all ANOVA's, and the abbreviation "GEE" is used when reporting statistics for generalized estimating equations. Supplementary Table 1 also lists all experimental groups referred to in the results section along with the number of subjects/experiments used in the statistical calculations and reported in the results.

RESULTS

Knockdown of $Na_v1.1$ expression

siRNA-mediated RNA interference was used to knockdown expression of *Scn1a*. siRNA complexes targeted to *Scn1a* (Scn1a-siRNA) were first validated *in vitro* using a rat neuroblastoma cell line expressing $Na_v1.1$. A significant reduction of *Scn1a* expression by 71% (control = 1.0+/−0.09, Scn1a = 0.288+/−0.019; mean +/− standard error) compared to a

negative control siRNA construct was observed 24 hours after transfection ($F(2,5)=28.24$, $p<.01$, n=3 per group; Fig. 2A) with a similar reduction of 73% (control = 1.0+/−0.09, Scn1a = 0.273+/−0.076) at the protein level after 48 hours ($t(4)=6.15$, $p<.01$, n=3 per group; Fig. 2B). This Scn1a-siRNA construct was then tested *in vivo*. siRNA complexes, prepared in a cationic liposomal vehicle, were injected into the MSDB once daily for 4 days (Fig. 1). siRNA complexes successfully entered cells (Supplementary Fig. 1) and suppressed $Na_v1.1$ expression (Fig. 2C-F). In the transfected region, 49% fewer cells (control = 41.0+/−1.02, Scn1a = 20.6+/−2.64) exhibited positive $Na_v1.1$ immunofluorescence ($t(7)=3.89$, $p<.01$, n=4 control, n=5 scn1a; Fig. 2F) and, among $Na_v1.1$ immunoreactive cells, the mean fluorescent intensity was reduced by 39% (control = 1.0+/−0.095, Scn1a=0.61+/−0.051) compared to control ($t(7)=3.89$, $p<.01$, n=4 control, n=5 scn1a; Fig. 2E).

Continuous EEG monitoring

Since a global loss of function of $Na_v1.1$ leads to spontaneous seizures, we monitored for seizure activity after local MSDB siRNA injections using continuous EEG monitoring. In one group (n=4), EEG was recorded from the cortical surface, and in a second group (n=4), intracranial EEG recordings were used to monitor for localized seizure activity in the MSDB and hippocampus. EEG was recorded for a minimum of 24 hours before the first siRNA injection and 24 hours again after the last siRNA injection – a duration that included the full time-course of the behavioral testing (see *Methods* and *Reaction to Novelty*). No seizure or epileptiform activity was observed with EEG monitoring (Fig. 3A-C), and no behavioral or electrographic seizure activity was observed during any subsequent experiments. Injection of the pro-convulsant agent bicuculline into the MSDB of one rat was also not sufficient to cause seizures, indicating that the MSDB may be less susceptible to generating seizures in general.

Reaction to novelty

The effect of $Na_v1.1$ knockdown in the MSDB was assessed on a reaction-to-novelty task. Rats were first allowed to voluntarily explore a “baseline” configuration of 3 objects located around the perimeter of a circular arena before (Day 1) and after (Day 6) siRNA injections (Fig. 4A). The time spent exploring the objects and the overall levels of exploration, measured as total distance travelled, were evaluated. When rats were re-exposed to the baseline configuration on Day 6, there was a trend towards a decrease in exploration in both groups (Fig. 4C); this finding was expected because the rats were being re-exposed to a familiar environment. Importantly, no differences in exploration distance ($F(1,10)=0.07$, $p>.05$, n=6 per group; Fig. 4F) or time spent exploring the objects ($F(1,10)=0.01$, $p>.05$, n=6 per group; Fig. 4C) were observed after siRNA treatment between groups. To assess potential changes in anxiety levels, the time spent exploring the center versus periphery of the arena was also measured, and no differences in either measure were found post-treatment between groups ($F(1,10)=0.23$, $p>.05$, n=6 per group; data not shown). Thus, siRNA treatment did not differentially affect overall exploration ability or anxiety levels in the arena.

Following re-exposure to the baseline configuration on Day 6, one object (object A) was moved to a new location, and the rats were allowed to explore this novel spatial arrangement. Rats administered control-siRNA showed a strong preference for the moved object, spending significantly more time exploring object A compared to objects B and C ($F(2,5)=12.92$, $p<.01$, n=6 per group; Fig. 4C). In contrast, rats receiving Scn1a-siRNA showed no preference for any object ($F(2,5)=.03$, $p>.05$, n=6 per group; Fig. 4C). Generalized estimating equations was used to compare the pattern of the responses over time, and revealed a significantly different response in the Scn1a-siRNA treated group to object A compared to controls (GEE: session × group, $p<.01$, n=6 per group; Fig. 4D).

Consistent with these results, the amount of overall exploration was also significantly reduced in Scn1a-siRNA treated rats compared to controls during the spatial session ($F(1,10)=9.83$, effect by group, $p<.05$, Bonferroni post-hoc correction for spatial, $t=3.36$, $p<.01$, $n=6$ per group; Fig. 4F). Together, these data indicate that Scn1a-siRNA treatment impaired reaction to spatial change.

In a final condition, object C was replaced with a novel object (object D) in the same location. During this condition, both groups showed increased exploration of object D (GEE: effect of session, $p<.001$; $n=6$ per group; Fig. 4C,E). Although there was a trend towards a weaker response in the Scn1a-siRNA treated group, this was not a significant difference (GEE: effect of group, $p>.05$; session × group $p>.05$, $n=6$ per group; Fig. 4C,E). Similarly, no difference was found in overall levels of exploration during this session ($F(1,10)=9.83$, effect by group, $p<.05$, Bonferroni post-hoc correction for identity, $t=1.63$, $p>.05$, $n=6$ per group; Fig. 4F). Therefore, reaction to object novelty was not significantly different between groups. Taken together, these data suggest that group differences during the spatial condition cannot be explained by a failure of the Scn1a-siRNA group to identify and distinguish objects, by a lack of motivation to respond to novelty, by a general deficit in exploration ability, or by a change in anxiety, indicating a specific impairment in reaction to spatial change.

Theta oscillations

Considering the critical role of the MSDB in regulating hippocampal theta oscillations during exploratory behavior, the effect of $Na_v1.1$ knockdown on theta oscillations was assessed during the reaction-to-novelty task (Fig. 5A-D). EEG was recorded in the dorsal CA1 region of the hippocampus (Fig. 1A) simultaneously with the behavioral testing. Since theta oscillations are known to be influenced by running speed, all analyses were adjusted for speed by using speed as a covariate in generalized estimating equations (see *Statistics*). There was no difference in theta power between groups (GEE: $p>.05$, $n=6$ per group, adjusted for speed; Fig. 5B). However, theta frequency was significantly altered after Scn1a-siRNA treatment (GEE: group × session, $p<.001$, $n=6$ per group, adjusted for speed; Fig. 5C,D). Although both groups showed a trend towards an increase in frequency during the spatial session from the previous baseline session, theta frequency was significantly reduced in the Scn1a-siRNA treated group compared to controls (GEE: effect between groups, $p<.05$, $n=6$ per group). In contrast, no differences were observed during novel object recognition (GEE: $p>.05$, $n=6$ per group).

To assess the relationship between theta frequency and performance, mean theta frequency was plotted versus percent time near the moved/novel object and a linear regression was performed (Fig. 5E,F). In rats treated with control siRNA, a significant relationship was found for spatial performance (Fig. 5E), with higher frequencies positively correlated to exploration of object A ($R^2=0.76$, $p<.05$, $n=6$). Interestingly, in rats receiving Scn1a-siRNA treatment, not only was theta frequency reduced (as seen in Fig. 5D), but the relationship of theta frequency with spatial performance was completely abolished ($R^2=0.005$, $p>.05$, $n=6$). The relationships between theta frequency and performance were significantly different between groups (GEE: effect of group, frequency and group × frequency, all $p<.01$, $n=6$ per group) and independent of speed (GEE: effect of speed, $p=.82$). Thus, theta frequency was significantly related to performance in the spatial session, and knockdown of $Na_v1.1$ in the MSDB caused a dysregulation of theta frequency that eliminated this relationship. In contrast, no relationship between frequency and performance was found for the novel object session (Fig. 5F) in either the controls ($R^2=0.08$, $p>.05$, $n=6$) or Scn1a-siRNA treated rats (($R^2=0.11$, $p>.05$, $n=6$), and there was no significant difference between groups (GEE: effect of group, frequency and group × frequency, all $p>.05$, $n=6$ per group).

DISCUSSION

In this study, we demonstrate that siRNA reduces $Na_v1.1$ expression *in vitro* and that this effect is reproduced *in vivo*. Reduction of $Na_v1.1$ in the MSDB caused a spatial memory impairment, indicated by a failure to respond to a change in object location during a reaction-to-novelty task. In contrast, no difference was found in response to a novel object or in more general measures of exploration. Therefore, this effect represents a selective spatial impairment rather than a more general impairment in the ability to recognize and distinguish objects, to explore the arena, or to respond to novelty. Interestingly, spatial performance was significantly related to hippocampal theta frequency in the control group, but this relationship was abolished after knockdown of $Na_v1.1$. This effect is consistent with the critical role of the MSDB in regulating hippocampal theta oscillations, but differs in that it represents a dysregulation of the rhythm rather than a permanent suppression of theta oscillations as is seen after MSDB inactivation. Moreover, continuous EEG monitoring demonstrated that this deficit was not caused by seizures. These results reveal a critical role for $Na_v1.1$ in the brain for the proper regulation of network oscillations and cognitive function.

The reaction-to-novelty task has been widely used to test spatial and non-spatial forms of cognition (Warburton and Brown, 2010). In this task, the ability to determine if an object configuration was previously encountered requires intact memory of the prior occurrence and involves different brain regions depending on the type of memory that must be formed. Object recognition depends on the perirhinal cortex, as lesions of this region eliminate reaction to object change (Mumby and Pinel, 1994; Ennaceur et al., 1996; Ennaceur, 1998). However, lesions of the hippocampus, MSDB or fimbria fornix, which disconnects the hippocampus from the MSDB, do not eliminate this response (Ennaceur et al., 1996; Warburton et al., 2000; Mumby, 2001; Winters et al., 2004). Recognition of a change in the location of an object, on the other hand, requires an intact hippocampus, MSDB and fimbria fornix (Thinus-Blanc et al., 1991; Ennaceur, 1998; Warburton et al., 2000; Winters et al., 2004; Warburton and Brown, 2010). Therefore, the specificity of the behavioral effect in our study for spatial recognition but not object recognition memory is in accordance with a specific deficit in the function of the MSDB and hippocampal network.

One mechanism by which the MSDB is involved in spatial cognition is through the regulation of hippocampal theta oscillations (Winson, 1978; Kelsey and Vargas, 1993; Buzsaki, 2002; Hangya et al., 2009). MSDB GABAergic and cholinergic neurons project to the hippocampus and are essential for the coordination of this rhythm (Stewart and Fox, 1989; Givens and Olton, 1990). The GABAergic projection neurons in the MSDB are believed to be involved in pacing theta oscillations (Brazhnik and Fox, 1999; Varga et al., 2008; Hangya et al., 2009), whereas the slow-firing cholinergic neurons are more likely to provide the excitatory drive required to activate intrinsic hippocampal oscillators and thereby influence theta amplitude (Fischer, 2004). In this study, we observed a dysregulation of theta frequency rather than a permanent suppression of the rhythm. This finding, therefore, could be explained in part by a deficit in the firing of GABAergic neurons. Indeed, other studies have revealed that $Na_v1.1$ loss of function affects the neuronal firing of GABAergic neurons in the hippocampus and cortex (Yu et al., 2006; Martin et al., 2010). However, since the siRNA treatment in this study is not cell-type specific, the effects of $Na_v1.1$ deficits on the different MSDB cell types warrants further investigation.

The observation that knockdown of $Na_v1.1$ in the MSDB induced a dysregulation of theta frequency rather than an overt suppression of theta power is also an important distinction between the findings in this study and other studies that lesion or inactivate the MSDB (Winson, 1978; Rawlins et al., 1979). We speculate that theta power was not reduced

because the excitatory drive to the hippocampus was not likely to be affected by the manipulation in this study. In support of this hypothesis, prior studies have shown that $Na_V1.1$ deficits spare slow-firing excitatory neurons (Yu et al., 2006). Additionally, theta amplitude is regulated primarily by the input from the entorhinal cortex and from the intrinsic theta oscillators in the hippocampus (Hagan et al., 1992; Buzsaki, 2002; Goutagny et al., 2009), neither of which was directly targeted in this study.

Although theta frequency was reduced on both post-injection baseline and spatial sessions, there was no difference between the treatment groups on the final object session. It is likely that the differential effect on theta frequency on Day 6 is related to how activity in the hippocampus is regulated. During baseline exploration, hippocampal theta oscillations are believed to be involved in sensorimotor integration, and the MSDB is actively involved in its generation and regulation (Bland and Oddie, 2001). Thus, $Na_V1.1$ downregulation in the MSDB would reasonably be expected to alter hippocampal theta during this baseline exploratory behavior. During the spatial session, the MSDB is required for proper performance on this task, and, therefore, an alteration in theta and an association with impaired performance is observed. In contrast, during the novel object session, neither the MSDB nor hippocampus are necessary (Ennaceur et al., 1996; Warburton et al., 2000; Mumby, 2001; Winters et al., 2004). Thus, a positive association between hippocampal theta frequency and performance during this task is not observed in either group. On the other hand, cortical input – particularly the perirhinal cortex – is required (Winters et al., 2004; Warburton and Brown, 2010). Because the hippocampus receives direct connections from the perirhinal cortex to the CA1 region and also has the potential to generate theta intrinsically (Hagan et al., 1992; Buzsaki, 2002; Goutagny et al., 2009), the regulation of theta during the object session likely involved input from this region, masking the effects of MSDB dysregulation. Since the injections were performed only in the MSDB, the capacity of the hippocampus to respond to other inputs would have remained intact, and thus theta oscillations could still be regulated when inputs from elsewhere were active, as is likely to be the case during the novel object session.

In any analysis of theta frequency, speed is a potential confounding variable, as theta frequency has been shown to positively correlate with running speed in multiple studies (McFarland et al., 1975; Sławińska and Kasicki, 1998; Wills et al., 2010). In this study, running speeds were low, between 10 to 15 cm/s on average (data not shown) and, thus, were likely to have a minimal effect on theta frequency compared to the larger ranges of speed typically observed in a linear track or running wheel (more than 50 cm/s). Importantly, we accounted for the possible contribution of changes in speed to changes in theta frequency by using speed as a covariate in all analyses. Both the differences observed in theta frequency, as well as the relationship between theta frequency and spatial performance, were found to be independent of running speed.

Although a causal link between theta oscillations and cognition cannot be concluded in this study, a dysregulation of theta frequency nonetheless suggests that the temporal integrity of normal network patterns is compromised due to a loss of $Na_V1.1$ expression. A similar magnitude reduction of theta frequency was also found by Chauvière et al., 2009 in association with an impaired reaction to spatial change in a temporal lobe epilepsy model (Chauvière et al., 2009). Consistent with our findings in rodents, an altered pattern of brain oscillations has been observed in children with DS characterized by a shift toward slower frequencies on the cortical EEG (Holmes et al., 2011). In adults with DS, the presence/absence of background alpha oscillations also significantly correlates with the severity of mental deficits (Akiyama et al., 2010). Together, these results suggest that dysregulation of brain oscillations in DS may be an important factor related to cognitive impairment.

An important difference between this study and prior studies using transgenic mouse models is that in the latter, $Na_v1.1$ is affected throughout the whole brain, whereas we have targeted a single structure. Since a global loss of function of $Na_v1.1$ leads to spontaneous seizures (Yu et al., 2006; Oakley et al., 2009), it is difficult to determine if impairments in these models are a secondary result of the detrimental impact of seizures or due to other underlying brain abnormalities. For these reasons, one cannot know whether factors other than seizures could contribute to adverse outcomes without isolating a single brain network and functional outcome. In this study, we reveal an impairment in cognitive function related to the loss of $Na_v1.1$ specifically in the MSDB. Furthermore, by avoiding the occurrence of seizures, we demonstrate that $Na_v1.1$ is required for normal cognitive function.

The absence of seizure activity after $Na_v1.1$ down-regulation in the MSDB is not surprising considering that this region has not been implicated as a primary site of seizure generation in epilepsy models. The reason for this may be that it lacks the same degree of recurrent excitatory connections found in the hippocampus and cortex, which are implicated in seizure generation (McCormick and Contreras, 2001). Therefore, it may be less susceptible to seizure activity in general. For example, even injection of the pro-convulsant agent, bicuculline, did not induce epileptiform activity when injected into the MSDB. Additionally, previous studies have shown that $Na_v1.1$ deficits only partially reduce GABAergic neuronal firing but do not abolish it (Yu et al., 2006; Martin et al., 2010); thus, this effect differs from complete blockade of GABAergic neurotransmission and may not be sufficient to provoke seizure activity when limited to the MSDB region.

Although we have not directly tested a disease model, these results may provide insight into the factors contributing to cognitive impairment in DS. In humans, *SCN1A* mutations are associated with a range of epilepsy disorders, including the milder Generalized Epilepsy with Febrile Seizures Plus (GEFS+) and the more severe DS. However, the severity of the epilepsy phenotype also correlates with the type and severity of the mutation in *SCN1A*, with loss-of-function mutations most often associated with DS (Zuberi et al., 2011). Considering that knockdown of $Na_v1.1$ in this study could be interpreted as a focal loss of function of the protein, these results are likely to relate primarily to DS. While the impact of seizures during development has been shown to negatively impact cognitive function in epilepsy models (Holmes et al., 1998; Ben-ari and Holmes, 2006; Khan et al., 2010), cognitive outcomes in patients with DS remain poor (Ohtsuka et al., 1991; Akiyama et al., 2010) despite antiepileptic treatment, suggesting that factors other than seizures may adversely affect cognition. As we show here, albeit in an isolated brain network, one of these factors may be the impact of $Na_v1.1$ deficits on the underlying neurobiological substrate supporting cognition. Furthermore, the finding that $Na_v1.1$ is required for cognitive function in adult rats implies that the potential deleterious effects of *SCN1A* mutations are likely to extend beyond the developmental period.

These results may also be relevant for understanding the contributions to cognitive dysfunction in Alzheimer's disease. As levels of the β-secretase enzyme become elevated in AD, a loss of function of $Na_v1.1$ is observed (Kim et al., 2007). It has been postulated that epileptic activity resulting from this $Na_v1.1$ deficit could contribute to cognitive decline in the early stages of the disease prior to neuronal degeneration (Huth et al., 2012). Considering that the region we targeted in this study, the MSDB, is indeed affected early in Alzheimer's disease, our results extend this hypothesis by implying that a deficit in $Na_v1.1$ expression could also directly impact cognition by compromising the ability of the septo-hippocampal network to support normal cognitive processes (Kim et al., 2007). It is important to recognize that the extrapolation of these results to human disease is also limited. However, they suggest that at a fundamental level $Na_v1.1$ is necessary for supporting this cognitive neural network.

In summary, knockdown of $Na_v1.1$ in the MSDB induces a spatial memory impairment associated with a dysregulation of hippocampal theta frequency. We propose that a loss of function of $Na_v1.1$ in DS, and possibly other disorders in which $Na_v1.1$ function is impaired, such as autism (Weiss et al., 2003; O'Roak et al., 2011) and Alzheimer's disease (Kim et al., 2007), may contribute to cognitive dysfunction. Importantly, our results demonstrate a link between $Na_v1.1$ deficits and cognitive impairment that is independent of seizures. New treatment strategies that target this underlying deficit in brain function may provide additional benefit for improving quality of life in patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- $Na_v1.1$ (*SCN1A*) deficits are linked to Dravet syndrome and other cognitive disorders
- RNA interference was used to knockdown $Na_v1.1$ expression in the basal forebrain region
- $Na_v1.1$ knockdown impaired spatial cognition in the absence of seizures
- Impaired spatial performance was associated with a dysregulation of theta frequency
- Nav1.1 deficits may impair network oscillations and cognition in human disorders

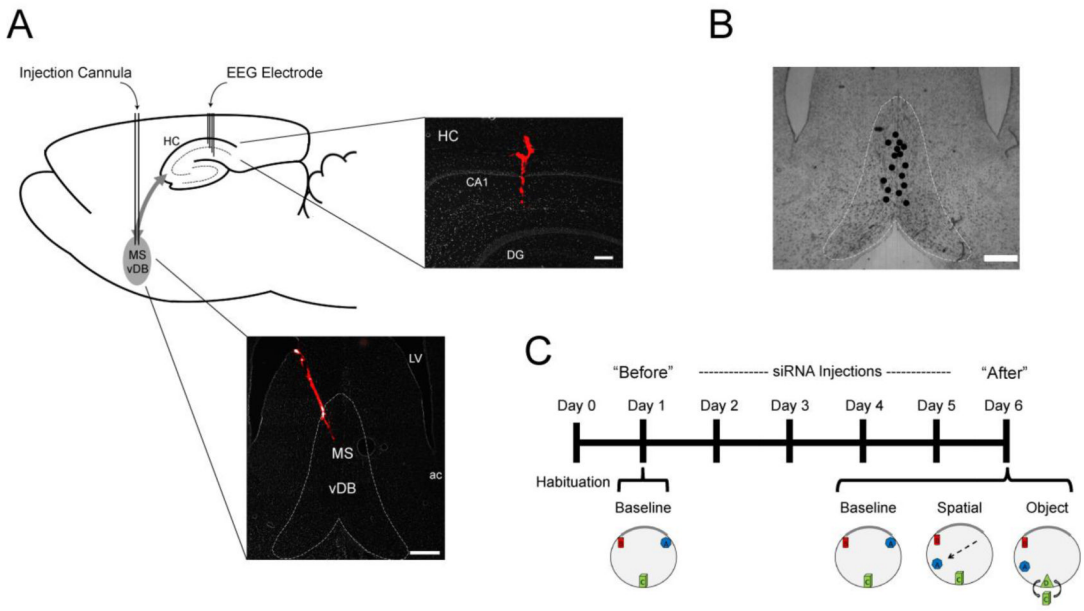

Figure 1.

Experimental design. (**A**) A guide cannula for siRNA injection was implanted into the MSDB and a custom EEG recording electrode was implanted into the CA1 region of the dorsal hippocampus. Insets show locations of injection and recording sites in coronal view from a cannula (lower) and electrode (upper) that were coated in DiI prior to implantation. Scale bars equal 500 μm (lower) and 200 μm (upper). (**B**) Accuracy of cannula placement. Black dots show locations of injection sites from 16 rats implanted during the course of the study. Scale bar equals 500 μm. (**C**) Timeline: Adult rats were injected with negative control- or Scn1a-targeted siRNA complexes over 4 days (Days 2-5) and then tested in a reaction-to-novelty task (Day 6). The "Baseline" session was performed on Day 1 and repeated on Day 6 (also see *Methods*). MS, medial septum; vDB, vertical limb of the diagonal band of Broca; LV, lateral ventrical; ac, anterior commissure; HC, hippocampus; CA1, field CA1 of hippocampus; DG, dentate gyrus.

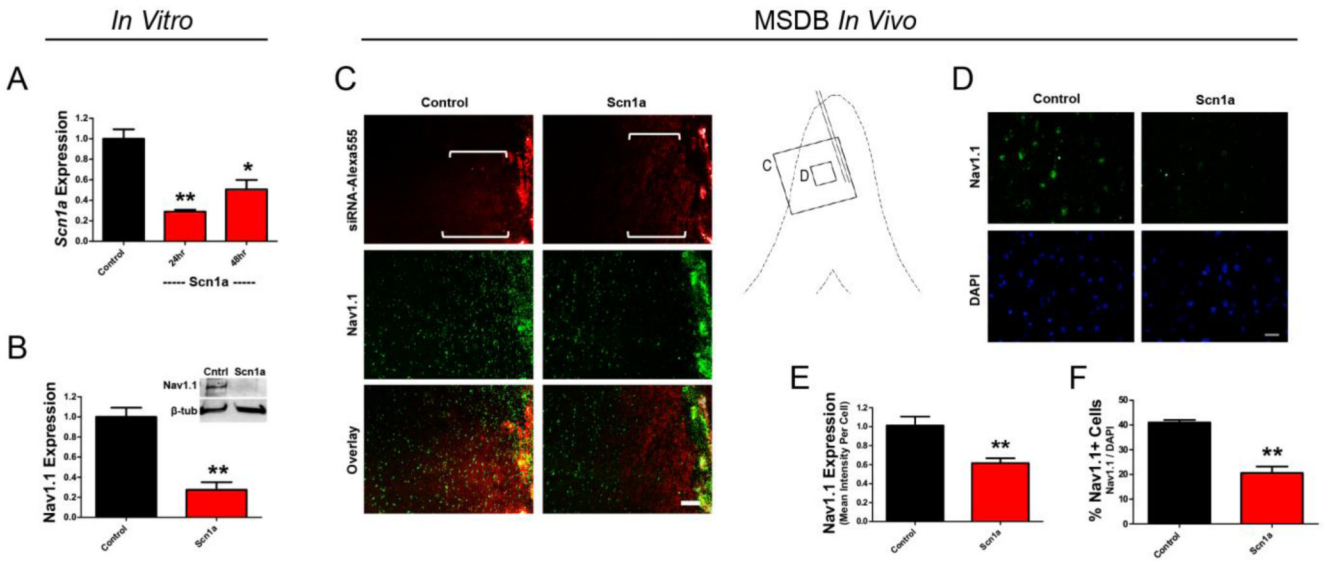

Figure 2.

siRNA-induced knockdown of $Na_v1.1$ expression. **(A)** Scn1a-siRNA constructs reduced *Scn1a* expression by 71% and 49% after 24 and 48 hours, respectively, in cultured rat neuroblastoma cells (*p<.05, **p<.01). Levels are expressed normalized to *GAPDH*. (**B**) $Na_v1.1$ protein expression was similarly reduced by 73% after 48 hours (**p<.01). Inset shows representative WB. (**C**) $Na_v1.1$ expression was assessed after 4 days injection of Alexa555-conjugated control- or Scn1a-siRNA complexes by immunofluorescence. Example shows reduced $Na_v1.1$ immunofluorescence in the Scn1a-siRNA group in the transfected region indicated by white brackets (identified by Alexa555 fluorescence). Artifact from the track of the cannula can be seen on the right side of the images. Scale bar equals 100 μm. (**D**) Examples of images taken at higher magnification revealing reduced $Na_v1.1$ staining after Scn1a-siRNA injections. Scale bar equals 20 um. (**E**) In the transfected region, the mean fluorescent intensity for $Na_v1.1$ immunoreactive cells was reduced by 39% (**p<.01), and (**F**) the proportion of $Na_v1.1$ immunoreactive cells (compared to DAPI) was decreased by 49% (**p<.01). Error bars represent standard error of the mean.

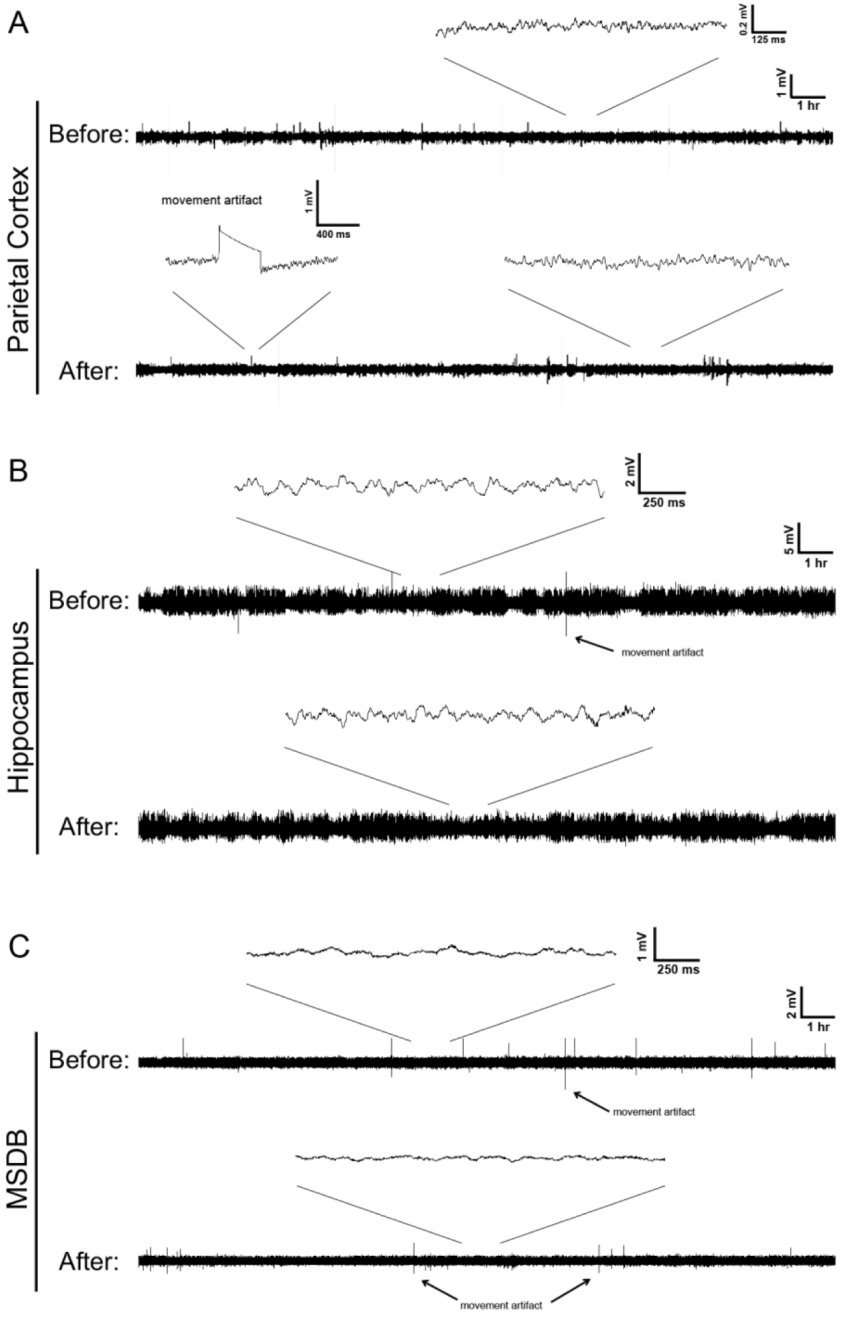

Figure 3.

Continuous EEG monitoring. Examples of 24 hr epochs of EEG recorded before and after Scn1a-siRNA administration. Each pair of a "Before" and "After" epoch is taken from the same animal. (A) In one group (n=4), EEG was recorded from the cortical surface. (B-C) In a second group (n=4), intracranial EEG recordings were used to monitor local activity bilaterally in the hippocampus (B) and in the MSDB (C). Continuous EEG monitoring was performed for a minimum of 24 hrs before and 24 hrs again after siRNA treatment. Insets show examples on shorter time scales of representative EEG signal and movement artifact. These artifacts occurred both before and after siRNA treatment in roughly equal proportion. No behavioral or electroencephalographic seizures were observed after siRNA treatment.

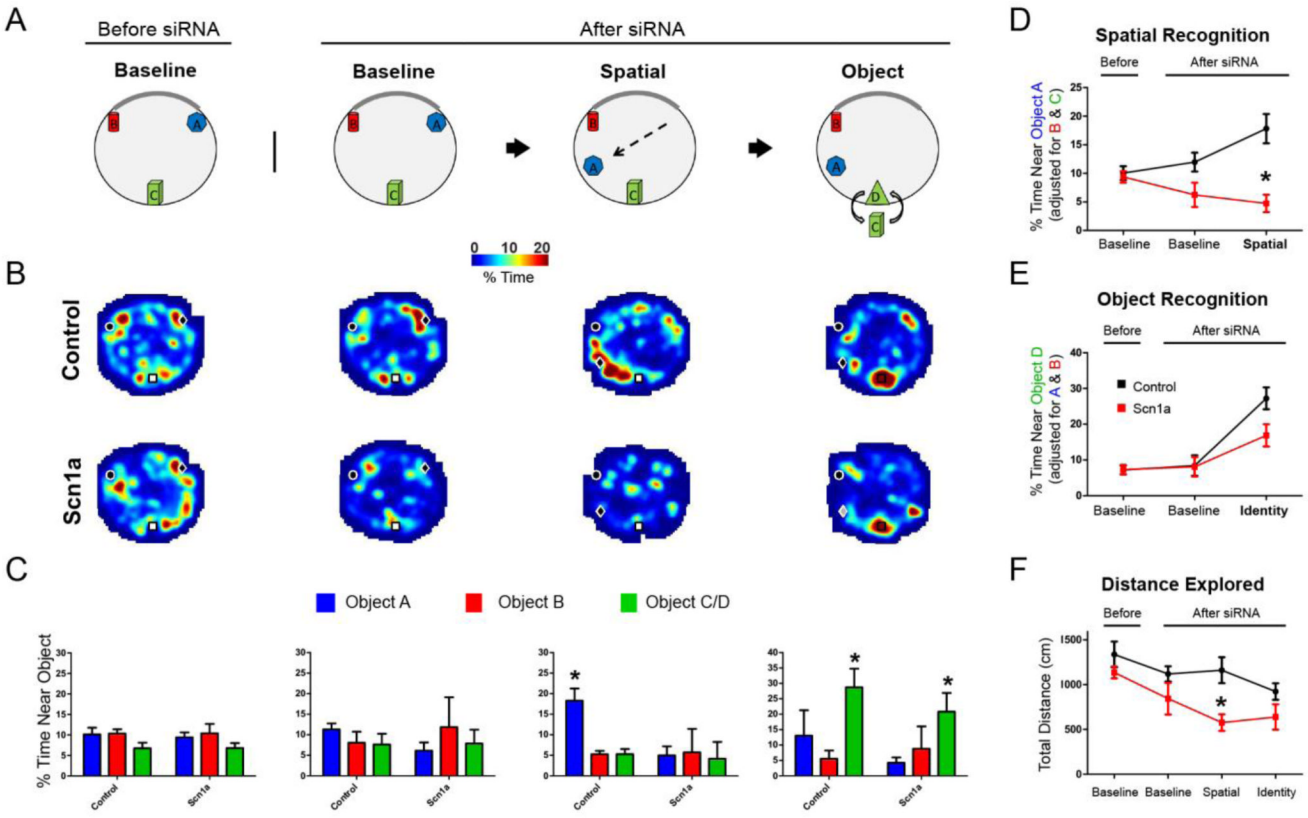

Figure 4.

Knockdown of $Na_v1.1$ in the MSDB impairs spatial memory. (**A**) Exploratory behavior was assessed in four sessions. Before siRNA injections (Day 1), rats were exposed to a "baseline" configuration of 3 objects. Then, after siRNA injections (Day 6) the rats were re-exposed to the same baseline session followed by a novel spatial session (object "A" was moved to a new location), and a novel object session (object "C" was replaced with object "D" in the same location). The response to spatial change, but not object change, was impaired in Scn1a-siRNA treated rats. (**B**) Mean exploration time for each group. Scale is expressed in percent time. (**C**) Mean percent time near each object. (**D**) Using generalized estimating equations to compare the pattern of the responses over time, the response of the controls, but not Scn1a-siRNA treated rats, to object A was significantly greater during the spatial session than during the prior baseline sessions, and this response was significantly different between groups (GEE: session × group, $p<.01$, n=6 per group). (**E**) In contrast, the responses to object "D" during the novel object recognition session were not significantly different between groups (group, $p>.05$; session × group $p>.05$, n=6 per group); both groups explored object "D" significantly more during the object identity session compared to prior sessions (session, $p<.001$, n=6 per group). (**F**) The total distance travelled was measured and a two-way repeated measures ANOVA with Bonferroni multiple comparisons was used to test for significance. Exploration was not significantly different between groups during either baseline sessions before or after siRNA treatment ($p>.05$). In agreement with panels B-E, exploration in controls was significantly greater than the Scn1a-treated rats during the spatial session ($F(1,10)=9.83$, effect by group, $p<.05$, Bonferroni post test for spatial, $t=3.36$, $p<.01$) but not significantly different during the novel object session ($p>.05$). Error bars represent standard error of the mean. *denotes significance by GEE.

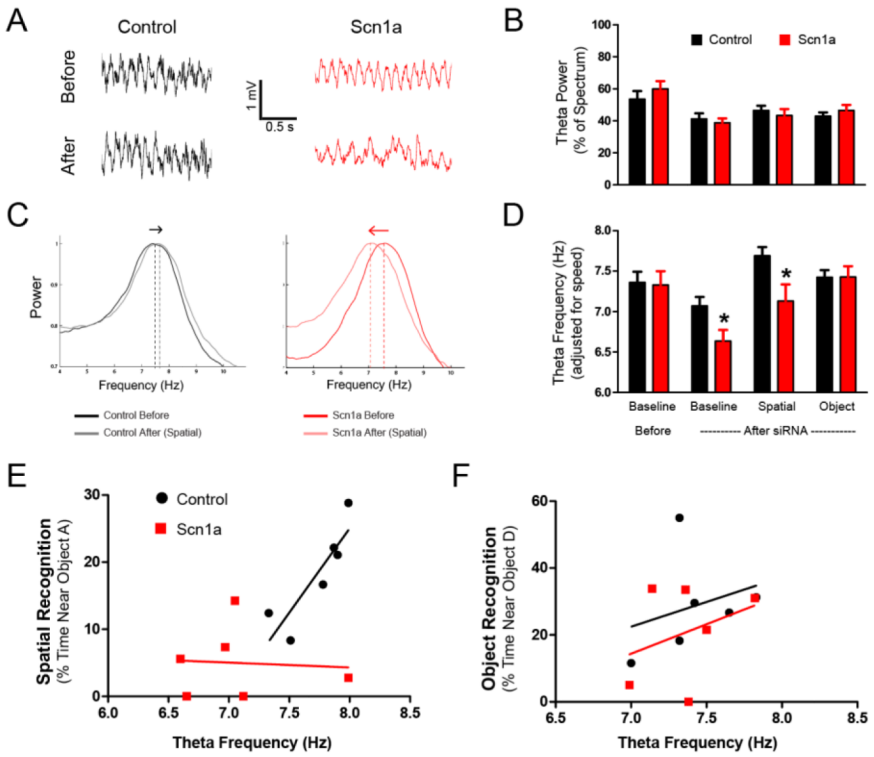

Figure 5.

Knockdown of $Na_v1.1$ in the MSDB alters hippocampal theta frequency. Hippocampal EEG recordings were performed simultaneously with behavioral testing. (**A**) Examples of EEGs recorded before and after siRNA injections. (**B**) No difference in hippocampal theta power was observed between groups (GEE: $p>.05$, n=6 per group, adjusted for speed). (**C**) Examples of EEG spectrograms from before and after siRNA administration recorded during periods of motion. Power is normalized to the peak of theta to show changes in frequency. A shift in theta frequency was observed, with controls increased during the spatial task and the Scn1a-siRNA rats decreased from Day 1. (**D**) Generalized estimating equations was used to compare changes in theta frequency with speed as a covariate. After siRNA administration, theta frequency was altered in Scn1a-siRNA treated rats (GEE: group × session, $p<.001$, n=6 per group). Scn1a-siRNA treated rats exhibited reduced theta frequency compared to controls during the post-exposure baseline and spatial sessions. Theta frequency values are shown adjusted for speed. Error bars represent standard error of the mean. *denotes significance by GEE. (**E**) Theta frequency is shown plotted versus percent time spent near object A during the post-treatment spatial session for each individual rat. Linear regression revealed a significant relationship between theta frequency and spatial performance in the controls ($R^2=0.76$, $p<.05$) that was absent in the Scn1a-siRNA treated group ($R^2=.005$, $p>.05$). (**F**) Theta frequency plotted versus percent time spent near object D during the post-treatment novel object session. No relationship was found in either group ($p>.05$).