Lenk Guy (Orcid ID: 0000-0001-8092-1405)

Scn8a antisense oligonucleotide is protective in mouse models of *SCN8A* Encephalopathy and Dravet Syndrome

Short title: ASO therapy for *SCN8A* encephalopathy

[1]Guy M. Lenk, [5]Paymaan Jafar-Nejad, [1,3]Sophie F. Hill, [2,3]Lucas D. Huffman, [1]Corrine E. Smolen, [1]Jacy L. Wagnon, [1]Hayley Petit, [1]Wenxi Yu, [4]Julie Ziobro, [4]Kritika Bhatia, [4]Jack Parent, [2,3,4]Roman J. Giger, [5] Frank Rigo, [1,3,4]Miriam H. Meisler

[1]Department of Human Genetics, [2]Department of Cell and Developmental Biology,

[3]Neuroscience Program and [4]Department of Neurology, University of Michigan,

Ann Arbor Michigan 48109 and [5]Ionis Pharmaceuticals, Carlsbad, California 92010

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ABSTRACT

Objective: *SCN8A* encephalopathy is a Developmental and Epileptic Encephalopathy (DEE) caused by *de novo,* gain-of-function mutations of sodium channel $Na_v1.6$ that result in neuronal hyperactivity. Affected individuals exhibit early-onset drug-resistant seizures, developmental delay and cognitive impairment. This study was carried out to determine whether reducing the abundance of the *Scn8a* transcript with an anti-sense oligonucleotide (ASO) would delay seizure onset and prolong survival in a mouse model of *SCN8A* encephalopathy.

Methods: ASO treatment was tested in a conditional mouse model with Cre-dependent expression of the pathogenic patient *SCN8A* mutation p.Arg1872Trp (R1872W). This model exhibits early onset of seizures, rapid progression and 100% penetrance. An *Scn1a*$^{+/-}$ haploinsufficient mouse model of Dravet Syndrome was also treated. ASO was administered by intracerebroventricular injection at postnatal day 2, followed in some cases by stereotactic injection at postnatal day 30.

Results: We observed a dose-dependent increase in length of survival from 15 days to 65 days in the *Scn8a*-R1872W/+ mice treated with ASO. EEG recordings were normal prior to seizure onset. Weight gain and activity in an open field were unaffected, but treated mice were less active in a wheel running assay. A single treatment with *Scn8a* ASO extended survival of Dravet Syndrome mice from 3 weeks to more than 5 months.

Interpretation: Reduction of *Scn8a* transcript by 25 to 50% delayed seizure onset and lethality in mouse models of *SCN8A* encephalopathy and Dravet Syndrome. Reduction of *SCN8A* transcript is a promising approach to treatment of intractable childhood epilepsies.

Keywords: epilepsy, encephalopathy, ASO, *SCN8A*, DEE, Dravet syndrome, oligonucleotide

INTRODUCTION

Developmental and epileptic encephalopathies (DEEs) are devastating early-onset disorders characterized by seizures and developmental delay (1,2). Monogenic causes have been identified for approximately one third of cases, with voltage-gated neuronal sodium channels accounting for 2% of the total (3-5). *SCN8A* encephalopathy (MIM#614588) is a DEE caused by *de novo* missense mutations in the *SCN8A* gene that encodes the neuronal sodium channel $Na_v1.6$ (6,7). Affected individuals are heterozygous for missense mutations that alter the biophysical properties of the channel, resulting in premature channel opening, delayed channel inactivation, and elevated persistent current (6,8). Since $Na_v1.6$ has a major role in excitatory neurons, elevated activity leads directly to increased neuronal excitability (6).

The average age of seizure onset in individuals with *SCN8A* encephalopathy is 4 months. The clinical course is severe, and approximately 50% of affected individuals remain nonambulatory and nonverbal. Long-term seizure control is rarely achieved with anti-epileptic drugs (7). We and others have studied recurrent patient mutations at arginine codon 1872. Substitution of arginine 1872 by the uncharged amino acids leucine, glutamine or tryptophan impairs inactivation of the $Na_v1.6$ channel (8,9). We generated a conditional mouse model of p.Arg1872Trp that recapitulates the early seizure onset and susceptibility to premature death that are characteristic of DEE (10).

Since the pathogenic mechanism of *SCN8A* encephalopathy is neuronal hyperexcitability due to gain-of-function mutations, reduction of transcript abundance is a logical therapeutic strategy. Antisense oligonucleotides (ASOs) hybridize by Watson-Crick base-pairing to mRNAs,

leading to degradation by RNaseH, inhibition of translation, or altered splicing. Dominant disorders can be treated with allele-specific ASOs that specifically target the mutant transcript, or with non-allele-specific ASOs that reduce both mutant and wildtype transcript (11, 12). The application of ASO therapy to neurological disorders is receiving increasing attention (13-15), and the FDA has recently approved treatment for Spinal Muscular Atrophy that uses intrathecal administration of an ASO at 6 month intervals (16). The goal of the current work is to evaluate ASO therapy for *SCN8A* encephalopathy. The results provide proof-of-principle that reduction of *Scn8a* transcript can reduce the severity of this devastating disorder as well as Dravet Syndrome, a DEE caused by haploinsufficiency of *SCN1A*.

METHODS

Mutant Mice. The conditional allele of *Scn8a* (*Scn8a*^{cond}) on background strain C57BL/6J is activated by Cre recombinase to express the patient mutation R1872W (10). The EIIA-Cre transgene on background strain C57BL/6J (JAX 003724) is expressed globally in preimplantation embryos and in mature oocytes. Homozygous male *Scn8a* $^{cond/cond}$ mice were crossed with female EIIA-Cre mice to generate 50% affected animals (*Scn8a*$^{cond/+}$,EIIA-Cre) and 50% unaffected *Scn8a*$^{cond/+}$ controls lacking Cre. Entire litters were randomly assigned to treatment with *Scn8a* or control ASO. Female EIIA-Cre mice were used for breeding to avoid the mosaic Cre expression observed in offspring of male EIIA-Cre mice (10). The *Scn1a* model of Dravet Syndrome carries a deletion of exon 1 that is maintained in heterozygous state in strain 129S6/SvEvTac (25). Experiments were carried out on affected F1 mice generated by crossing with strain C57BL/6J

(27). Mice were housed and cared for in accordance with NIH guidelines in a 12/12h light/dark cycle with standard mouse chow and water available ad libitum. Experiments were approved by the Committee on the Use and Care of Animals at the University of Michigan. Open field activity and wheel running were assayed in the Michigan Mouse Metabolic Phenotyping Center (NIH U2CDK110768).

Antisense Oligonucleotides. ASOs were synthesized by Ionis Pharmaceuticals as described (28). The ASOs are 20 bp 'gapmers' with five 2′-*O*-methoxyethyl-modified nucleotides at each end and 10 DNA nucleotides in the center. The *Scn8a* ASO 5' GACGA TTAGT GACAT AGGCT 3' is complementary to the 3' UTR that does not differ between wildtype and mutant transcript (Figure 1A). The control ASO 5' CCTAT AGGAC TATCC AGGAA 3' does not match any transcript encoded by the mouse genome and was shown to be well tolerated in the mouse (28).

Intracerebroventricular (ICV) administration of ASOs. At postnatal day 2, mice received 2 ul of ASO in PBS injected manually into the cerebral ventricle (29). Adult mice received 2.5 ul of ASO in PBS by stereotaxic injection into the left ventricle using an automated injector pump (Stoetling) connected to a 5-µl Hamilton syringe (30). Coordinates for stereotaxic injection into the cerebral ventricle were M/L=1.0 mm, A/P = 0.3 mm, V/L=3.0 mm.

qRT-PCR. *Scn8a* transcripts in mouse brain and cultured neurons were quantified using TaqMan gene expression assays (10). Total *Scn8a* transcript was detected with the FAM-labelled assay Mm00488110_m1 using as endogenous control the Tata binding protein mRNA (VIC labelled Mm01277042_m1). Relative transcript quantity was calculated by the ΔΔCT method (31). Data represent the average result from two independent cDNA preparations for each animal.

Western blotting. Purified membrane protein was prepared from whole brain and analyzed by Western blotting essentially as described (33). The primary antibodies were rabbit anti-Nav1.6 (Millipore #5580, lot 3188705) diluted 1:500, and mouse anti-tubulin (Millipore # MAB 1637 lot 2080833) diluted 1:1,000.

RESULTS

The *Scn8a* ASO targets a sequence in the proximal 3' UTR of mouse *Scn8a* (Figure 1A). Addition of ASO to the culture medium of wildtype primary mouse neurons generates a dose-dependent reduction in transcript abundance (Figure 1B). Wildtype mice of strain C57BL/6J were treated on postnatal day 2 (P2) by intracerebroventricular (ICV) injection of ASO, and the abundance of *Scn8a* transcript in brain RNA was measured at P21. Control ASO did not reduce the *Scn8a* transcript (Figure 1C), while a dose-dependent reduction of *Scn8a* mRNA was obtained in mice treated with 15 to 45 μg of *Scn8a* ASO (Figure 1C). Spinal cord Scn8a transcript was also reduced to 0.6 ± 0.1 relative to wildtype (n=5). Treatment with 45 μg of ASO did not interfere with weight gain in the postnatal period, with no evidence of muscle wasting; at P21, body weight was 9.7 ± 1.4 g in untreated WT mice (mean ± SD, n=21) and 10.6 ± 1.2 (n= 18) in mice treated with 45 ug of ASO at P2 (p=0.2, unpaired t-test).

The therapeutic effect of the ASO was evaluated in mice expressing a conditional allele of *Scn8a*-p.Arg1872Trp (10). Homozygous conditional mice were crossed with mice expressing the global EIIA-Cre transgene to activate the conditional allele. The resulting (untreated) *Scn8a*$^{cond/+}$,EIIA-Cre offspring exhibited sudden onset of convulsive seizures at P14-P16, followed

by death within 24 hours (10). Prior to seizure onset, these mice develop normally and do not exhibit epileptiform EEG discharges (10).

ASO was administered by ICV injection at P2 to litters containing 50% affected *Scn8a*$^{cond/+}$,EIIA-Cre mice and 50% unaffected *Scn8a*$^{cond/+}$ controls lacking Cre. Treatment of *Scn8a*$^{cond/+}$,EIIA-Cre mice with control ASO did not prevent seizure onset and death at P14-P16 (Figure 2A). Administration of 15 μg to 45 μg of *Scn8a* ASO resulted in a dose-dependent increase in survival up to 7 weeks of age (Figure 2A, B). To test the efficacy of repeated doses of ASO, mice were treated with 30 μg of ASO on P2 followed by a second dose of 100 μg on postnatal day 30, administered by stereotactic injection. The second treatment further increased median survival from 6 weeks for 30 μg ASO to approximately 9 weeks (Figure 2A), indicating that repeated ASO administration might be effective as a long-term treatment, as is the case for spinal muscular atrophy (16).

In mice treated with 45 μg ASO on P2, seizure onset begins at 6 weeks of age (Figure 2A). To determine the duration of the effect of ASO treatment on transcript level, and the relationship between transcript level and seizure onset, we measured *Scn8a* transcripts at three time points after treatment at P2 with 45 μg ASO (Figure 2D). Transcript levels in treated mice at each point were compared with age-matched untreated wildtype controls. The level of transcript at 3 weeks of age was 50% of control (Figure 2D, also shown in Figure 1C). At 5 weeks post treatment, *Scn8a* transcript level was increased to 80% of control, but no seizures were observed (Figure 2D). At the time of seizure onset, transcripts in the mice with seizures had reached 100% of the level of untreated controls. Thus the reduction of *Scn8a* transcripts by ASO

treatment persists for approximately 6 weeks and is correlated with seizure protection. The reduction of Nav1.6 protein at 3 weeks post treatment is demonstrated in Figure 2E.

To investigate the possibility that treated mice experience sub-threshold electrographic seizures during the period before seizure onset, mice were injected with 45 μg of ASO at P2 and monitored by EEG recording continuously for eight days, from P31 to P39. Five of the six treated mice exhibited normal EEGs throughout this period, which ended near the age of seizure onset (Figure 3). One treated mouse developed a convulsive seizure at the age of 37 days, followed within 30 seconds by death. The EEG recording of this seizure (Figure 3) is similar to that previously reported for untreated mutant mice at P14-16 (10). Thus treatment with *SCN8A* ASO delayed seizure onset but did not prevent the very rapid progression from initial seizure to death that is characteristic of mice expressing the R1872W mutation.

Since *SCN8A* is an essential gene (17), it was important to evaluate potential adverse effects of ASO treatment. From previous work in the mouse we know that reduction of brain transcripts below 5% of the wildtype level is lethal (17,18) and reduction to 10% of wildtype level results in dystonia, muscle wasting and reduced body weight (17-19). In contrast, reduction to 50% of wildtype level is well tolerated in the mouse, with only mild behavioral abnormalities (20). Similarly, many haploinsufficient patients with 50% of wildtype expression have mild intellectual disability (21, 22). These earlier observations predicted that the ASO treatment that prevented seizures, with approximately 50% of wildtype expression remaining in brain (Figure 1), would have only small effects on movement.

To detect potential impairment of motor function, we compared ASO-treated mutant mice with untreated wildtype littermates. We examined motor activity during the period of extended survival after ASO treatment. In the open field test, mice treated with 45 μg of ASO exhibited normal activity at 6 weeks of age. Total distance travelled, % time spent in the center of the open field, and number of rearing events did not differ from wildtype controls (Figure 4A).

In the wheel running test, the ASO treated mutant mice were slower than the wildtype mice (Figure 4B). There was no difference between the two groups in the time spent running, but the distance covered was reduced by approximately one third, due to a slower average speed of 1.2 km/h for treated mutant mice compared with 2.0 km/h for wildtype controls. Cerebellar function was assessed by analysis of ledge-walking, hind limb clasping, gait and kyphosis (23). The only deficit observed was impaired ledge-walking in 3 of the 5 treated mutant mice (Figure 4C-F, Figure 2C). The normal posture and home cage activity of ASO treated mice is demonstrated in Figure 2C and Supplemental Videos 1-3. Thus reduction of *Scn8a* transcript to a level sufficient for delay of seizure onset results in only minor effects on movement. Overall, the ASO treated mice remained alert and active until the onset of seizures.

Since Nav1.6 is a major determinant of the firing properties of excitatory neurons, reduction of *Scn8a* expression could have a general ameliorating effect on seizure disorders of various causes (24). To test this possibility, we examined the effect of the *Scn8a* ASO in a mouse model of Dravet Syndrome (DS), a DEE caused by loss-of-function mutations of *SCN1A*. Reduction of *Scn8a* by other methods was previously shown to be protective against acutely induced seizures in a Dravet mouse (25, 26). We used the Kearney mouse model of DS that is

haploinsufficient due to deletion of exon 1 of *Scn1a* (27). The untreated DS mice exhibited onset of spontaneous seizures by 4 weeks of age and 50% penetrance of seizures and lethality (Figure 6A), consistent with previous reports (27). In contrast, DS mice treated with a single dose of 45 μg of *Scn8a* ASO on P2 survived beyond 5 months of age without behavioral seizures (Figure 5A). The *Scn8a* ASO resulted in 50% reduction of *Scn8a* transcript in brain and spinal cord of DS mice with no effect on the *Scn1a* transcript (Figure 5B). Five consecutive days of EEG recordings of ASO treated DS mice recorded at 5 months of age did not detect any sub-threshold electrographic abnormalities or seizures (Figure 5C). This extended period of protection after a single treatment suggests that ASO administration during a critical period of postnatal development might give long term seizure control in Dravet patients. A transient developmental window of interneuron dysfunction has been described in Dravet mice (32). Expansion of future testing of the *Scn8a* ASO to other seizure models will be of great interest.

DISCUSSION

The experiments described here provide the first preclinical evidence that therapeutic reduction of *Scn8a* transcript could delay the onset of spontaneous convulsive seizures in patients with *SCN8A* encephalopathy. ASO treatment did not result in major adverse effects, suggesting that it may be well tolerated in patients with *SCN8A* encephalopathy or Dravet Syndrome. Further investigations will be required to determine whether initiation of ASO treatment *after* seizure onset is effective. In these animal models, death follows within minutes to days of the first seizure; the resulting inability to evaluate post-onset treatment is a limitation

of the present study. Clinical application will also require definition of a therapeutic window in human to prevent the deleterious effects of more extensive reduction of *SCN8A* expression. Our observations on the Dravet Syndrome mouse support the exciting possibility that treatment with *SCN8A* ASO could be protective in Dravet Syndrome and in other seizure disorders with a variety of underlying etiology.

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Potential Conflicts of Interest: P.J.-N. and F.R. are employed by Ionis Pharmaceuticals, a for-profit company that develops ASO therapies.

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FIGURE LEGENDS

Figure 1. Scn8a ASO treatment reduces the abundance of Scn8a transcript in wildtype neurons and brain. (A) Cartoon of the 3' end of the Scn8a gene. The last two exons are represented by boxes separated by a line representing the last intron. Coding sequences within the exons are shaded. The position of the stop codon that terminates translation is marked as 'stop'. The sequence of the 20 bp Scn8a ASO is shown. The ASO sequence is identical to the genomic sequence of the 3'UTR at a position approximately 500 bp downstream from the translation stop codon within the last exon of the gene. (**B)** Primary cortical neurons from E14 embryos of strain C57BL/6J were cultured for 3 days with the indicated concentration of *Scn8a* ASO. (**C***)* The indicated dose of ASO was administered to C57BL/6J mice on postnatal day 2 and the abundance of *Scn8a* transcript in brain RNA was measured at P21. Five or six animals were treated with each dose; each symbol represents data from one animal.

Figure 2. *Scn8a* ASO delays seizure onset and prolongs survival of mutant mice expressing the pathogenic mutation *SCN8A*-R1872W. (A) *Scn8a*$^{cond/+}$, EIIA-CRE mice were treated on postnatal day 2 by ICV injection with the indicated mount of control or *Scn8a* ASO. A second dose of 100 µg of *Scn8a* ASO at P35 further extended survival ($p<0.001$) (dotted line). **(B**) Dose-dependence of mean survival; values are mean +/- SEM. **(C)** *Scn8a*$^{cond/+}$, EIIA-CRE mice treated with 45 ug exhibit normal posture without hind limb clasping. **(D)** Duration of the effect of *Scn8a* ASO on transcript level. Brain RNA was prepared from *Scn8a*$^{cond/+}$, EIIA-CRE mice treated

with 45 μg of ASO and untreated age-matched wildtype mice and analyzed by qRT-PCR. **(E)** Reduction of $Na_v1.6$ protein in brain at 3 weeks of age in mice treated with 45 μg of ASO as in (D).

Figure 3. ASO treatment protects against electrographic seizures. Mutant mice of genotype *Scn8a*$^{cond/+}$*,EIIA Cre* were treated with 45 μg *Scn8a* ASO by ICV at P2 and monitored from the age of P31 to P39 by 24 hour video EEG recording. Traces recorded from right and left cortical electrodes of six mice are shown. Five mice were completely protected from electrographic and behavioral seizures. Mouse #2 exhibited a single, fatal, generalized tonic-clonic seizure at P37 that closely resembled the EEG profile in untreated mutants at P15 (10). (P37 corresponds to the age of the earliest seizure in the cohort of 10 mice treated with 45 ug of ASO in Figure 2.)

Figure 4. Motor activity of ASO treated mice. *Scn8a*$^{cond/+}$*,EIIA Cre* mice were treated with 45 μg *Scn8a* ASO at P2. A) Activity in an open field was monitored for 30 min at 45 days of age. Treated mutant mice did not differ significantly from wildtype mice in average running speed (Student's t test, p=0.12) or percent of time spent in the center of the open field (Two-way Anova, p=0.83). Rearing behavior of mutant and wildtype mice was also comparable.
B) Wheel running activity was monitored 24 hours/day during the 4 day interval between P31 and P39, after habituation to the running cage for 10 days. The distance travelled by the treated mutant mice during the 96 hours of monitoring was significantly smaller than for wildtype mice. There was no significant difference in the time spent running, but the average speed was lower for the mutant mice. C-F) Cerebellar function was assessed at 5 weeks of age by analysis of

ledge-walking, hind limb clasping, gait and kyphosis as described (23). The only deficit observed was impaired ledge-walking in 3 of the 5 treated mutant mice. Each symbol represents one animal and values are the mean of triplicate assays. WT, wildtype C57BL/6J mice untreated with ASO; Mutant, *Scn8a*$^{cond/+}$,*EIIA Cre* mice treated with 45 μg ASO;
p values are for the comparisons between WT and mutant mice (Student's t-test).

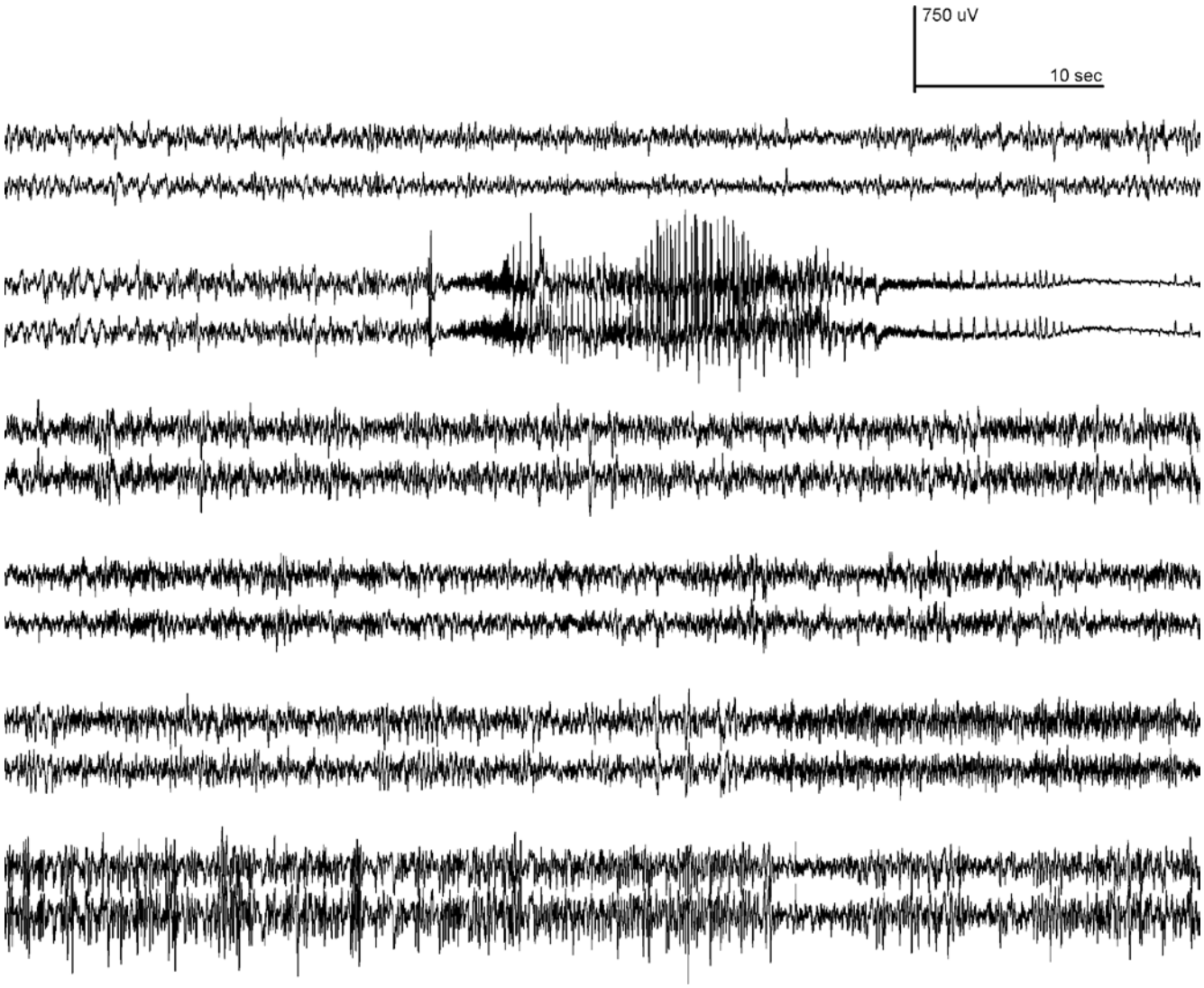
Figure 5. *Scn8a* ASO rescues survival of Dravet Syndrome mice. *Scn1a*$^{+/-}$ mice were treated on P2 with 45 μg *Scn8a* ASO. **(A)** For untreated *Scn1a*$^{+/-}$ mice, median survival was 26 days with 50% penetrance of the lethal phenotype, consistent with the original description of this Dravet model (27). In contrast, all of the ASO treated mice have survived beyond 5 months of age (p=0.004, Mantel-Cox Log-rank test). Tick marks represent living mice. **(B)** The *Scn8a* ASO reduces *Scn8a* transcript level with no effect on *Scn1a* transcript level. Brain RNA was prepared from untreated wild-type P21 (solid symbols) or mice treated with 45 ug *Scn8a* ASO (open symbols). **(C)** The *Scn8a* ASO protects against electrographic seizures in *Scn1a*$^{+/-}$ mice. Four ASO treated mice, 5 months of age, were monitored for 5 successive days with 24-hour EEG recording. No electrographic seizures or other EEG abnormalities were observed. Two traces for each animal, from left and right cortex.

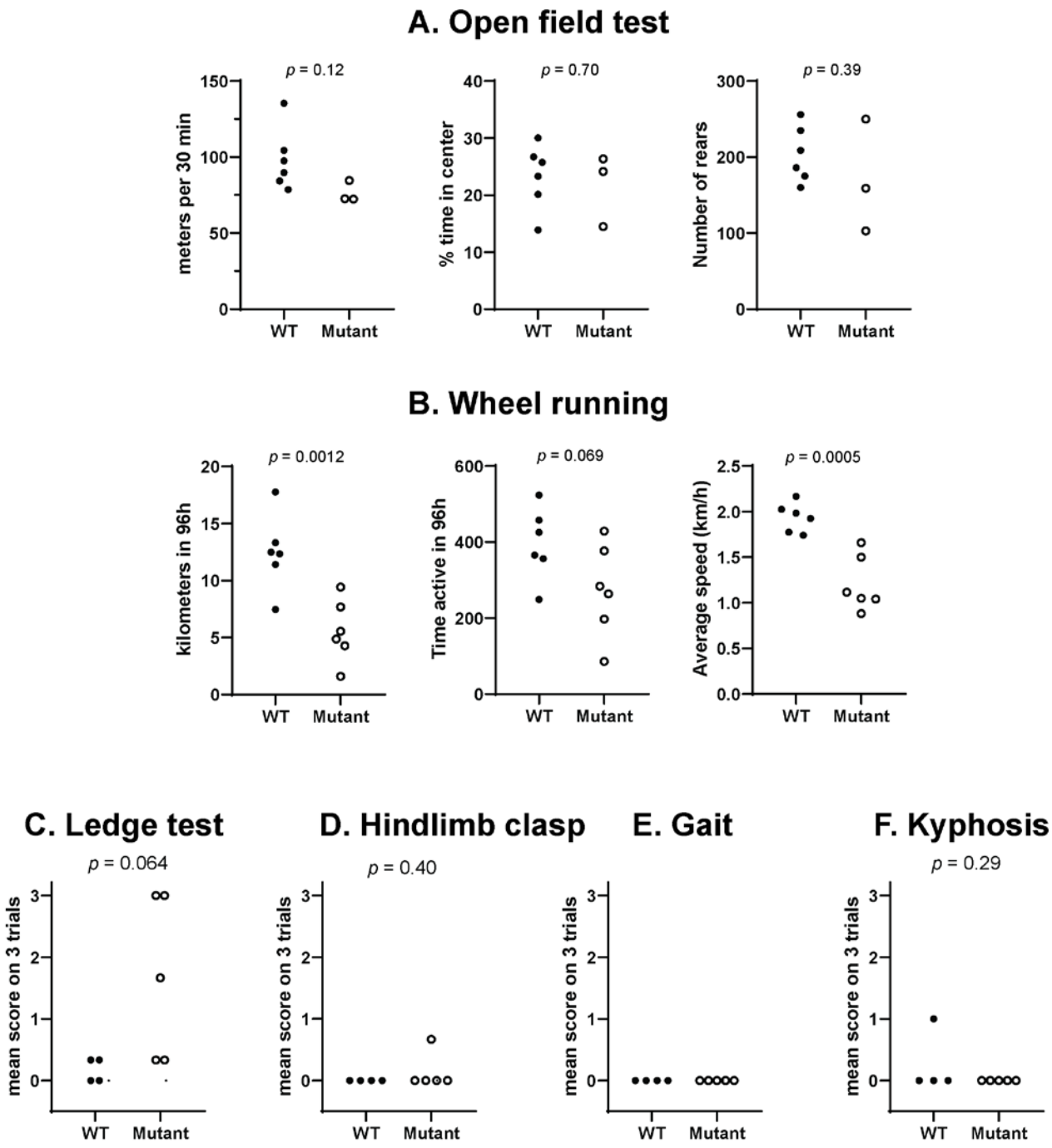
Supplemental videos. *Scn8a* $^{cond/+}$*, EIIA-Cre* mice were treated on P2 with 30 μg of *Scn8a* ASO and assessed at P21, one week beyond the survival without ASO.

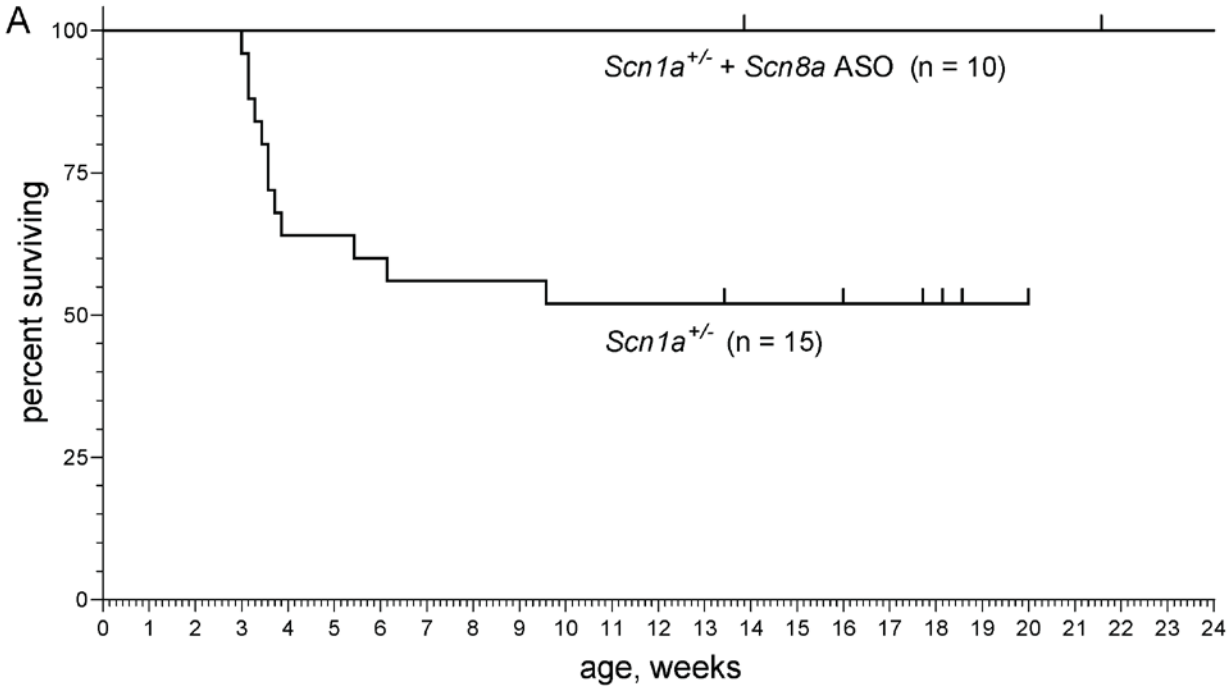
Video 1. Normal gait and movement, side view. 30 sec.

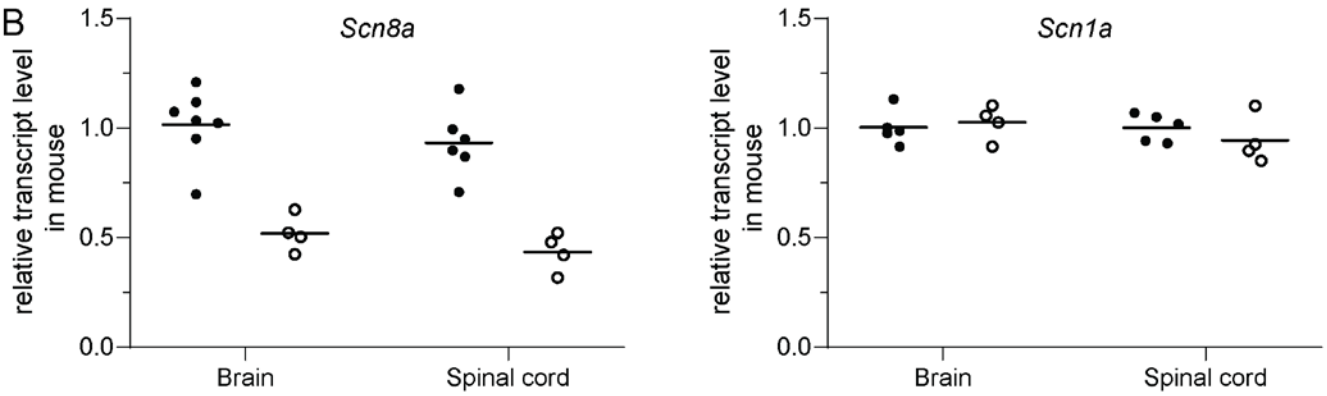
Video 2. Normal gait and activity, overhead view.

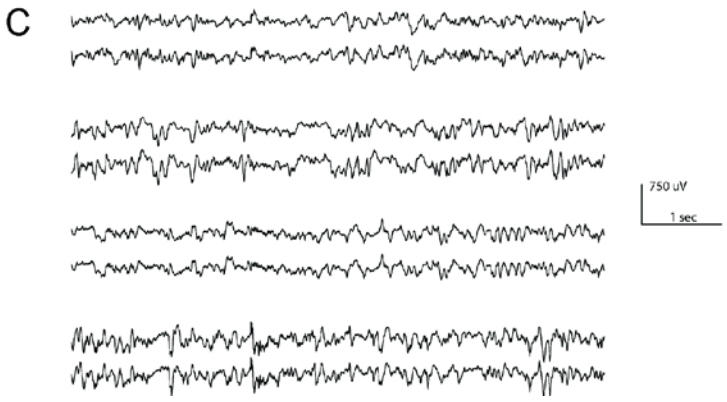
Video 3. Absence of hind-limb clasping in the treated mutant mice.











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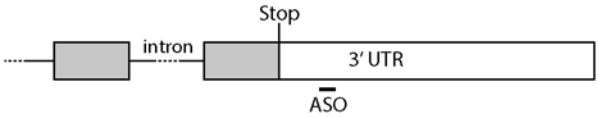

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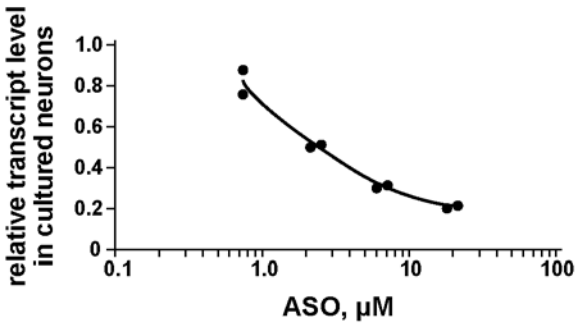

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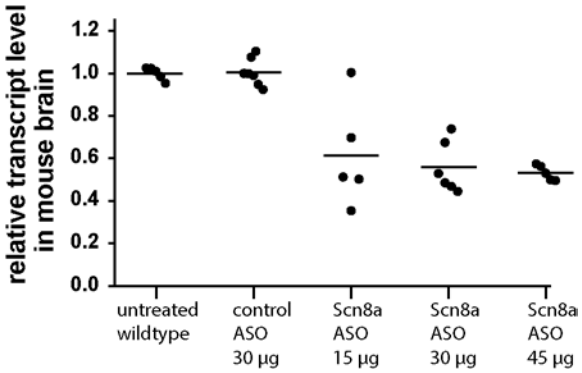

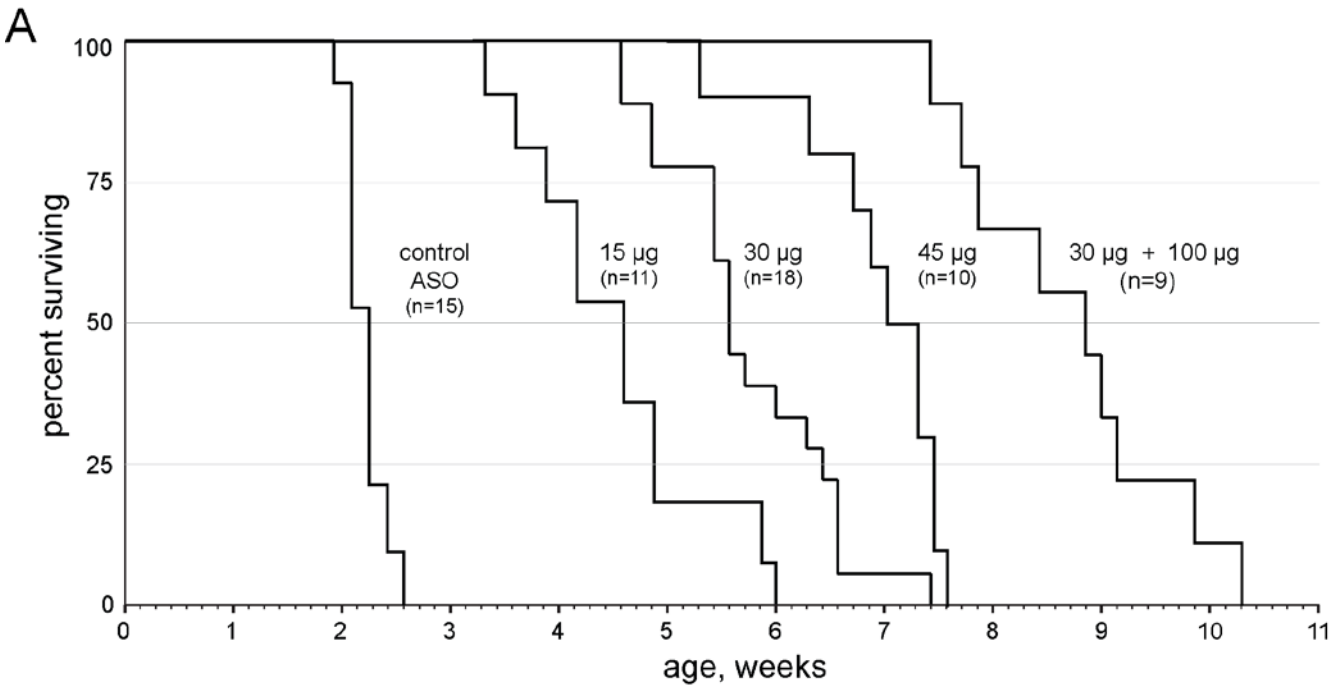

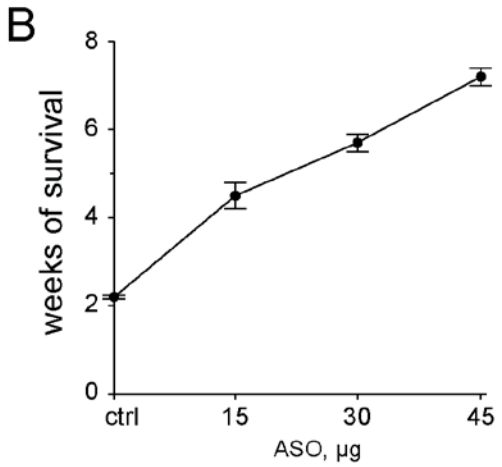

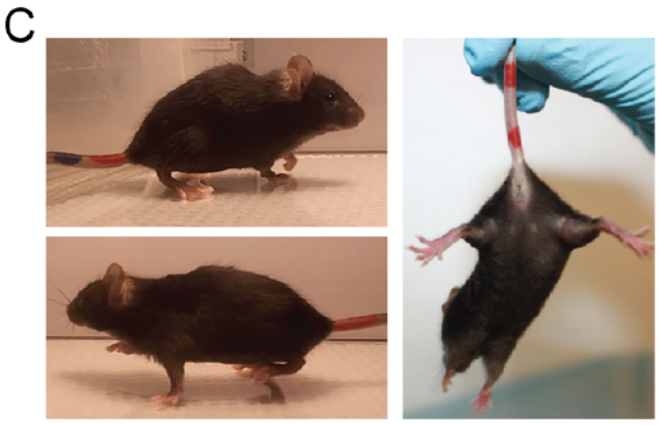

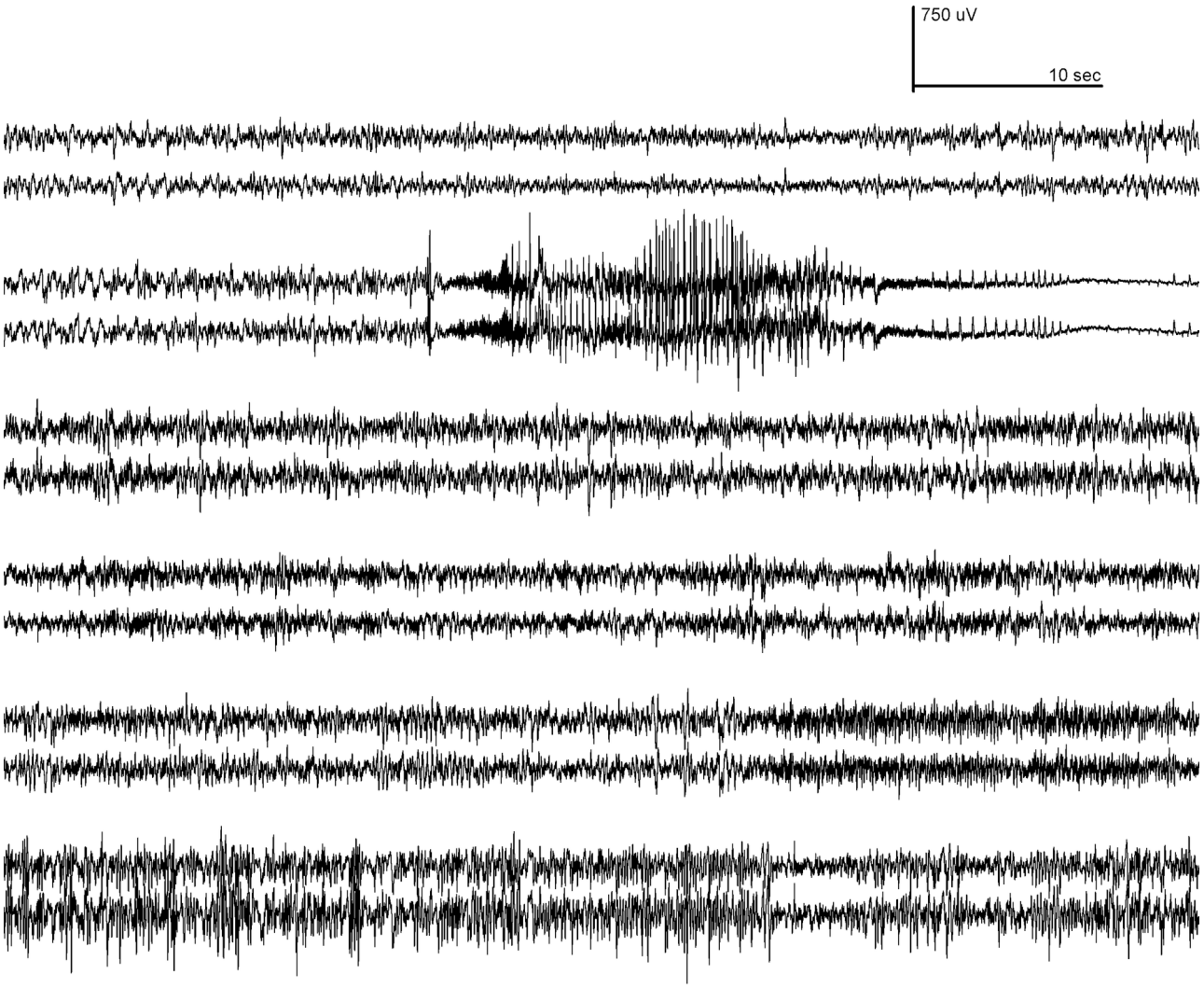

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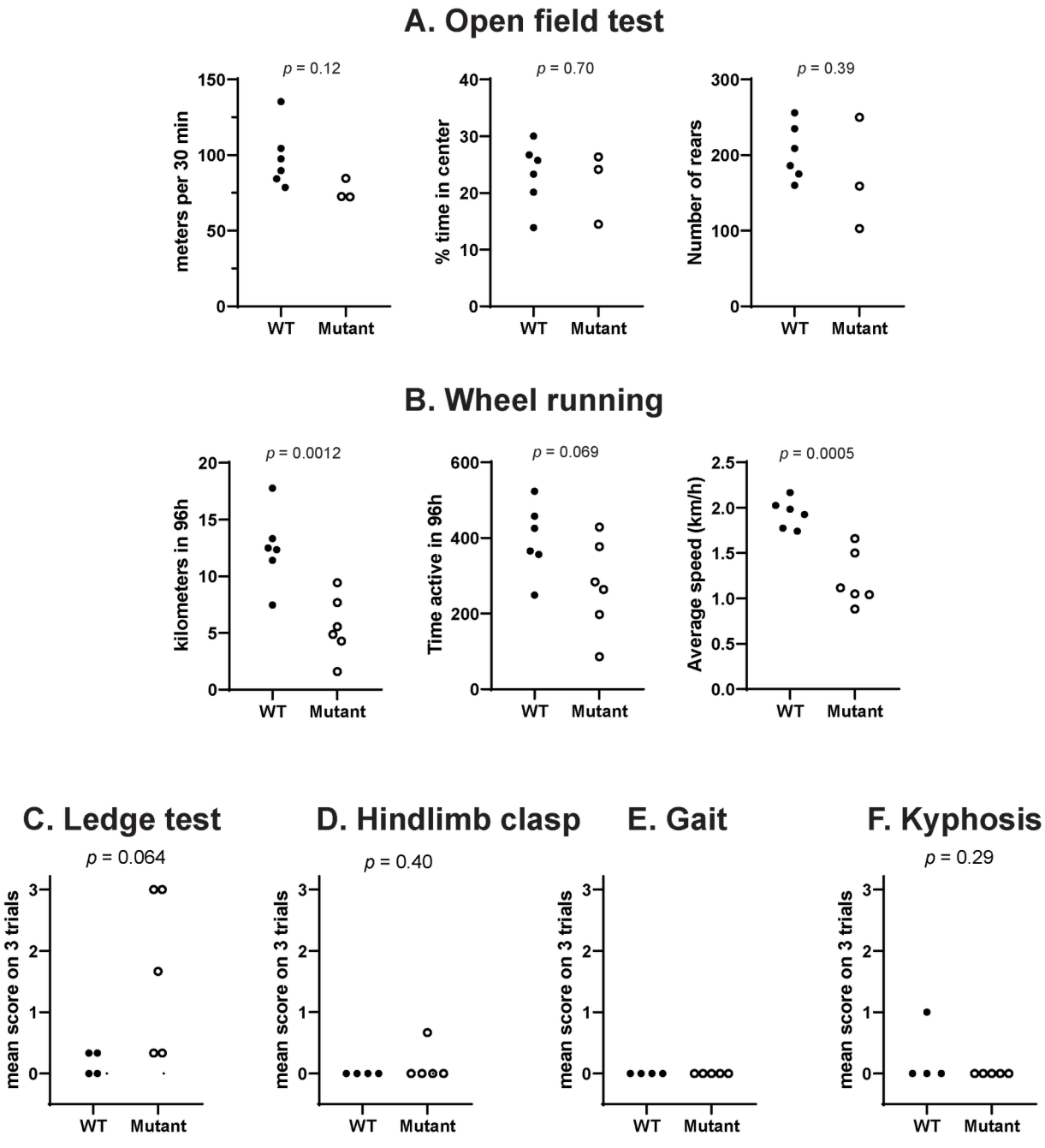

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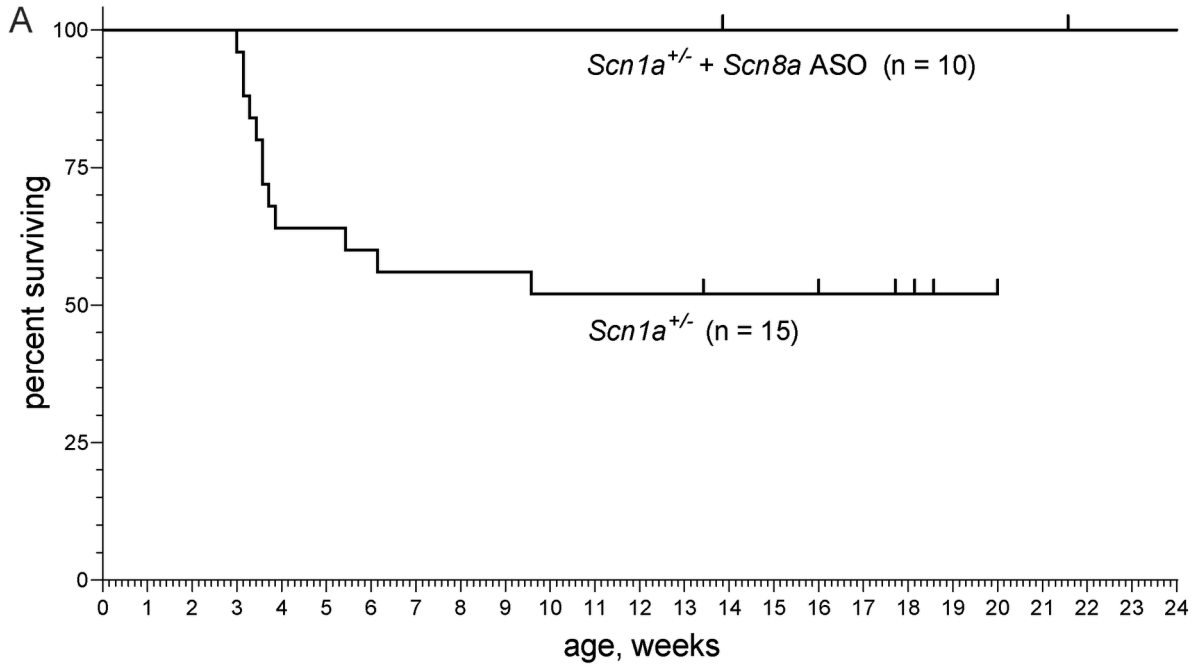

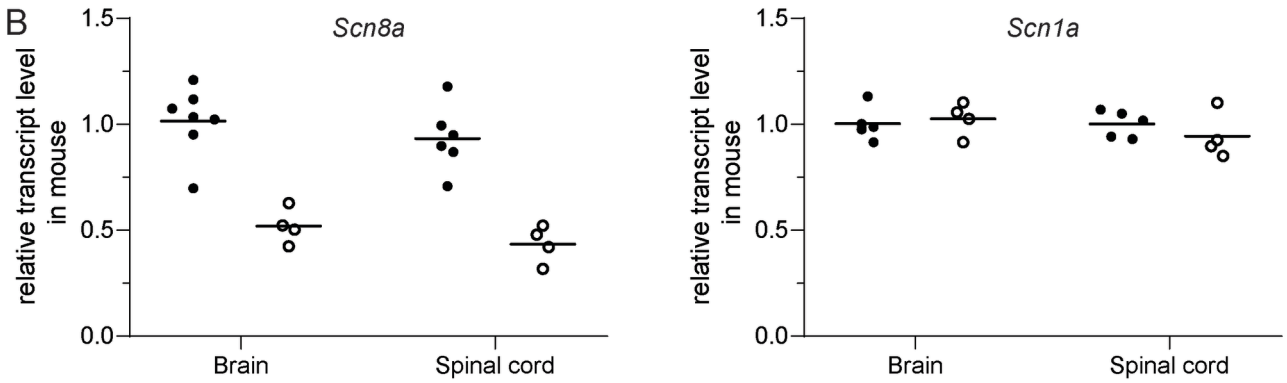

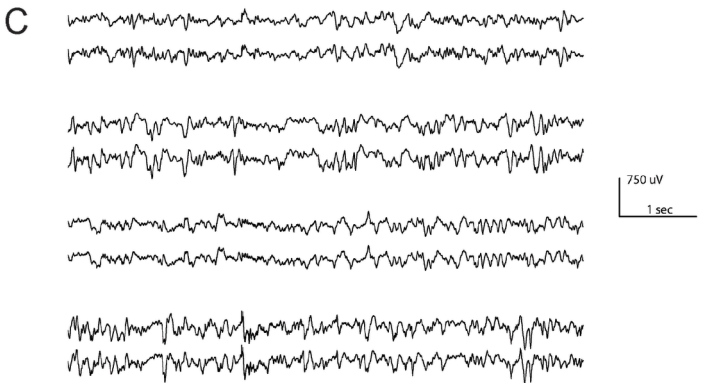

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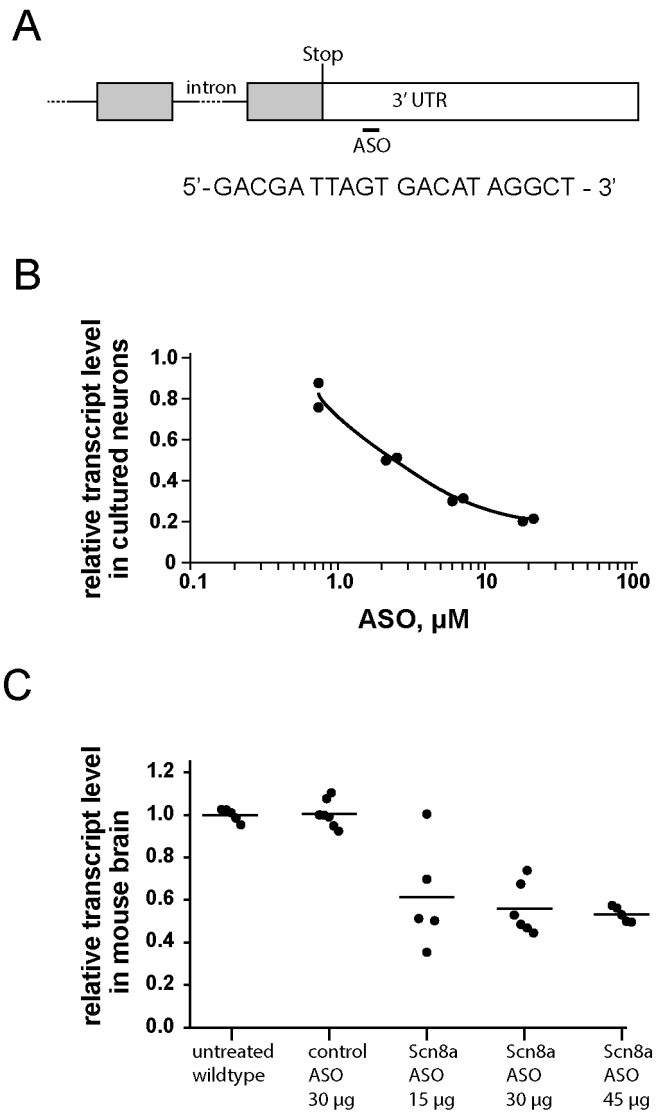

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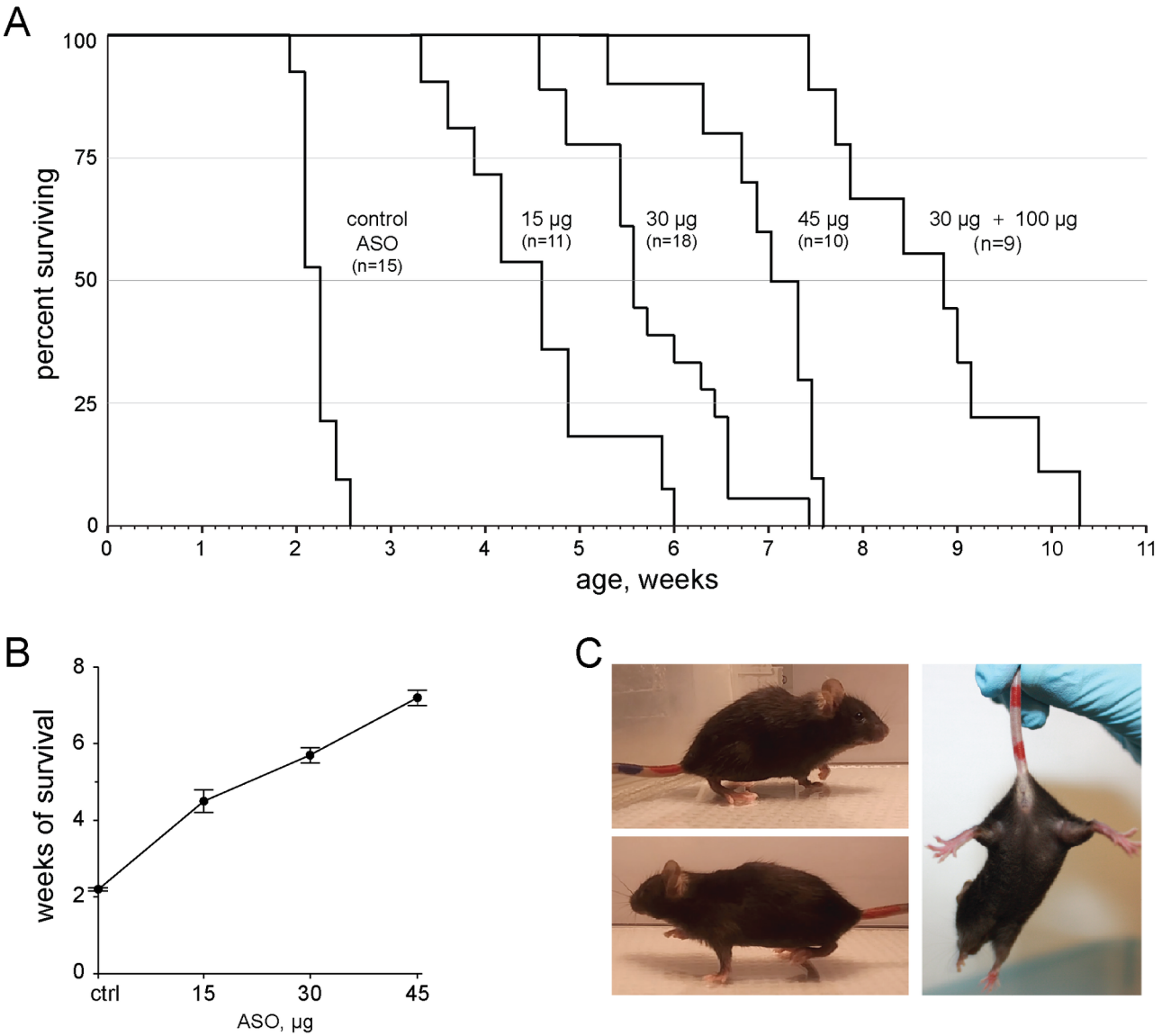

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