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ORIGINAL ARTICLE

Genes, Brain and Behavior

Generation and basic characterization of a gene-trap knockout mouse model of *Scn2a* with a substantial reduction of voltage-gated sodium channel $Na_v1.2$ expression

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Abstract

Large-scale genetic studies revealed *SCN2A* as one of the most frequently mutated genes in patients with neurodevelopmental disorders. *SCN2A* encodes for the voltage-gated sodium channel isoform 1.2 ($Na_v1.2$) expressed in the neurons of the central nervous system. Homozygous knockout (null) of *Scn2a* in mice is perinatal lethal, whereas heterozygous knockout of *Scn2a* (*Scn2a*$^{+/-}$) results in mild behavior abnormalities. The $Na_v1.2$ expression level in *Scn2a*$^{+/-}$ mice is reported to be around 50–60% of the wild-type (WT) level, which indicates that a close to 50% reduction of $Na_v1.2$ expression may not be sufficient to lead to major behavioral phenotypes in mice. To overcome this barrier, we characterized a novel mouse model of severe *Scn2a* deficiency using a targeted gene-trap knockout (gtKO) strategy. This approach produces viable homozygous mice (*Scn2a*$^{gtKO/gtKO}$) that can survive to adulthood, with about a quarter of $Na_v1.2$ expression compared to WT mice. Innate behaviors like nesting and mating were profoundly disrupted in *Scn2a*$^{gtKO/gtKO}$ mice. Notably, *Scn2a*$^{gtKO/gtKO}$ mice have a significantly decreased center duration compared to WT in the open field test, suggesting anxiety-like behaviors in a novel, open space. These mice also have decreased thermal and cold tolerance. Additionally, *Scn2a*$^{gtKO/gtKO}$ mice have increased fix-pattern exploration in the novel object exploration test and a slight increase in grooming, indicating a detectable level of repetitive behaviors. They bury little to no marbles and have decreased interaction with novel objects. These *Scn2a* gene-trap knockout mice thus provide a unique model to study pathophysiology associated with severe *Scn2a* deficiency.

KEYWORDS
behavior, channelopathy, genetic variants, gene-trap knockout, mouse model, Nav1.2, nesting, neurodevelopmental disorder, open field, SCN2A gene, voltage-gated sodium channel

1 | INTRODUCTION

SCN2A encodes for the type II alpha subunit of the voltage-gated sodium channel isoform ($Na_v1.2$), which is a major type of sodium channel expressed in the neurons of the central nervous system.[1,2] The $Na_v1.2$ channel is known to be responsible for the initiation, propagation, and backpropagation of action potentials.[3-5] During development (the first through second year in humans or the first through third week in mice), $Na_v1.2$ is the main sodium channel isoform expressed in the axon of neurons, thereby making it the dominant player in the initiation and propagation of action potentials in the central nervous system.[6]

Genetic variants in *SCN2A* are strongly associated with neurodevelopmental disorders such as autism spectrum disorder (ASD) and intellectual disability. Indeed, recent large-scale human genetic studies have indicated that *SCN2A* is one of the leading genes associated with ASD.[7,8] A majority of *SCN2A* variants identified from ASD cases are protein-truncating variants or have been experimentally verified to result in loss-of-function (*SCN2A* deficiency).[9,10] *SCN2A* variants are also found in patients with epileptic encephalopathy, schizophrenia, or other diseases with comorbidities including sleep irregularities, gastrointestinal disturbances, and motor discoordination.[7,8,11-16] An estimated 400 cases of *SCN2A*-related disorders are born each year in the United States[15]; unfortunately, there is no curable treatment available for *SCN2A*-related disorders. The development of effective therapeutic interventions for people with *SCN2A* deficiency will rely on a deeper understanding of cellular, circuital, and behavioral consequences of *SCN2A* deficiency in disease models.

To study disorders associated with *SCN2A* deficiency, *Scn2a* knockout mouse models were generated.[17,18] Canonical knock-out (null, 100% knockout) of *Scn2a* in C57BL/6 mice is perinatal lethal,[17] therefore existing works focus on heterozygous *Scn2a* knockout mice ($Scn2a^{+/-}$) as the main model for study. However, $Scn2a^{+/-}$ mice display mild behavioral abnormalities, showing minimal differences from wild-type (WT) mice in body weight, olfactory sensitivity, auditory startle, thermal sensitivity, nesting, and marble burying.[5,18-20] $Na_v1.2$ protein expression level in $Scn2a^{+/-}$ mice is reported to be around 50–60% of the WT level,[21] which indicates that a $\sim$ 50% protein level reduction of $Na_v1.2$ is not likely to be sufficient enough to lead to major behavioral phenotypes in mice in the C57BL/6 strain. Notably, nonsense or loss-of-function mutations of *SCN2A* in one allele can produce disease phenotypes in humans. This discrepancy may imply that mice are likely more tolerant of *Scn2a* deficiency. We reasoned that if we could substantially reduce the *Scn2a* expression level without eliminating it completely, the residual $Na_v1.2$ may allow the mice to survive. Meanwhile, the substantial reduction of *Scn2a* could render severe phenotypes, providing a robust model for the study of *Scn2a* deficiency. To this end, we have characterized a novel *Scn2a* deficient mouse model using a gene-trap knockout (gtKO) approach.

The gene-trap knockout is achieved by inserting a "trapping cassette" into an intron of the gene of interest,[22,23] thus trapping the upstream exons by splicing the cassette and truncating the transcripts (Figure 1(A)). The trapping cassette contains a LacZ reporter flanked by a splice acceptor and polyadenylation signals, as well as site-specific recombination sites for further genetic modification of the allele. Mouse strains with a trapping cassette are designated as tm1a, knockout-first, or gene-trap knockout (gtKO). Studies have found that for certain "gtKO" mouse models, a residual amount of wild-type mRNA and protein can still be expressed via splicing around the trapping cassette (Figure 1(A)).[22,24] This feature of the gtKO strategy allows for the generation of viable homozygous mice that otherwise cannot be obtained via a classic knockout (null) approach.[22,24] Indeed, using this gtKO strategy, we generated viable homozygous $Scn2a^{gtKO/gtKO}$ mice that can survive to adulthood. We confirmed a significant reduction in *Scn2a* expression and examined the health, sensory, and basic phenotypes of this gtKO mouse model. Our data demonstrates that $Scn2a^{gtKO/gtKO}$ mice could be a valid new mouse model for in-depth studies of disorders associated with *SCN2A* deficiency.

2 | MATERIAL AND METHODS

2.1 | Animals

All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. C57BL/6N-$Scn2a1^{tm1aNarl}$/Narl (here we refer to as $Scn2a^{WT/gtKO}$) mice were generated from the National Laboratory Animal Center, National Applied Research Laboratories (NLAC, Narlabs), a member of International Mouse Phenotyping Consortium (IMPC), based on a modified gene-trap design.[22] A detailed sequence of the trapping cassette is available at the following link (https://www.mousephenotype.org/imits/targ_rep/alleles/44588/escell-clone-genbank-file). The targeting construct (tm1a trapping cassette) was electroporated into C57BL/6N embryonic stem (ES) cells. Targeted ES cells were microinjected into recipient blastocysts. Details of targeted ES cells are available at the following link (https://www.mousephenotype.org/imits/targ_rep/targeted_alleles/44588). The resulting chimeric mice were then crossed with C57BL/6N mice to obtain founders. Therefore, the founder mice represent a pure C57BL/6N background. We backcrossed them to C57BL/6N mice for six generations to segregate away any unintended genetic alterations that may have arisen in the targeted ES cells. Since our founders were produced and maintained in a pure C57BL/6N genetic background, the "flanking allele problem" does not apply.[25,26]

Heterozygous ($Scn2a^{WT/gtKO}$) mice were used as breeding pairs to generate mice for experiments. Pups were weaned 21–28 days after birth. To increase the survival of smaller mice by reducing diarrhea and improving hydration after weaning, a high-calorie diet was recommended by our veterinary staff. To treat all mice the same, mice with all genotypes were given a nutritionally fortified dietary gel supplement (DietGel 76A; ClearH_2O, Portland, ME) from 21–35 days old. Mice were same-sex housed in mixed-genotype groups (3–5 mice per cage) on vented cage racks with 1/8" Bed-o-cobb bedding (Anderson, Maumee, OH) and > 8 g

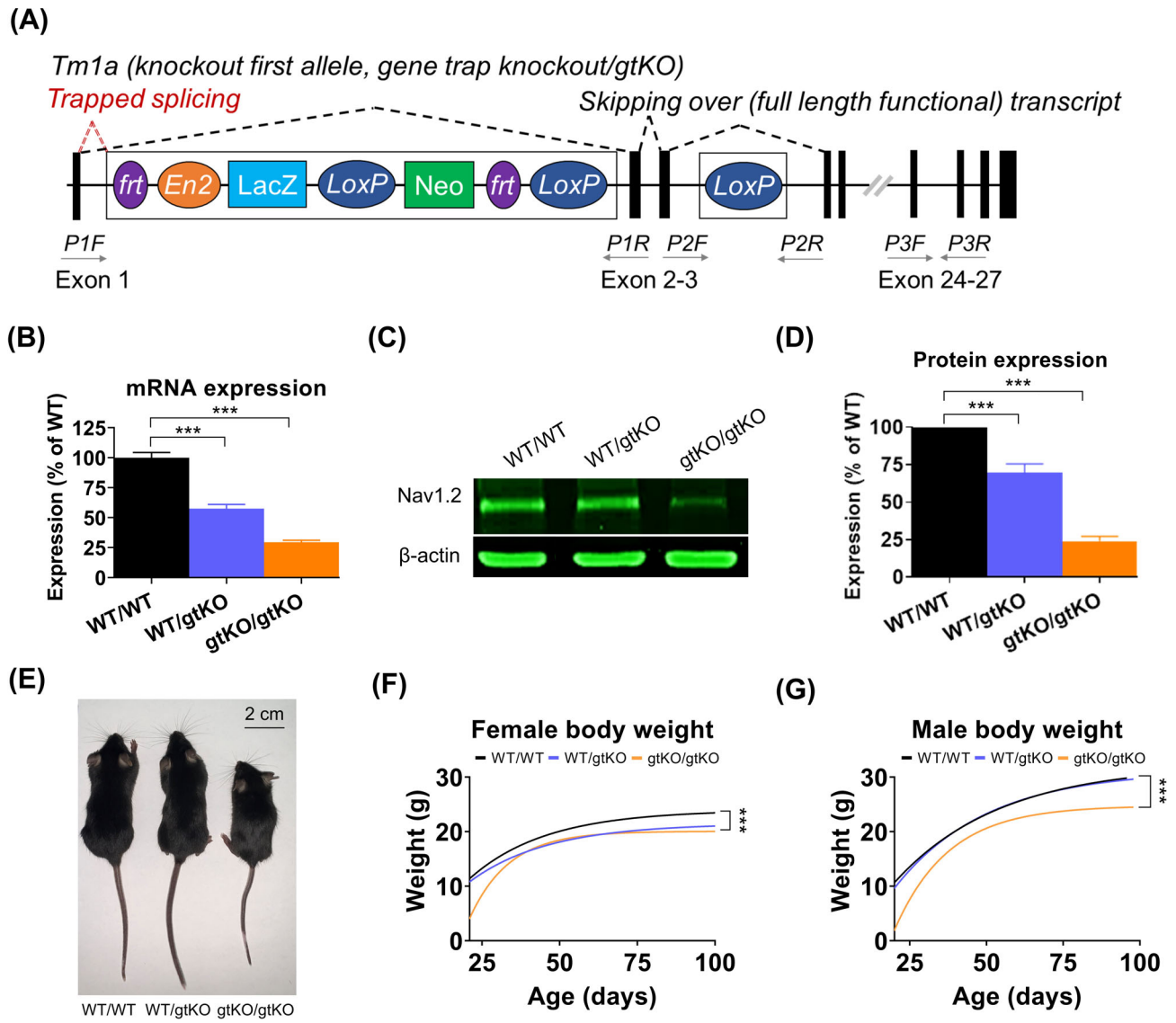

FIGURE 1 *Substantial reduction of Nav1.2 expression in Scn2a$^{gtKO/gtKO}$mice.* (A) Diagram of the tm1a gene trap cassette between Exon 1 and Exon 2 of *Scn2a* gene. Abbreviations: Frt, Flp recognition target (purple); En2, engrailed-2 splice acceptor (orange); LacZ, lacZ β-galactosidase (light blue); LoxP, locus of X-over P1 (dark blue); and Neo, neomycin (green). P1F and P1R: Primer set to check "skipping over" transcripts; P2F and P2R: Genotyping primer set; P3F and P3R: qPCR primer set. (B) Expression levels of *Scn2a* are reduced in heterozygous and homozygous *Scn2a* gene trap knock-out (gtKO) mice normalized to WT littermates (n = 3 mice for each genotype). (C) Representative Western Blot of Nav1.2 with β-Actin control. (D) Reduced expression levels of Nav1.2 in heterozygous and homozygous *Scn2a* gtKO mice normalized to WT littermates (n = 6 mice for each genotype). (E) Representative image of 40-days-old male littermates (left, WT; middle, *Scn2a$^{WT/gtKO}$*; right, *Scn2a$^{gtKO/gtKO}$*). (F) Female body weight with a one-phase decay least squares line of best fit with rate constants of 0.0405, 0.0396, and 0.07892 with R^2 of 0.7971, 0.7432, and 0.8815 for WT, *Scn2a$^{WT/gtKO}$*, and *Scn2a$^{gtKO/gtKO}$* mice, respectively (n = 14, 18, and 17 for WT, *Scn2a$^{WT/gtKO}$*, *Scn2a$^{gtKO/gtKO}$*). (G) Male body weight with a one-phase decay least squares line of best fit with rate constants of 0.0290, 0.0333, and 0.0569 with R^2 of 0.9181, 0.9032, and 0.9228 for WT, *Scn2a$^{WT/gtKO}$*, and *Scn2a$^{gtKO/gtKO}$*, respectively (n = 9, 14, and 13 for WT, *Scn2a$^{WT/gtKO}$*, *Scn2a$^{gtKO/gtKO}$*). Data are presented as mean value with SEM error bars. For molecular biology, significance was calculated from one-way ANOVA with post-hoc Bonferroni corrections with p<0.001 (***) for mRNA and protein. For weight, significance was calculated based on weight at 100 days from one-way ANOVA with post-hoc Bonferroni corrections with p<0.001 (***)

of nesting material as enrichment (shredded paper, crinkle-cut paper, and/or cotton nestlet) on a 12 hr light cycle. Food (2018S Teklad from Envigo) and reverse osmosis water was given ad lib.

All work was performed blinded from genotype at the Purdue Animal Behavioral Core during the dark cycle. Mice of both sexes (3–5 months old) were habituated to the behavior room 45–60 minutes before testing. A minimum of six mice of each sex and genotype were tested for behavior. A minimum of three mice of each genotype were tested for molecular analysis. To reduce the number of mice needed, multiple experiments were conducted on the same animal based on least to most stressful with one-week intervals between tests. Both sexes were tested at the same time. We used seven (a-g) different cohorts of mice for different behavioral experiments: a, weight, SHIRPA, nesting, 10 min open field, and molecular analysis; b,

metabolism; c, von Frey, hot/cold static/dynamic plate (randomized order, consecutive days); d, marble, olfactory, and quinine; e, auditory startle; f, Y-maze and grooming; g, 30 min open field and novel object exploration. All testing equipment was cleaned with 70% isopropanol/water solution before and after each animal.

2.2 | Genotyping and molecular analysis

Mice were genotyped at weaning (21–28 days) via ear punch. Genotyping for the tm1a cassette was performed using gene-specific polymerase chain reaction (PCR) on DNA extracted from ear tissue using a tissue DNA extraction kit (Macherey-Nagel, Bethlehem, PA, USA) with primers (forward 5′ to 3′: GAGGCAAAGAATCTGTACT-GTGGGG, reverse: GACGCCTGTGAATAAAACCAAGGAA). PCR product for the wild type allele is 240 base pairs (bp) and the tm1a allele is 340 bp. qPCR was performed on whole brains using an RNA extraction kit (Qiagen, Hilden, Germany) and a cDNA kit (ThermoFisher). Primers for qPCR were ATTTTCGGCTCATTC TTCACACT (forward, on Exon 24) and GGGCGAGGTATCGGTT TTTGT (reverse, on Exon 25). Western Blot was performed using an anti-*SCN2A* antibody (Catalog #ASC-002, Alomone, Jerusalem, Israel) and a β-actin control (Catalog #3700S, Cell Signaling Technology, Danvers, MA). For each sample, 40 μg of total protein (determined by Nanodrop, Thermo Scientific) were loaded to the gel. Immunoreactive bands were scanned by LiCor Odyssey and quantitatively analyzed by densitometry with Image Studio Lite 5.2 (https://www.licor.com/bio/image-studio-lite/d5). Each sample was normalized to its β-actin value, then normalized with the corresponding WT littermate. The primers to detect "skip-over" expression were CGCCTGTCTTGGCTTTTTC (forward) and ACGCATTGCAGAAGAGAAAGC (reverse). Data from mice of both sexes were included in the molecular analysis.

2.3 | Metabolism

Mice were singly-housed in a metabolism monitoring system (Techniplast, West Chester, PA, USA) in the same housing room. The system consists of a water bottle holder, side food hopper, a wire floor to allow fecal matter and crumbs to fall through, and a urine funnel which lead to a different collection tube. Fecal boli were separated from the food crumbs. Standard food pellets (20 g) were provided with water ad lib. Plastic huts were used in lieu of nesting material. Measurements (weight of food, weight of crumbs, weight of total fecal matter, and number of individual fecal boli) were taken daily at the same time each day and averaged over 4 days for each animal as its final value.

2.4 | SmithKline Beecham/Harwell/Imperial College/Royal London Hospital Phenotype Assessment (SHIRPA)

A modified SHIRPA was conducted as a phenotypic screen of muscle and lower motor neuron, spinocerebellar, sensory, neuropsychiatric, and autonomic function.[27] Genotype-blind researchers gave a score (0–5) using criteria outlined in Table S1. Body position, spontaneous activity, and tail posture were assessed after 5 minutes of home cage observation. By holding by the base of the tail, mice were then removed from the cage. Trunk curl, limb grasping, and postural reflex were assessed. Next, mice were placed so that their front paws were gripping the end of the wire food hopper to measure grip strength. When mice were slowly lowered back in their cage, their extension was observed. Mice were placed on their backs to assess their righting reflex.

2.5 | Open field

Mice were placed in an open field box with dimensions 40 × 40 × 40 cm (Maze Engineers, Boston, MA). Two versions of the open field paradigm were performed on two different cohorts of mice with one for 10 minutes at 60 lux[19,28] and the other for 30 minutes at 250 lux.[29] Only one open field paradigm was performed on each mouse. The center was defined as a 20 × 20 cm square in the middle of the field. Distance traveled, center duration, immobile duration (freezing) were recorded by EthoVision XT (Noldus, Leesburg, VA). Self-grooming was hand-scored in the open field as well.

2.6 | Olfactory sensitivity

Olfactory sensitivity by analyzing cinnamon over water preference (sniffing time) was tested as described previously.[30] Mice were placed in a standard cage bottom with no bedding for 5 minutes. After habituation, filter paper covered with 1% cinnamon extract (McCormick & Company, Sparks Glencoe, MD) was placed at one end with a filter paper covered in water at the other end.

2.7 | Quinine avoidance

The quinine avoidance test was conducted using a previously defined protocol.[31] Mice were habituated to two 50 mL conical bottles with sippers on them for 10 days (both bottles containing water) in a two-grommet cage. After habituation, the bottles were replaced with one 0.6 mM quinine[32,33] in reverse osmosis (RO) water and one of just RO water on random sides alternating daily for four continuous days. Volume was recorded daily at the same time each day. A cage without a mouse was used as a control to measure the daily drip rate, which was subtracted from the total volume to obtain the total volume drank.

2.8 | Auditory startle hearing test

Auditory startle was conducted in SR-LAB startle response chambers (San Diego Instruments, San Diego, CA) based on previous studies.[34-36] Mice were habituated to the container and 70 dB background white noise for 5 min. Then eight blocks of five 40 msec stimuli

(75, 85, 95, 105, 115 dB) at 4 kHz were presented in a pseudo-random order with a random inter-stimulus interval between 10–30 second (mean 20 second), and a continuous background white noise of 70 dB throughout the experiment. The total session time was approximately 15 minute.

2.9 | Thermal response

For heat tolerance, mice were placed on a 50°C hot/cold plate system model PE34 (IITC Life Sciences, Woodland Hills, CA). Cold tolerance was at 7.5°C. Dynamic hot plate thermal sensitivity started at 30°C and increased to 60°C at a rate of 2°C/second. Dynamic cold plate cold sensitivity started at 30°C and decreased to 5°C at a rate of 2°C/second. Temperatures were determined based on previous studies[37-39] and Institutional Care and Use Committee guidelines. Time (second) from placing the mouse until nocifensive behavior was observed was recorded using a stopwatch of two genotype-blind observers. Nocifensive behavior was defined as flicking, biting, or licking of the arms, legs, or tail. Animals were removed at the first sign of nocifensive behavior or after 20 seconds if no signs were observed to prevent tissue damage.[40]

2.10 | von Frey mechanical sensitivity

A simplified up-down von Frey test was performed as previously described.[41] Mice were placed in a sectioned chamber with a mesh bottom for 5 minutes to habituate. Von Frey fibers (BioSeb Lab Instruments, Vitrolles Cedex, France), starting with 2.0 g up to 4.0 g, were pressed on the mouse's hind paw pad until fiber started to bend and held for 3 seconds. A response would be a withdrawal, flicking, or flinching. If we tested a fiber and there was no response from the mouse, then a thicker fiber would be tested. Conversely, if we tested a fiber and a response from the mouse was detected, then a thinner fiber would be used until they did not have a response. The average of the thicker fiber that they responded to and the thin fiber that the mouse did not respond to was recorded as the sensitivity for that mouse.

2.11 | Nesting behavior

Nesting behavior was assessed using a modified protocol based on literature.[42] Mice were singly housed in clean cages for 2 hours before the start of the dark cycle with bedding and ad lib food and water. One 5 by 5 cm square condensed cotton nestlet (Ancare, Bellmore, NY, USA) was added to the front-right corner of the cage 1 hour before the start of the dark cycle. Height and quality of the nest were recorded at hour 36 after the addition of the nestlet. Height was recorded as the average of the four corners of the nest. Nest quality score was the average score rated by three genotype-blind researchers based on a point scale (0–5) according to the following criteria as defined previously[42]: **0** = nesting material is untouched; **1** = nesting material is largely untouched (> 90% intact); **2** = nesting material is partially torn up (50–90% intact); **3** = nesting material is mostly shredded with no identifiable nest site (< 50% of the nesting material is intact but < 90% is within a quarter of the cage floor area); **4** = an identifiable, but flat nest (> 90% of the nesting material is torn up into a nest within a quarter of the cage floor area); **5** = a near-perfect or perfect nest (nest is crater or cocoon-shaped with defined walls higher than 50% of the mouse's circumference).

2.12 | Marble burying

The marble burying test was modified from a previous protocol.[43] 15 black glass marbles (1.5 cm diameter) arranged in a 3 by 5 array were placed in a clean home cage with 5 cm deep bedding. The number of marbles at minimum two-thirds buried in a 30-minute session was recorded by three blind investigators, then averaged.

2.13 | Novel object exploration

Mice were placed in an open field box with dimensions 40 × 40 × 40 cm (Maze Engineers, Boston, MA) for 10 minutes to habituate to the chamber. Then four novel objects (2 mL centrifuge tube, binder clip, lid, and button) were placed at each corner of the familiar chamber and video recorded for 5 minutes using EthoVision XT. Interaction time with the object was defined as the period in which the mice has its nose facing the object and within a 2 cm radius around the object. An alternation is defined as the mouse exploring all four objects consecutively which includes an overlapping pattern. For example, a pattern of A–C–B–C–A consists of two alternations.[44] Percentage of fixed-pattern alternations were calculated from the number of alternations divided by the total number of exploration events minus two (% alternation = number of alternations / [total number of exploration events −2] * 100) based on literature.[44] Fixed-pattern alternations can be used as a measure of high-order repetitive behavior because it involves the repetitive exploration.[44]

2.14 | Spontaneous and induced grooming

Spontaneous and induced grooming were assayed using published methods.[45-47] Mice were placed in a standard cage bottom with no bedding (to eliminate digging). They were video recorded for 5 min then misted with reverse osmosis water and recorded again for another 5 min. Grooming duration and number of bouts before and after spraying with water were hand-scored from recorded video.

2.15 | Y-maze spatial working memory test

The Y-maze is a two-phase spatial reference/working memory task[44] that uses a plastic apparatus with three arms of 5 cm wide × 35 cm

long with 20 cm tall walls placed 120° apart (Maze Engineers). Black shapes (dots, stripes, and squares) were positioned at the end of each arm to serve as cues. The training phase consists of blocking a random arm and placing the mouse at the end of an unblocked arm, facing the cue. After 5 min of exploration, each mouse was placed back in its home cage. Two hours later, the retention phase was conducted, during which the mouse was placed in the same spot, with all the arms open. The duration in each arm, number of arm entries, and distance were recorded using EthoVision XT. Preferential exploration of the novel arm (blocked arm in the training phase) was used as an indicator of spatial working memory as previously defined.[44]

2.16 | Statistical analysis

Behavior data was analyzed using a two-way analysis of variance (ANOVA) to identify any genotype, sex, or genotype x sex interaction effects, then analyzed using a post-hoc with Bonferroni corrections unless noted otherwise. A survival curve was calculated using survival proportions and compared using a log-rank Mantel-Cox test. Auditory startle and induced grooming data were analyzed using a two-way repeated-measures ANOVA. Weight was also analyzed using one-way ANOVA since there is an inherent sex difference. Molecular biology data was analyzed using a one-way ANOVA. GraphPad Prism 8.3.0 (GraphPad Software Inc, La Jolla, CA) was used for statistical analysis and figure generation. Results are presented as mean ± standard error of the mean (SEM). If there was a significant genotype x sex interaction or sex effect, then the data was separated by sex and genotype. If there was no significant genotype x sex interaction or sex effect, then data from both sexes were combined. Significance was determined when $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

3 | RESULTS

3.1 | Validation of gene-trap knockout of Scn2a

We characterized a *Scn2a*-deficient mouse model generated with a modified targeted gene trap (gtKO) strategy.[22] gtKO of *Scn2a* is achieved by inserting a gene-trap cassette into Intron 1 of the *Scn2a* gene, thus "trapping" the splicing of Exon 1 of *Scn2a* exons to produce a LacZ fusion transcript and truncating the wild-type transcript (Figure 1(A)). In theory, since no exon sequences are removed, residual wild-type mRNA and protein could be expressed via splicing around the trapping cassette. Using two primers (in Exon 1 and Exon 2 respectively) flanking the inserted trapping cassette, which captures the "skip-over" full-length transcripts, we sequenced the RT-PCR products. We identified transcripts containing correctly assembled Exon 1 and Exon 2 sequences, supporting the notion that full-length non-truncated *Scn2a* transcripts exist. We also identified transcripts containing Exon 1 spliced to LacZ sequences of the trapping cassette, further confirming that gene trapping is functioning, but does not eliminate wild-type transcripts entirely.

Although skipping-over occurred to produce full-length transcripts in gtKO strategy, the extent of wild-type transcript and protein may vary and must be determined empirically. To measure the residual amount of *Scn2a*, we assayed both mRNA and protein expression levels of *Scn2a* in each genotype. Both heterozygous ($Scn2a^{WT/gtKO}$) and homozygous ($Scn2a^{gtKO/gtKO}$) adult mice expressed significantly less levels of *Scn2a* mRNA ($F_{2,3} = 94.56$, $p = 0.002$; $Scn2a^{WT/gtKO}$ vs. WT $p = 0.0002$; $Scn2a^{gtKO/gtKO}$ vs. WT $p < 0.0001$). mRNA levels were approximately 57.5 ± 3.5% and 29.4 ± 1.7% of the value of WT control in $Scn2a^{WT/gtKO}$ and $Scn2a^{gtKO/gtKO}$ mice, respectively (Figure 1(B)). Similarly, the whole brain protein levels of $Na_v1.2$ were approximately 69.6 ± 5.9% and 24.0 ± 3.1% relative to the WT control in adult $Scn2a^{WT/gtKO}$ and $Scn2a^{gtKO/gtKO}$ mice ($F_{2,12} = 80.31$, $p < 0.0001$; $Scn2a^{WT/gtKO}$ vs. WT $p = 0.0008$; $Scn2a^{gtKO/gtKO}$ vs. WT $p < 0.0001$) (Figure 1(C) and (D)). Thus, we demonstrate that $Scn2a^{gtKO/gtKO}$ mice have substantially reduced expression of *Scn2a* mRNA and $Na_v1.2$ protein, supporting the use of this transgenic mouse strain as a model of severe *Scn2a* deficiency.

3.2 | Health and Survival

In our laboratory, we attempted multiple breeding pairs of $Scn2a^{gtKO/gtKO}$ mice without success. From observation, it appears that male $Scn2a^{gtKO/gtKO}$ mice do not display any mounting behavior. Therefore, it is unknown if $Scn2a^{gtKO/gtKO}$ mice can produce healthy offspring. To maintain our transgenic line, we mated heterozygous mice. According to Mendelian genetics, the theoretical ratio of a $Scn2a^{WT/gtKO}$ x $Scn2a^{WT/gtKO}$ breeding pair is 1:2:1 (WT: $Scn2a^{WT/gtKO}$: $Scn2a^{gtKO/gtKO}$). Our mice colony produced 839 pups across 100 litters with an average rate of 1:1.65:0.89 (WT: $Scn2a^{WT/gtKO}$: $Scn2a^{gtKO/gtKO}$) with no sex ratio differences. A soft, high-calorie diet was provided at weaning to reduce mortality of $Scn2a^{gtKO/gtKO}$ due to dehydration from diarrhea. The survival rate to 100 days for females was 100% for WT, 94.8% for $Scn2a^{WT/gtKO}$, and 81.1% for $Scn2a^{gtKO/gtKO}$ (Supplemental Figure S1A, $Scn2a^{gtKO/gtKO}$ vs. WT $p < 0.0001$, log-rank Mantel-Cox test for survival curve). For males, the survival rate to 100 was 100% for WT, 97.3% for $Scn2a^{WT/gtKO}$, and 75.8% for $Scn2a^{gtKO/gtKO}$ (Supplemental Figure S1B, $Scn2a^{gtKO/gtKO}$ vs. WT $p < 0.0001$, log-rank Mantel-Cox test for survival curve). $Scn2a^{gtKO/gtKO}$ mice died or were euthanized per IACUC guidelines and veterinary recommendations due to severe diarrhea (14/212), penile prolapse (9/212), rectal prolapse (2/212), hydrocephalus (5/212), or were considered moribund due to shallow breathing, slow heartbeat, and unable to stay upright from a gentle poke (9/212). Other cases of $Scn2a^{gtKO/gtKO}$ mortality were categorized into sudden unexpected death of unknown causes noted by animal care staff during their daily morning check. $Scn2a^{gtKO/gtKO}$ mice were significantly smaller than WT mice ($Scn2a^{gtKO/gtKO}$ vs. WT female $p < 0.0001$, male $p < 0.0001$) in both sexes (female $F_{2,34} = 12.76$, $p < 0.0001$; male $F_{2,19} = 78.88$, $p < 0.0001$) (Figure 1(E)-(G)). Interestingly, female, but not male, $Scn2a^{WT/gtKO}$ weighed less than wild type (female $Scn2a^{WT/gtKO}$ vs. WT $p = 0.0103$).

To test if the decreased survivability and reduced weight of $Scn2a^{gtKO/gtKO}$ mice were due to food/water consumption or

excessive excretion, we monitored the animal's food and water intake and fecal production. Data was normalized based on the individual's weight and is shown in Table S2. The water intake was greater in *Scn2a*$^{WT/gtKO}$ and *Scn2a*$^{gtKO/gtKO}$ mice compared to WT mice (genotype $F_{2,55} = 4.02$, $p = 0.0236$; sex $F_{1,55} = 16.79$, $p = 0.0001$) with females having increased water intake overall. Food intake was greater in *Scn2a*$^{gtKO/gtKO}$ mice compared to WT mice (genotype $F_{2,55} = 6.60$, $p = 0.0027$; sex $F_{1,55} = 11.02$, $p = 0.0016$) with females having increased food intake overall. Weight of all the fecal matter (fecal mass) was similar among all genotypes but *Scn2a*$^{gtKO/gtKO}$ male mice had a significantly higher number of individual fecal boli than any other group (genotype x sex interaction $F_{2,55} = 5.11$, $p = 0.0094$; genotype $F_{2,55} = 8.99$, $p = 0.0004$; sex $F_{1,55} = 18.52$, $p < 0.0001$). Together, these results suggest that *Scn2a*-deficiency may cause changes in food/water consumption and fecal output which may represent metabolic abnormalities.

To measure general muscle and lower motor neuron, spinocerebellar, sensory, neuropsychiatric, and autonomic function, a preliminary SmithKline Beecham/Harwell/Imperial College/Royal London Hospital Phenotype Assessment (SHIRPA) was conducted.[27] The SHIRPA is a qualitative analysis (rated on a qualitative scale, see Table S1) as an initial assessment. Among the nine behaviors observed, body position, trunk curl, grip, and postural reflex were significantly different between WT and *Scn2a*$^{gtKO/gtKO}$ mice after post-hoc analysis. Specifically, *Scn2a*$^{gtKO/gtKO}$ mice had a lower body position rating (more hunched) with an average score of 3.13 ± 0.23 compared to 3.43 ± 0.33 for *Scn2a*$^{WT/gtKO}$ mice and 3.67 ± 0.37 for WT mice (genotype $F_{2,30} = 11.71$, $p = 0.0002$; *Scn2a*$^{WT/gtKO}$ vs. WT $p = 0.0565$; *Scn2a*$^{gtKO/gtKO}$ vs. WT $p < 0.0001$). *Scn2a*$^{gtKO/gtKO}$ mice had less trunk curl when held by the tail with a score of 1.08 ± 1.51 compared to 1.61 ± 1.16 for *Scn2a*$^{WT/gtKO}$ mice and 2.42 ± 0.90 for WT mice (genotype $F_{2,30} = 3.95$, $p = 0.0297$; *Scn2a*$^{gtKO/gtKO}$ vs. WT $p = 0.0178$). They also had decreased grip ability (slight grip attempted) of 1.40 ± 1.64 compared to 4.32 ± 0.82 for *Scn2a*$^{WT/gtKO}$ mice and 4.17 ± 1.40 for WT mice (genotype $F_{2,30} = 21.99$, $p < 0.0001$; *Scn2a*$^{gtKO/gtKO}$ vs. WT $p < 0.0001$). *Scn2a*$^{gtKO/gtKO}$ mice had less of a postural reflex when held by the tail (some head raise, front paws curled inward, some spread of hind limbs) with a score of 3.86 ± 0.83 compared to 4.41 ± 0.50 for *Scn2a*$^{WT/gtKO}$ mice and 4.48 ± 0.51 for WT mice (genotype $F_{2,30} = 5.86$, $p = 0.0048$; *Scn2a*$^{gtKO/gtKO}$ vs. WT $p = 0.0058$). Activity, tail posture, limb grasping, extension, and righting reflex were similar between WT, *Scn2a*$^{WT/gtKO}$, and *Scn2a*$^{gtKO/gtKO}$ mice. There were no significant sex differences in any of the nine SHIRPA parameters. These results suggest notable differences in basic phenotypes between *Scn2a*$^{gtKO/gtKO}$ mice and WT mice.

3.3 | Open field test

Aversion to a novel open space and general motor ability were assessed by the open field test. We performed two versions of the open field assays. The first paradigm was for 10 min at 60 lux[19,28] and the second was a 30 min test at 250 lux paradigm.[29] Center duration in the open field has commonly been used to assess anxiety-like behaviors in mice.[48] In the 10 min paradigm in low-light conditions, *Scn2a*$^{gtKO/gtKO}$ mice, but not *Scn2a*$^{WT/gtKO}$ mice, spent significantly less time in the center than WT regardless of the sex (Figure 2(A) and (B); $F_{2,39} = 8.3850$, $p = 0.0009$; *Scn2a*$^{gtKO/gtKO}$ vs. WT $p = 0.0103$). A significant genotype x sex interaction was identified in the measurement of total distance traveled ($F_{2,39} = 5.24$, $p = 0.0096$). Specifically, female *Scn2a*$^{gtKO/gtKO}$ traveled longer distances compared to WT ($p = 0.0103$), but male WT, *Scn2a*$^{WT/gtKO}$, and *Scn2a*$^{gtKO/gtKO}$ mice traveled the same distance (Supplemental Figure S2A). All mice had a similar duration of freezing (Supplemental Figure S2B).

In the 30 min test in bright-light conditions, the *Scn2a*$^{gtKO/gtKO}$ mice also had a significant decrease in time spent in the center of the open field (genotype $F_{2,30} = 16.180$, $p < 0.0001$; *Scn2a*$^{gtKO/gtKO}$ vs. WT $p = 0.0009$) (Figure 2(C)), similar to the 10 min, 60 lux assay. *Scn2a*$^{gtKO/gtKO}$ mice have a decreased distance traveled (genotype $F_{2,30} = 6.17$, $p = 0.0057$; female *Scn2a*$^{gtKO/gtKO}$ vs. WT $p = 0.0327$) (Supplemental Figure S2C). Unlike the 10 min test at 60 lux, *Scn2a*$^{gtKO/gtKO}$ mice had a significant increase in freezing time (genotype $F_{2,30} = 18.440$, $p < 0.0001$; *Scn2a*$^{gtKO/gtKO}$ vs. WT $p < 0.0001$) (Supplemental Figure S2D). Together, we observed a decrease in center duration from both paradigms in *Scn2a*$^{gtKO/gtKO}$ mice, suggesting a phenotype of higher anxiety-like behavior in novel, open space.

3.4 | Sensory Modalities

Sensory sensitivity disorders are common comorbidities of many neurodevelopmental disorders.[49,50] To test the response to thermal and cold stimuli, mice were placed on temperature-controlled plates. We tested both temperature tolerance and sensitivity. We define tolerance as the latency of response on a plate of static temperature, whereas sensitivity is the latency of response to scaled temperatures on the dynamic plate paradigm. Temperatures were determined based on previous studies.[37-39] The thermal/cold tolerance test was a plate kept at a constant, static temperature (50°C for hot and 7.5°C for cold). *Scn2a*$^{gtKO/gtKO}$ mice (*Scn2a*$^{gtKO/gtKO}$ vs. WT $p = 0.0082$) but not *Scn2a*$^{WT/gtKO}$ had a significant decreased tolerance for heat ($F_{2,30} = 6.10$, $p = 0.0060$) (Figure 3(A)). Using the static cold plate to measure tolerance, *Scn2a*$^{gtKO/gtKO}$ mice (*Scn2a*$^{gtKO/gtKO}$ vs. WT $p < 0.0001$) and *Scn2a*$^{WT/gtKO}$ mice (*Scn2a*$^{WT/gtKO}$ vs. WT $p = 0.0098$) had a lower tolerance for cold temperature (genotype $F_{2,30} = 31.29$, $p < 0.0001$; sex $F_{1,30} = 4.47$, $p = 0.0428$) (Figure 3(B)). Thermal/cold sensitivity was a dynamic hot/cold plate that started at 30°C and ramped (2°C/sec) to a maximum/minimum temperature (60°C for hot and 5°C for cold). WT, *Scn2a*$^{WT/gtKO}$, and *Scn2a*$^{gtKO/gtKO}$ mice all performed similarly in the dynamic hot plate for thermal sensitivity (Figure 3(C)). However, *Scn2a*$^{gtKO/gtKO}$ mice had an increased sensitivity (response at a higher temperature) during the dynamic cold plate (*Scn2a*$^{gtKO/gtKO}$ vs. WT $p = 0.0239$) (Figure 3(D)). Mechanical sensitivity was measured by applying von Frey fibers to the hind paw pad using an up-down protocol until the mouse responded to the fiber.[41]

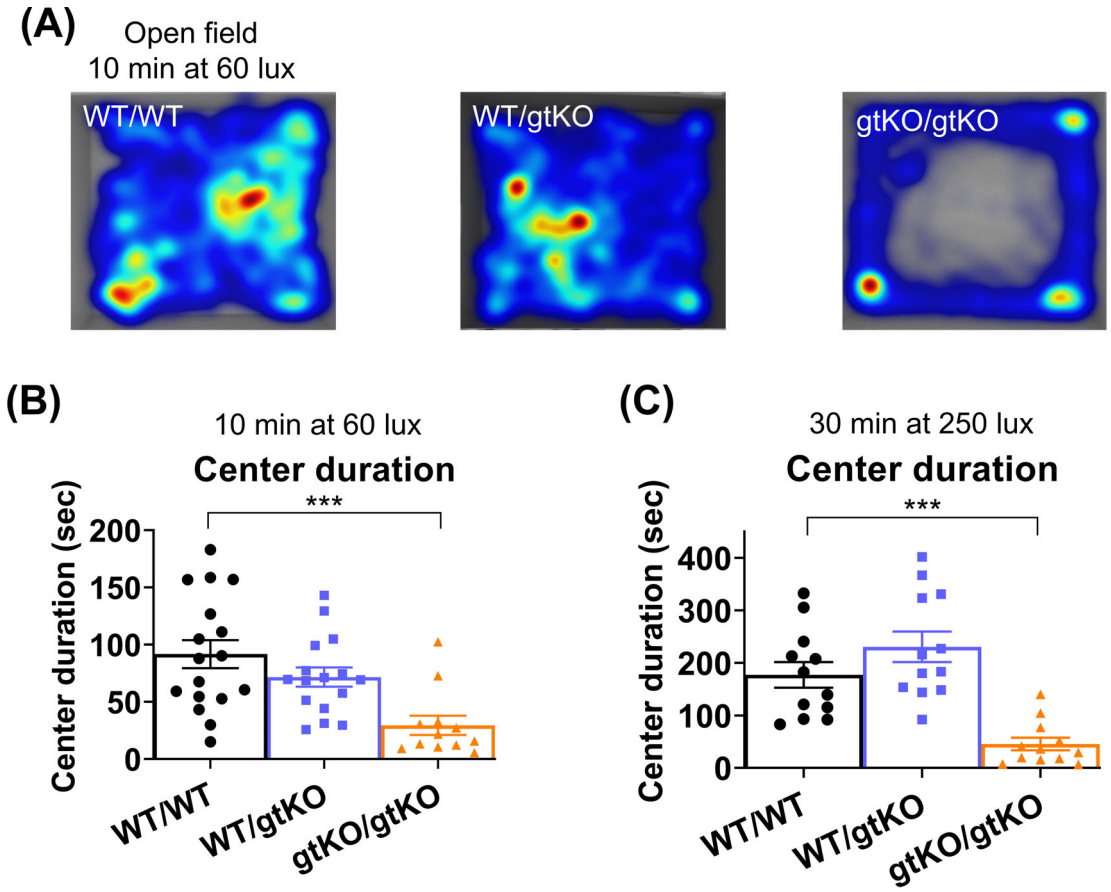

FIGURE 2 *Scn2a*$^{gtKO/gtKO}$ *mice have elevated anxiety-like behaviors in the open field.* (A) Representative heatmaps of 10 minute at 60 lux open field illustrating a WT and *Scn2a*$^{WT/gtKO}$ mouse which spent more time in the middle of the chamber than the *Scn2a*$^{gtKO/gtKO}$ mouse, who spent most of the time in the corners or by the sides suggesting anxiety-like behaviors. Warm colors (red, orange, and yellow) represent spots more frequently traveled by the mice whereas cool colors (blue and green) represent less frequently traveled areas. (B-C) Center duration is decreased in *Scn2a*$^{gtKO/gtKO}$ mice for the 10 minute at 60 lux (B) and the 30 minute at 250 lux (C) open field paradigm. For 10 minute at 60 lux, n = 8/9, 11/6, and 6/6 for WT, *Scn2a*$^{WT/gtKO}$, *Scn2a*$^{gtKO/gtKO}$ female/male. For 30 minute at 250 lux, n = 6 of each sex of each genotype. Data are presented as mean value with SEM error bars. Significance was calculated from two-way ANOVA with post-hoc Bonferroni corrections with $p<0.05$ (*) and $p<0.001$ (***)

There were no mechanical sensitivity differences between WT, *Scn2a*$^{WT/gtKO}$, and *Scn2a*$^{gtKO/gtKO}$ mice (*Scn2a*$^{gtKO/gtKO}$ vs. WT $p = 0.0912$). Therefore, we concluded that *Scn2a*$^{gtKO/gtKO}$ mice had lower tolerances for both hot and cold temperatures, but no major deficits in thermal, cold, or mechanical sensitivity.

Next, we tested olfaction, taste aversion, and auditory abilities. Odor discrimination by comparing the time spent interacting (i.e. sniffing, licking) with a cinnamon solution versus water as described previously.[30] WT, *Scn2a*$^{WT/gtKO}$, and *Scn2a*$^{gtKO/gtKO}$ mice had a similar level of cinnamon preference (sex $F_{1,30} = 4.72$, $p = 0.0379$), but *Scn2a*$^{gtKO/gtKO}$ mice had a non-significantly higher total interaction time. These results of longer odor discrimination time with no changes in discrimination accuracy are consistent with a previous study on a mouse model with knockdown of $Na_v1.2$ in granule cells using short hairpin RNA.[51]

SCN2A has been identified along with other ion channels in sweet, bitter, umami, and sour taste cell populations and is suggested to play a role in sensing initial taste receptor activation.[52] To test whether *Scn2a*$^{gtKO/gtKO}$ mice can detect bitter taste, mice were given two bottles, one with reverse osmosis water and the other with quinine in which they could drink ad lib for four days. There were no significant differences in quinine avoidance between WT, *Scn2a*$^{WT/gtKO}$, and *Scn2a*$^{gtKO/gtKO}$ mice, suggesting that these *Scn2a*$^{gtKO/gtKO}$ mice do not have a significant deficit in discriminating bitter tastes.

$Na_v1.2$ is also expressed in unmyelinated efferent axons in the organ of Corti and ear hair cells.[53] The hearing of the mice was tested using an acoustic startle hearing test at five volumes ranging from 75–115 dB at the same frequency (4 kHz) at 10 dB steps. At 75 dB (5 dB above background), *Scn2a*$^{gtKO/gtKO}$ mice had a significantly less startle response (*Scn2a*$^{gtKO/gtKO}$ vs. WT $p = 0.0107$) (genotype $F_{2,30} = 4.97$, $p = 0.0136$) (Supplemental Figure S3). However, no other significant genotype or interaction effects were observed at the other volumes. From these measures, we concluded that there were no gross deficits in hearing, but there could be a small deficit in male *Scn2a*$^{gtKO/gtKO}$ deciphering sounds that are very close to background noise.

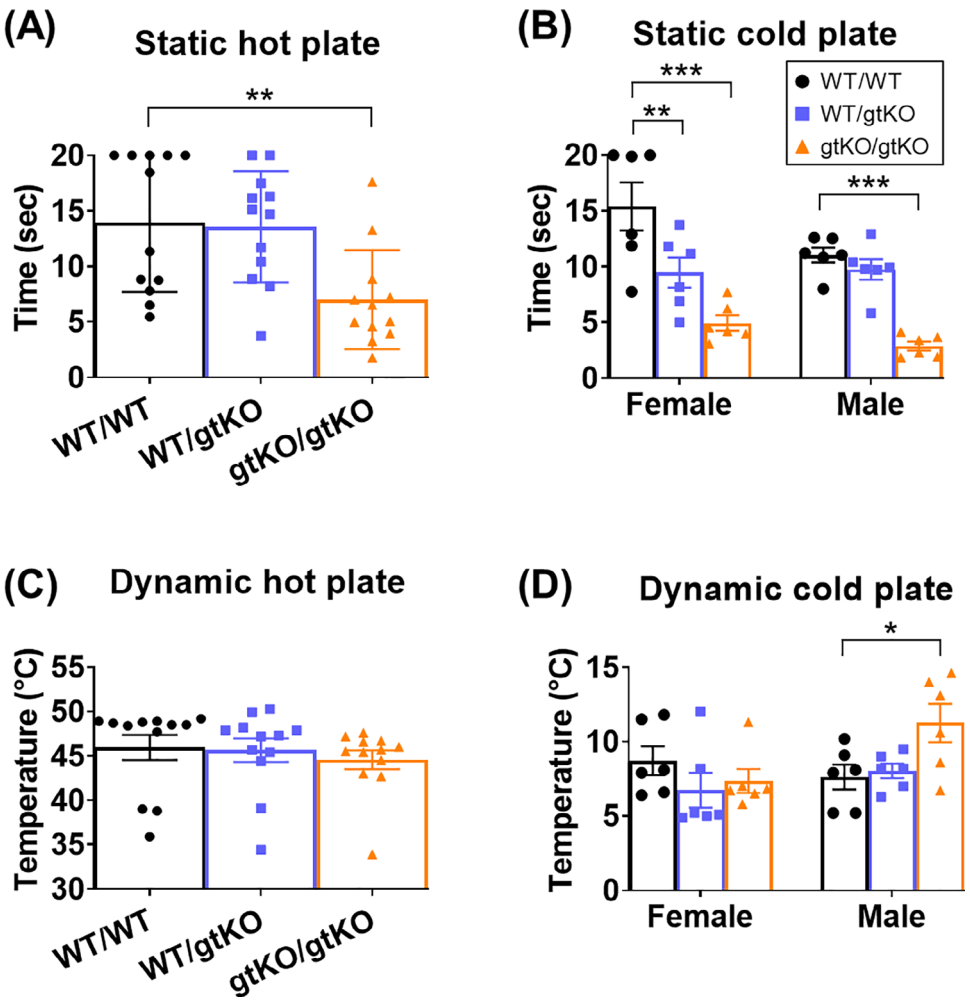

FIGURE 3 *Scn2a*$^{gtKO/gtKO}$ *mice have deficits in thermal and cold tolerance but not sensitivity.* (A) Response to a static hot plate was significantly faster in *Scn2a*$^{gtKO/gtKO}$ mice. (B) Female *Scn2a*$^{WT/gtKO}$ mice and *Scn2a*$^{gtKO/gtKO}$ mice had a decreased latency to respond to the cold plate while only male *Scn2a*$^{gtKO/gtKO}$ mice had a decreased latency compared to WT. (C) Responses to scaled temperature from 30 to 55°C on the hot plate were similar among mice with all genotypes. (D) Male *Scn2a*$^{gtKO/gtKO}$ mice had an increase in cold sensitivity during the scaled dynamic cold plate of decreasing temperature from 20 to 0°C. The sample size was 6 mice per sex per genotype. Data are presented as mean value with SEM error bars. Significance was calculated from two-way ANOVA with post-hoc Bonferroni corrections with $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***)

3.5 | Innate behavior

Building a nest is a normal, innate behavior of mice.[42] Mice gain territorial security and warmth from their nests. Remarkably, *Scn2a*$^{gtKO/gtKO}$ mice have little to no nesting activity compared to WT measured by nest quality (genotype $F_{2,39} = 155.00$, $p < 0.0001$) (Figure 4(A) and (B)) and nest height (genotype $F_{2,39} = 36.12$, $p < 0.0001$; sex $F_{1,39} = 5.48$, $p = 0.0244$) after 36 hours (Figure 4(C)). In particular, *Scn2a*$^{gtKO/gtKO}$ mice make nests of significantly less quality (*Scn2a*$^{gtKO/gtKO}$ vs. WT $p < 0.0001$) as rated on a scale of 0–5 (see Materials and Methods), and a much smaller nest height (*Scn2a*$^{gtKO/gtKO}$ vs. WT $p < 0.0001$). *Scn2a*$^{WT/gtKO}$ mice build nests similar to WT in both quality and height. Together, nesting deficits in *Scn2a*$^{gtKO/gtKO}$ mice may represent a major impairment in innate behavior.

3.6 | Other behaviors related to neurodevelopment

Repetitive behaviors, attention deficit, lack of interest for novelty, or neophobia are also commonly found in mouse models related to neurodevelopmental disorders[54]; Studies suggested that grooming was not altered in *Scn2a*$^{+/-}$ mice.[5,18] In both open field paradigms, however, *Scn2a*$^{gtKO/gtKO}$ mice displayed increased grooming with no significant sex or genotype effects. We found a significant increase in grooming between *Scn2a*$^{gtKO/gtKO}$ vs. WT mice in the 10 min/60 lux open field (genotype $F_{2,39} = 3.09$, $p = 0.0583$; *Scn2a*$^{gtKO/gtKO}$ vs. WT $p = 0.0427$) and a non-significant increase in grooming in the 30 min/250 lux open field (genotype $F_{2,30} = 2.77$, $p = 0.0786$; *Scn2a*$^{gtKO/gtKO}$ vs. WT $p = 0.0538$) (Figure 5(A) and (B)). To further understand the grooming behaviors,, we performed the home-cage induced grooming test.[45-47] Our data revealed that *Scn2a*$^{gtKO/gtKO}$ mice had a slightly increased induced (water mist) grooming duration and bouts (Figure 5(C) and (D)). We also performed a four-object novel object exploration test.[55,56] *Scn2a*$^{gtKO/gtKO}$ mice interacted significantly less overall with all the objects (genotype $F_{2,30} = 7.79$, $p = 0.0019$; *Scn2a*$^{WT/gtKO}$ vs WT $p = 0.0587$; *Scn2a*$^{gtKO/gtKO}$ vs. WT $p = 0.0009$) (Figure 5(E)), and had less interaction time with the objects during each interaction bout. Moreover, we found that *Scn2a*$^{gtKO/gtKO}$ mice have increased alternations/fix-pattern exploration (genotype $F_{2,30} = 5.34$, $p = 0.0104$; *Scn2a*$^{gtKO/gtKO}$ vs. WT $p = 0.0058$) (Figure 5(F)), which could suggest an elevated high-order repetitive behavior. We also tested marble burying, in which *Scn2a*$^{gtKO/gtKO}$ mice strikingly buried little to no marbles (genotype

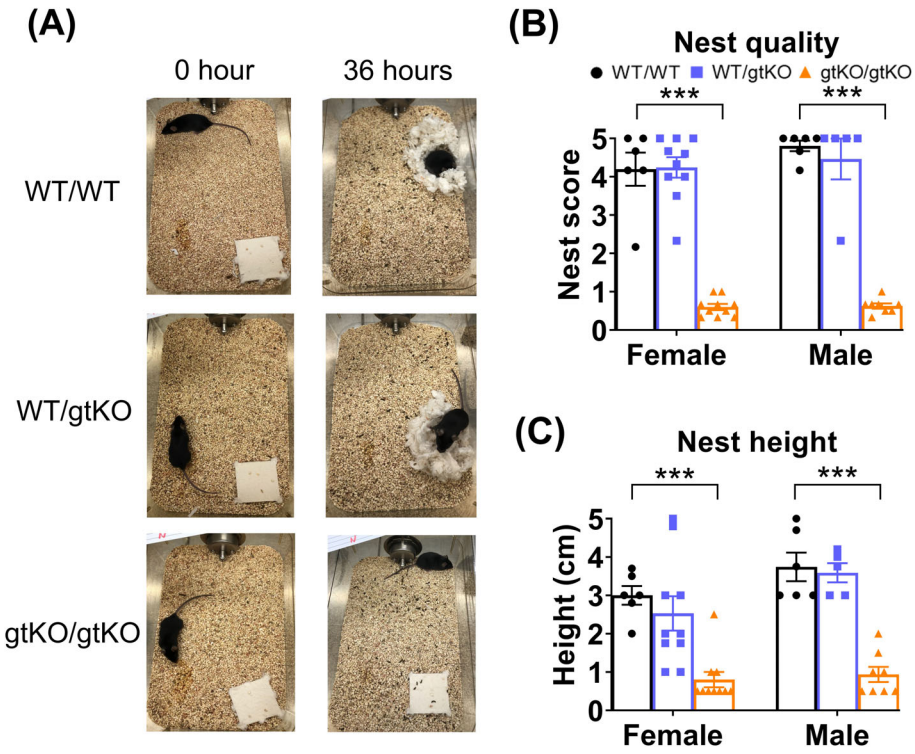

FIGURE 4 *Scn2a*$^{gtKO/gtKO}$ *mice have severely impaired nesting behavior.* (A) Representative nests at 0 hours (start) and after 36 hours. The top right image (WT after 36 hours) shows an example of a quality rating of 5 (on a scale from 0–5), which is defined as a near-perfect nest with all of the nesting material in a crater-shape with walls higher than the mouse. The bottom right image (*Scn2a*$^{gtKO/gtKO}$ mice after 36 hours) shows a quality rating of 0 with little to no nesting material disturbed. (B) Quantification of nest quality revealed that both sexes of *Scn2a*$^{gtKO/gtKO}$ mice have significantly decreased nesting quality. (C) Nest height was also significantly decreased in both sexes of *Scn2a*$^{gtKO/gtKO}$ mice. n = 6/6, 10/5, and 10/8 (WT, *Scn2a*$^{WT/gtKO}$, *Scn2a*$^{gtKO/gtKO}$ females/males). Data are presented as mean value with SEM error bars. Significance was calculated from two-way ANOVA with post-hoc Bonferroni corrections with p<0.001 (***)

$F_{2,30}$ = 130.90, p < 0.0001; sex $F_{1,30}$ = 6.67, p = 0.0149; *Scn2a*$^{gtKO/gtKO}$ vs. WT p < 0.0001 for both females and males), while *Scn2a*$^{WT/gtKO}$ buried a similar number to WT mice (Figure 5(G) and (H)). The *Scn2a*$^{gtKO/gtKO}$ mice still dug around the marbles but did not deliberately bury them like WT mice. Decreased novel object exploration duration, together with little to no marble burying behavior, may together indicate a likelihood of attention deficit, lack of interest for novelty, or neophobia[57,58] of *Scn2a*$^{gtKO/gtKO}$ mice.

3.7 | Assessment for spatial working memory

Spatial working memory was assessed using a Y-maze.[44] The mice were trained with one of the marked arms closed off. 2 hours later, they were put back in the maze, with the closed arm now open. WT mice readily explore the novel arm since they should remember that they have already explored the other arms. We did not observe any significant genotype effects in the duration, number of entries, latency to the novel arm, or preference for the novel arm; however there were significant sex effects for the duration in the novel arm ($F_{1,30}$ = 5.56, p = 0.0251), entries ($F_{1,30}$ = 9.88, p = 0.0038), and latency ($F_{1,30}$ = 6.75, p = 0.0146), but no preference for novel arm (Supplemental Figure S4). Additionally, male *Scn2a*$^{WT/gtKO}$ and *Scn2a*$^{gtKO/gtKO}$ mice have a slight increase in latency, decreased number of entries, decreased duration, and decreased preference (genotype $F_{2,30}$ = 2.65, p = 0.0870; *Scn2a*$^{gtKO/gtKO}$ vs. WT p = 0.0834) for the novel arm. These findings could indicate a mild deficit in spatial working memory in male *Scn2a*$^{WT/gtKO}$ and *Scn2a*$^{gtKO/gtKO}$ mice, while female *Scn2a*$^{WT/gtKO}$ and *Scn2a*$^{gtKO/gtKO}$ mice are likely to have normal spatial working memory in the Y maze.

4 | DISCUSSION

In this study, we characterized a mouse model with severe *Scn2a* deficiency. We provide evidence to suggest that *Scn2a*$^{gtKO/gtKO}$ mice may recapitulate certain aspects of behavioral deficits and could serve as a model for *SCN2A* loss-of-function/nonsense channelopathies. Our results show that *Scn2a*$^{gtKO/gtKO}$ mice have little-to-no mating and nesting, which could suggest innate behavior impairments. They exhibited increased anxiety-like behaviors in the open field as well as increased repetitive behavior. *Scn2a*$^{gtKO/gtKO}$ mice have normal olfactory, taste, hearing, and mechanical sensitivity but a decrease in thermal and cold tolerance. Our *Scn2a*^{gtKO} model can be used to further understand the etiology of *SCN2A*-related disorders, and to develop and test interventions.

Mouse models have been a cornerstone for our understanding of in vivo biological mechanisms and have provided valuable insights for many genetic disorders. Mechanistic investigations to gain critical knowledge of *SCN2A* function at the cellular, circuital, and behavioral levels are essential to deeply understand the role of $Na_v1.2$ in neurodevelopmental disorders. Current mouse models of *SCN2A* provide useful but limited insights. Canonical *Scn2a* null mice (MGI: 2180186, *Scn2a*^{tm1Mml}) were generated by removing Exon 2 (Ex2).[17] Initial studies of the Ex2 *Scn2a* mouse model demonstrated that homozygous (*Scn2a*$^{-/-}$) knockout is perinatal lethal, whereas heterozygous (*Scn2a*$^{+/-}$)

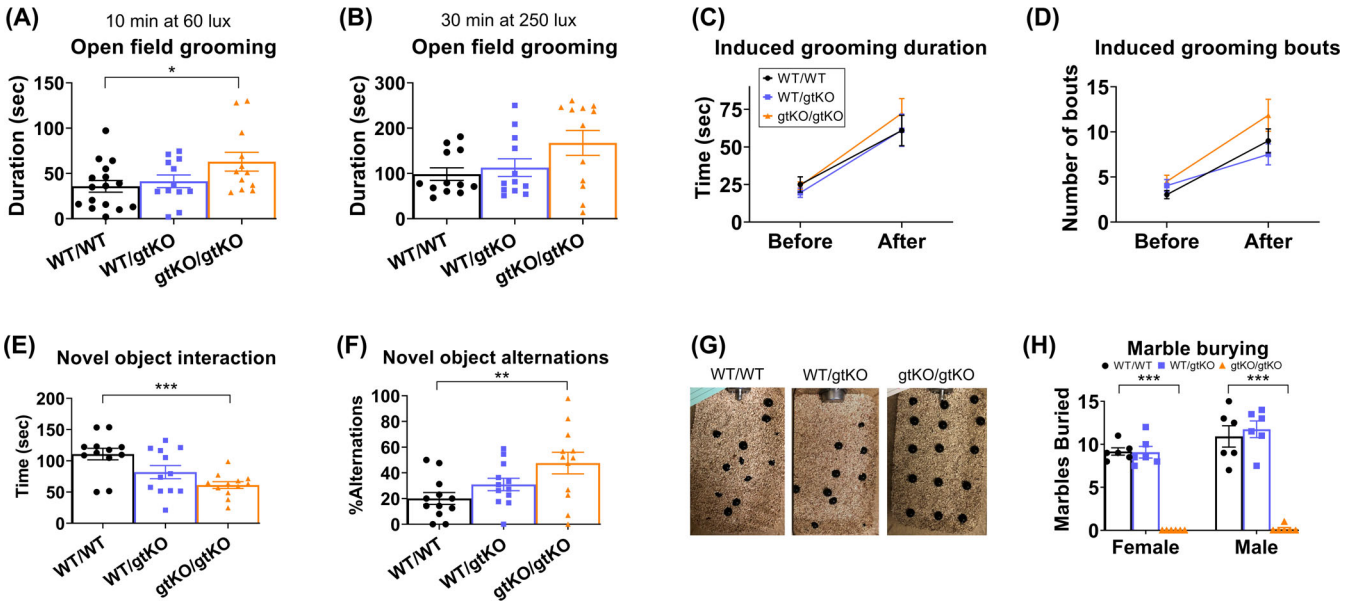

FIGURE 5 *Scn2a*$^{gtKO/gtKO}$ *mice have increased repetitive behaviors with little to no marble burying behavior.* (A) During the 10 minute at 60 lux open field, *Scn2a*$^{gtKO/gtKO}$ mice have increased grooming. (B) During the 30 minute at 250 lux open field, *Scn2a*$^{gtKO/gtKO}$ mice have a slight increase in grooming. For 10 minute at 60 lux, n = 8/9, 11/6, and 6/6 for WT, *Scn2a*$^{WT/gtKO}$, *Scn2a*$^{gtKO/gtKO}$ female/male. For 30 minute at 250 lux, n = 6 of each sex of each genotype. (C-D) Home cage grooming duration (C) and the number of grooming bouts (D) were scored before (spontaneous) and after (induced) spraying with water. Grooming sample size was n = 10/10, 10/10, and 12/10 (WT, *Scn2a*$^{WT/gtKO}$, *Scn2a*$^{gtKO/gtKO}$ females/males). A slight increase in induced grooming in *Scn2a*$^{gtKO/gtKO}$ mice was observed in the home cage. (E) Interaction time with novel objects was significantly less in *Scn2a*$^{gtKO/gtKO}$ mice during the novel objective exploration test. (F) Percent of alternation/fixed-pattern exploration of novel objects was significantly increased in *Scn2a*$^{gtKO/gtKO}$ mice during the novel objective exploration test. (G) Representative image of marble buried at the end of 30 minutes. 15 marbles are arranged in a 3 × 5 grid in their standard home cage with 5 cm of bedding. Marbles were counted after 30 minutes and counted buried if >2/3rds were under the bedding. (H) *Scn2a*$^{gtKO/gtKO}$ mice buried significantly fewer marbles than *Scn2a*$^{WT/gtKO}$ mice and WT mice. Novel object and marble burying sample size were 6 mice per sex per genotype. Data are presented as mean value with SEM error bars. Significance was calculated from two-way ANOVA with post-hoc Bonferroni corrections with $p<0.01$ (**) and $p<0.001$ (***)

mice are viable and fertile with no detectable phenotypical abnormalities compared to WT mice (*Scn2a*$^{+/+}$).[17]

More recent studies performed with this traditional knock-out mouse model revealed mild phenotypes. In particular, Dr. Mantegazza's team at the University Cote d'Azur and CNRS, France assessed the behavior of this heterozygous *Scn2a*$^{+/-}$mice (male only). They found that adult *Scn2a*$^{+/-}$ male mice have no changes in olfaction or marble burying behaviors,[20] similar to our results for *Scn2a*$^{WT/gtKO}$ mice. Although they detected some behavioral abnormalities in juveniles, these behavioral abnormalities disappear in adulthood. In contrast, our behavioral tests were all performed on adult mice. Juvenile behavior of *Scn2a*$^{gtKO/gtKO}$ mice remains to be tested. Also, Dr. Yamakawa's team at RIKEN Institute in Japan has conducted an extensive behavioral analysis in which most of the assays reveal no difference between WT and *Scn2a*$^{+/-}$ (males only) in body weight, hot plate, and auditory startle.[19] Similarly, our study of *Scn2a*$^{WT/gtKO}$ males shows no statistically significant differences in these tests. However, we noted that female *Scn2a*$^{WT/gtKO}$ have reduced body weight, highlighting the importance of studying both females and males in neuroscience research.[59] Meanwhile Dr. Bender's group at the University of California, San Francisco focused on electrophysiological properties, which revealed differences in neuronal excitability and synaptic functions between WT and *Scn2a*$^{+/-}$ mice (both males and females); however, no impaired nesting of *Scn2a*$^{+/-}$ mice was observed.[5] Similarly, we do not see any changes in *Scn2a*$^{WT/gtKO}$ nesting. Only *Scn2a*$^{gtKO/gtKO}$ mice display strong deficits in nesting.

A second *Scn2a* knockout mouse model was generated by the deletion of exons 4–6 (MGI: 6407391, *Scn2a*$^{tm1.2Bcgen}$), which was studied by Dr. Kim's lab at the Korea Advanced Institute of Science and Technology (KAIST) in Daejeon, South Korea.[18] Homozygous *Scn2a* knock-out in this mouse model also died perinatally,[18] further illustrating that global *Scn2a* null mice cannot develop into adulthood. Their phenotypic analysis of heterozygous mice again supported the notion that most behaviors of *Scn2a*$^{+/-}$ mice are relatively normal compared to WT (only tested males as well). Both *Scn2a*$^{WT/gtKO}$ mice (Figure 1) and *Scn2a*$^{+/-}$ mice express ~50% of *Scn2a* protein expression levels compared to WT mice,[21] collectively indicating that a 50% reduction of *Scn2a* protein may not be sufficient to render major behavioral phenotypes in adult mice.

Notably, there are some inconsistencies in these studies regarding the open field results of these *Scn2a*$^{+/-}$ mice. Possibly, small differences in handling, maintenance, experimental conditions, or genetic background of these mice may contribute to the subtle and variable

phenotypes observed in these studies.[60] Total distance traveled was increased in *Scn2a*$^{+/-}$ male adult mice compared to WT mice of Yamakawa's study[19]; however in Mantegazza's study,[20] there was no difference in distance between male adult *Scn2a*$^{+/-}$ and WT mice. In Bender's study, female adult *Scn2a*$^{+/-}$ mice had a slight increase in total distance traveled while male adult *Scn2a*$^{+/-}$ mice had a similar distance to WT.[5] We did not observe any significant differences in distance traveled between male or female adult *Scn2a*$^{WT/gtKO}$ and WT mice in the 10 minute/60 lux or the 30 minute/250 lux open field test. However, the *Scn2a*$^{gtKO/gtKO}$ female adult mice had an increase in distance traveled in the 10 minute/60 lux open field test, but a decrease in distance traveled in the 30 minute/250 lux open field test. This difference could be because *Scn2a*$^{gtKO/gtKO}$ female adult mice had an increased time not moving (immobile/freezing duration) in the 30 minute/250 lux open field but a similar freezing duration to WT in the 10 minute/60 lux open field test. For anxiety-like behavior, Mantegazza group found juvenile male *Scn2a*$^{+/-}$ mice spent a slightly increased time in the center compared to WT with no differences in center time in male adult *Scn2a*$^{+/-}$,[20] while the Yamakawa group reported that adult *Scn2a*$^{+/-}$ mice spent an increased time in the center.[19] Bender's group found that male *Scn2a*$^{+/-}$ mice had a significant decrease in the proportion of movement in the center, while female *Scn2a*$^{+/-}$ mice had a slight increase in the proportion of movement in the center when compared to WT.[5] We observed that both male and female adult *Scn2a*$^{gtKO/gtKO}$ mice display a significant decrease in the time spent in the center of the open field in both the 10 minute/60 lux and the 30 minute/250 lux paradigms, strongly indicating anxiety-like behavior in the open field test. Additionally, we tested grooming in these two open field paradigms, together with induced grooming in the home cage.[45-47] Although increased grooming was found in these different paradigms, a statistically significant difference was only observed in the 10 minute/60 lux open field test, suggesting that these mice may only have mild low-order repetitive behaviors. However, we detected a notable difference in fixed-pattern exploration of *Scn2a*$^{gtKO/gtKO}$ mice in the novel object exploration test,[55,56] which on the other hand, likely indicates a stronger "high-order" repetitive behavior of these mice.

Canonical homozygous knockout (null,100% knockout) of *Scn2a* in mice leads to pups dying 1–2 days after birth with complications including dehydration, hypoxia, ~16% loss in body weight, and reduced milk content in the stomach.[17] Our *Scn2a*$^{gtKO/gtKO}$ mice had a similar ~15% reduced body weight compared to WT through adulthood, but close to 80% of *Scn2a*$^{gtKO/gtKO}$ mice survived to adulthood. A residual amount of full-length functional *Scn2a* protein is expressed in *Scn2a*$^{gtKO/gtKO}$ mice, which could be the reason why a majority of *Scn2a*$^{gtKO/gtKO}$ mice survive to adulthood. Another mouse model of neurodevelopmental disorder, *Foxp1*$^{-/-}$, also had failed to thrive at weaning unless a soft, high-calorie diet was provided.[61] We similarly provided a gel-based high-calorie food source. The combined phenomenon of failure to thrive in adolescence and overeating in adulthood in humans is also evident in Prader-Willi syndrome.[62] Interestingly, *Scn2a*$^{gtKO/gtKO}$ mice seem to have gastrointestinal issues including penile/vaginal and rectal prolapse and diarrhea. *Scn2a* is suggested to be expressed in the enteric nervous system of humans, particularly increased during embryonic 12–16 weeks.[63] Single-cell RNA sequencing in mice revealed *Scn2a* expression in nitrergic and cholinergic enteric neurons.[64] In guinea pigs, a study reported that *Scn2a* expressed in the small and large intestines contributes to the somal action potential of enteric sensory neurons.[65] However, whether the gastrointestinal issues of *Scn2a*$^{gtKO/gtKO}$ mice is due to the reduced expression of *Scn2a* outside of the central nervous system would require further investigation.

A portion of individuals with ASD have a decreased tolerance threshold to sudden changes in cold and heat stimuli, but no altered sensitivity was detected when the temperature was scaled.[66] This phenotype was interestingly recapitulated in our model in that *Scn2a*$^{gtKO/gtKO}$ mice had a significantly decreased thermal tolerance but no difference in thermal sensitivity. The heterozygous mouse model studied by the Yamakawa group had a similar latency to response on a static thermal hot plate compared to WT.[19] Some tactile and mechanical abnormalities in people with ASD have been observed in clinical literature.[67] Meanwhile, mouse models of ASD-associated genes *Mecp2*, *Gabrb3*, *Shank3*, and *Fmr1* have altered tactile discrimination and hypersensitivity.[68] *Scn2a*$^{gtKO/gtKO}$ mice, however, do not have a difference in tactile sensitivity. The underlying mechanism of these distinct phenotypes remains to be determined.

Innate behavior such as nesting has been observed to be abnormal in mouse models of neurodevelopmental disorders.[69,70] Our *Scn2a*$^{gtKO/gtKO}$ mice, strikingly, have little-to-no nest building activity. Another common phenotypic test performed on mouse models of neurodevelopmental disorders is marble burying, although its behavioral implication has been under debate.[71] Studies have suggested that marble burying could be either a neophobic response,[57] repetitive/compulsive,[72,73] or just a normal behavioral routine of inherent burying behavior.[74] Regardless, our mice surprisingly buried almost no marbles during the test, which is remarkably different compared to the WT mice. Decreased nesting and marble burying were also observed in a Fragile-X mouse model.[75] Considering the decreased novel object exploration duration, we interpret this lack of marble burying as a possible attention deficit, lack of interest for novelty, neophobic response.

In conclusion, the results from this study support the use of the *Scn2a*$^{gtKO/gtKO}$ mice to model *SCN2A*-deficiency to study genetic sodium channelopathies. Importantly, the phenotypes between *Scn2a*$^{gtKO/gtKO}$ mice and WT mice are more distinct than those between *Scn2a*$^{WT/gtKO}$ and WT mice. Our data shows a reduced center duration of *Scn2a*$^{gtKO/gtKO}$ mice of both sexes, indicating anxiety-like behaviors in novel, open spaces. Moreover, other behavioral deficits (e.g., impairments in marble burying and nesting) in *Scn2a*$^{gtKO/gtKO}$ mice are also profound, highlighting the importance of this *Scn2a*$^{gtKO/gtKO}$ mouse model for the study of *Scn2a*-deficiency. Further studies will explore other behavioral, biochemical, and electrophysiological properties of neurons in these mice at different developmental stages. Since we have observed sex-specific abnormalities in this mouse model, more studies are needed to determine the role of *Scn2a* on hormone regulation and sexual maturation, which are suggested to

play a role in neurodevelopment.[76,77] Studies performed on these mice will help to discern mechanisms underlying how *SCN2A*-deficiency leads to neurodevelopmental disorders.[78]

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CONFLICT OF INTEREST

All authors declare no potential conflict of interest.

AUTHOR CONTRIBUTION

Muriel Eaton, Anthony C. Park, Emma Lietzke, Chloé M. Romero, Emily R. Coleman, Ji Hea Lee, Sophia Palant, and Huynhvi P. Nguyen performed behavioral experiments and analysis, as well as husbandry. Jingliang Zhang, Zhixiong Ma, Xiaoling Chen, Tiange Xiao, and Yushuang Liu performed the molecular biology and analysis. Muriel Eaton, Zhefu Que, Shirong Lai, Jiaxiang Wu, Zhuo Huang, William C. Skarnes, Wendy A. Koss, and Yang Yang participated in data analysis and experimental design. Yang Yang supervised the project and edited the paper. Muriel Eaton wrote the paper with inputs from all authors.

DATA AVAILABILITY STATEMENT

Additional results can be found in the supplemental material. The data that supports the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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