Social Deficits and Cerebellar Degeneration in Purkinje Cell Scn8a Knockout Mice

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Mutations in the SCN8A gene encoding the voltage-gated sodium channel α-subunit Nav1.6 have been reported in individuals with epilepsy, intellectual disability and features of autism spectrum disorder. SCN8A is widely expressed in the central nervous system, including the cerebellum. Cerebellar dysfunction has been implicated in autism spectrum disorder. We investigated conditional Scn8a knockout mice under C57BL/6J strain background that specifically lack Scn8a expression in cerebellar Purkinje cells (Scn8a<sup>flox/flox</sup>, L7Cre<sup>+</sup>) mice. Cerebellar morphology was analyzed by immunohistochemistry and MR imaging. Mice were subjected to a battery of behavioral tests including the accelerating rotarod, open field, elevated plus maze, light-dark transition box, three chambers, male-female interaction, social olfaction, and water T-maze tests. Patch clamp recordings were used to evaluate evoked action potentials in Purkinje cells. Behavioral phenotyping demonstrated that Scn8a<sup>flox/flox</sup>, L7Cre<sup>+</sup> mice have impaired social interaction, motor learning and reversal learning as well as increased repetitive behavior and anxiety-like behaviors. By 5 months of age, Scn8a<sup>flox/flox</sup>, L7Cre<sup>+</sup> mice began to exhibit cerebellar Purkinje cell loss and reduced molecular thickness. At 9 months of age, Scn8a<sup>flox/flox</sup>, L7Cre<sup>+</sup> mice exhibited decreased cerebellar size and a reduced number of cerebellar Purkinje cells more profoundly, with evidence of additional neurodegeneration in the molecular layer and deep cerebellar nuclei. Purkinje cells in Scn8a<sup>flox/flox</sup>, L7Cre<sup>+</sup> mice exhibited reduced repetitive firing. Taken together, our experiments indicated that loss of Scn8a expression in cerebellar Purkinje cells leads to cerebellar degeneration and several ASD-related behaviors. Our study demonstrated the specific contribution of loss of Scn8a in cerebellar Purkinje cells to behavioral deficits.
INTRODUCTION

Autism spectrum disorder (ASD) is a behaviorally defined pervasive neurodevelopmental disorder, characterized by persistent impairment of social communication, restricted interests and repetitive behaviors (Varghese et al., 2017). In addition to these core symptoms, there may be psychiatric or neurological comorbidities, of which attention-deficit/hyperactivity disorder (ADHD), anxiety, depression and epilepsy are most common (Lord et al., 2020). It is estimated that the prevalence of ASD is 1–2% in the general population (Wisniowiecka-Kowalnik and Nowakowska, 2019). Over the past decade, genomic technologies have enabled rapid progress in the identification of genes linked to ASD (Abrahams and Geschwind, 2008; Arnett et al., 2019). Although much effort has centered on the genetic delineation of syndromic forms of ASD, the underlying molecular mechanisms remain incompletely understood (Hampson and Blatt, 2015).

Several lines of evidence implicate cerebellar dysfunction in the development of ASD. Post-mortem studies have demonstrated a reduced number and density of cerebellar Purkinje cells (PC) in patients with ASD, and isolated cerebellar injury has been associated with a higher incidence of ASD (Wang et al., 2014; Hampson and Blatt, 2015). The cerebellum has been consistently implicated in several monogenic syndromes associated with ASD (Fatemi and Folsom, 2015; Sundberg and Sahin, 2015; Varghese et al., 2017). Recent studies suggest that PC dysfunction caused by mutations in Tsc1, Tsc2, Shank2, and PTEN during a critical developmental period may contribute to behavioral deficits relevant to ASDs (Tsai et al., 2012, 2018; Reith et al., 2016). Similar to findings in PC-specific Tsc1 and PTEN knockout mice, PC-specific Scn8a knockout mice had a lower frequency of spontaneous firing of PC indicative of lower excitability (Raman et al., 1997; Khalil et al., 2003; Levin et al., 2006).

Motivated by these studies implicating the PC of Scn8a mutant mice in ASD related traits, we used the Cre-loxP recombination system to generate conditional knockout mice in which Scn8a inactivation is restricted to PC. We found that PC-specific Scn8a knockout mice of C57BL/6 strain background exhibited late-onset cerebellar degeneration and deficits in motor coordination and social interaction, increased repetitive behavior, anxiety and abnormal activity of PC, demonstrating the specific contribution of PC to these Scn8a-dependent phenotypes.

METHODS

Subjects

All animal experimental procedures were performed in accordance with the animal protocol approved by the Institutional Animal Care and Use Committee at the School of Medicine, Shandong University. Scn8a<sup>fl</sup>ox<sup>fl</sup> mice (Levin and Meisler, 2004) have been maintained on a C57BL/6J strain background since 2004 in the Meisler lab at the University of Michigan, and were imported to Shandong University in 2017. L7/PCP2-Cre transgenic mice (stock number: J006207) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China), where they had also been maintained on the C57BL/6J strain background. Mice were housed in a 12-h light/dark cycle (lights on 7:00 AM) with controlled temperature and humidity and ventilated with a dedicated system. All mice had <i>ad libitum</i> access to sterile food and water. We crossed Scn8a<sup>fl</sup>ox<sup>fl</sup>, L7Cre<sup>−</sup> female mice and Scn8a<sup>fl</sup>ox<sup>fl</sup>,L7Cre<sup>−</sup> male mice to generate Scn8a<sup>fl</sup>ox<sup>fl</sup>,L7Cre<sup>−</sup> (control) and Scn8a<sup>fl</sup>ox<sup>fl</sup>,L7Cre<sup>+</sup> (mutant) mice on the C57BL/6J strain background using the previously described breeding scheme (Levin et al., 2006).

Immunohistochemistry

Mice were intraperitoneally anesthetized with 4% chloral hydrate and transcardially perfused with PBS and then 4% paraformaldehyde (PFA). Brains were extracted, postfixed in 4% paraformaldehyde (PFA) over night in 4% PFA, dehydrated, embedded in paraffin, and sectioned at 3 µm. The sections were submerged into EDTA antigenic retrieval buffer (pH 8.0) and microwaved for antigenic
retrieval. Sections were blocked with 10% goat serum and 0.5% Triton X-100 in 1 × PBS for 20 min. Slides were incubated in primary antibody solution overnight at 4°C. Sections were washed in 3 × PBS and incubated with secondary antibody for 1 h at room temperature, then washed in 3 × PBS and incubated with DAPI. Finally, anti-fluorescence quencher was used to seal the sections (Wuhan goodbio technology CO., LTD, Wuhan, China). Fluorescence images were acquired using a Nikon Eclipse Ti-SR Inverted Microscope. Images were then processed and analyzed using CaseViewer software (3D Histech Ltd, Budapest, Hungary).

The following primary antibodies were used: Calbindin (1:100; ab82812, Abcam), Na±,K1.6 (1:100; ab65166, Abcam), Caspase3 (1:250; GB11532, Wuhan goodbio technology CO., LTD, Wuhan, China). Secondary antibodies were: goat anti-mouse Cy3-IgG (1:300; GB21301, Wuhan goodbio, Wuhan, China), goat anti-rabbit 488 (1:400; GB25303, Wuhan goodbio, Wuhan, China), goat anti-mouse Cy3-1gG (1:300; GB21301, Wuhan goodbio, Wuhan, China), goat anti-rabbit Cy3-IgG (1:300; GB21303, Wuhan goodbio, Wuhan, China).

**MRI Scanning Parameters**
MRIs were collected on a 3-tesla MRI scanner (GE Healthcare, USA) using a standard birdcage head coil. Before the functional MRIs were collected on a 3-tesla MRI scanner (GE Healthcare, China), goat anti-mouse Cy3-IgG (1:300; GB21301, Wuhan goodbio, Wuhan, China), goat anti-rabbit 488 (1:400; GB25303, Wuhan goodbio, Wuhan, China), goat anti-mouse Cy3-1gG (1:300; GB21301, Wuhan goodbio, Wuhan, China).

**Patch Clamp Recording**
Six to eight week old mice were anesthetized with urethane (1.2 g/kg, i.p.) and decapitated. Parasagittal cerebellar slices were prepared with vibratome (VT 1000S, Leica, Germany) with a thickness of 300 μm. The slices were incubated in ACSF (saturated with 95% O2 to 5% CO2) for at least 1 h before recording.

After incubation, the slices were promptly transferred to the recording chamber placed on the stage of a modified upright Olympus microscope and continuously perfused with ACSF (95% O2 to 5% CO2). The patch electrodes (3–7 MΩ) were pulled on a multistage micropipette puller (P-97, Sutter Instrument, USA), and the pipette solution contained (in mM): KCl 140, MgCl2 2, EGTA 10, HEPES 10, Mg-ATP 2, buffered to pH = 7.4 with KOH. After the whole-cell clamp configuration was formatted, the cells were stabilized for 5 min before recording. Then the PCs were depolarized by current steps to evoke action potential at a holding potential of −70 mV.

**TUNEL Staining Assay**
Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was carried out on cerebellar slices according to the manufacturer’s instructions (Roche). TUNEL-positive and DAPI-positive nuclei were examined using a fluorescence light inverted microscope (Nikon Eclipse Ti-SR). The ratio of TUNEL-positive to total DAPI positive cells was calculated in six visual fields at ×100 magnification.

**Behavioral Tests**
All behavioral tests were performed during the light cycle between 07:00 and 19:00. Male and female 5–9-week old mice were used. Similar numbers of male and female mice for each genotype were included. It is possible that genotype effects may have been underestimated or overlooked if they were sex dependent or if the baseline differences between female and male mice increased the variance in the data. Therefore, analysis of variance (ANOVA) models was used to test for the sex dependence of the genotype effects. A three-way repeated measures ANOVA with between-subject factors for genotype and sex and a repeated measure for training day was applied to accelerating rotarod and water-T maze data; a three-way ANOVA with factors for genotype, sex and pairing group was used for three-chambered test, and a two-way ANOVA with factors for genotype and sex was utilized for open-field test, light-dark transition box, elevated plus maze, grooming and male-female interaction. These analyses did not show any measures in which there was a significant effect of sex or a sex-genotype interaction. All behavioral assays were performed with the examiner blind to mouse genotypes. All the videos were analyzed by Smart software (Pan Lab, Harvard Apparatus).

**Motor Function**
**Gait Analysis**
The gait of Scn8a<sup>flax/flax</sup> L7Cre<sup>+</sup> mutant mice was compared with Scn8a<sup>flax/flax</sup> L7Cre<sup>−</sup> control mice by footprint analysis as previously described (Carter et al., 1999). Briefly, to obtain footprints the fore and hind paws of the mice were coated with red and black non-toxic, water-soluble paint, respectively. Footprint patterns were analyzed using a runway (50 cm × 9 cm wide) with white paper on the bottom. The average length and width of the steps were measured.

**Rotarod Test**
Motor coordination and balance were tested with the accelerating rotarod (Panlab, Harvard apparatus) as described previously (Buitrago et al., 2004). Animals were tested over 5 consecutive days, each day consisting of 3–5 trials. The mice were placed on a 3 cm diameter rod which began rotating at 4 rpm and accelerated to 40 rpm over a period of 2 min. Latency to fall was recorded. Animals were tested at 5–6 weeks of age.

**Locomotor Activity and Anxiety**
**Open Field Test**
Exploratory locomotor activity was measured in an open field as previously described (Burne et al., 2005). Each mouse was placed in an opaque open field (30 cm × 30 cm × 30 cm), under dim light. Mice were placed in the chamber for a 15 min period. Distance traveled in 1 min time bins was recorded using a centrally placed video camera and automated video tracking software (Smart software, Pan Lab/Harvard Apparatus). To assess anxiety-related behaviors, the number of entries in the center zone and percent of time in the center of the chamber was also recorded (Bailey and Crawley, 2009). Measurements were taken from animals aged 6 weeks.
Elevated Plus-Maze Tests
The elevated plus maze is a plus-shaped apparatus consisted of two open arms $8 \times 25$ cm and two closed arms $(8 \times 25 \times 25$ cm) with $8 \times 8$ cm central area, elevated 50 cm from the floor. Mice were placed in the central area facing one of the open arms, and allowed to freely explore the maze for 5 min. The number of entries and time spent in open or enclosed arms was measured as a parameter of anxiety-like behavior using an overhead camera and tracking system (SMART®; Panlab, Harvard Apparatus).

Light-Dark Box Test
To further measure anxiety-like responses, the light-dark box test was performed as described previously (Tang et al., 2017). The light/dark box was constructed of plexiglass $(45 \times 27 \times 27$ cm) consisting of two chambers, a black chamber $(18 \times 27$ cm) and a light chamber $(27 \times 27$ cm). Mice were placed into the dark box and allowed to freely move between the light box and dark box for 5 min. The amount of time spent in the dark side and the total number of transitions between the light and dark sides were recorded.

Social Behaviors

Social Interaction
The automated three-chambered social approach task is commonly employed as a standard test for assaying sociability in mice (Yang et al., 2011). The apparatus consists of a rectangular, three-chambered box made from clear polycarbonate. Retractable doorways within the two dividing walls allowed access to the side chambers. The number of entries and time spent in the chambers were automatically recorded using an overhead camera and tracking system (Smart software, Panlab/Harvard Apparatus). The subject mouse was allowed to habituate in the apparatus for 20 min before the sociability test, first for 10 min in the central chamber, followed by 10 min of free exploration in the entire empty arena with both doors open. In the social interaction testing period, a novel object (an inverted wire mesh cup) was placed in one of the side chambers and a novel mouse (with different genetic background matched to the subject mouse by sex and age) was placed inside an identical inverted wire cup in the other side chamber. In the social novelty testing period, another novel mouse was placed inside the empty wire cup. The apparatus was cleaned with 70% ethanol and water between subjects. Time spent interacting with the novel animal and with the object was recorded by an examiner with a stopwatch (Crawley, 2007). Animals were tested between 7 and 8 weeks of age.

Olfaction
We evaluated the ability of the mouse to detect novel odors and social odors as previously described (Yang et al., 2011; Tsai et al., 2012). Animals were placed in an empty, clean observation cage containing a thin layer of clean bedding and a hole on the flat filter top lid for inserting a cotton-tipped swab. Mice were habituated for 30 min with a clean cotton swab and then presented sequentially with non-social odors and social odors. Odors were presented in three consecutive trials per odorant stimulus (2 min per trial) in the following order: water, almond extract (1:100), banana extract (1:100), social odor 1, social odor 2. Social odors were created by swabbing the cotton tip in a zigzag fashion in previously soiled bedding from cages containing unfamiliar gender and age-matched animals the experimental animal had not interacted with. Time spent sniffing the swab with each presentation for each 2 min trial was measured by an investigator with a stopwatch. Measurements were taken from animals aged 7–8 weeks.

Male-Female Interaction
The procedure was adapted from a previously described protocol (Cupolillo et al., 2016). The test for male-female interaction was performed in a clean testing cage (Plexiglass box, $25 \times 40 \times 18.5$ cm). Each male mouse was habituated to the testing cage for 15 min, after which an unfamiliar female of the same genotype was placed into the testing cage with a single layer of corncob bedding. An experimenter blind to the mouse genotype measured the cumulative time (by means of a stopwatch) that the male mouse spent in close contact with the female. Social interaction behavior included close following at the same speed behind the female, touching, nose-to-nose sniffing, anogenital sniffing and/or mouthing and licking the fur of the female. The cumulative time was measured (using a stopwatch) by the investigator and calculated as total time spent in contact. Animals were tested between 7 and 8 weeks of age.

Repetitive Behavior and Reversal Learning

Grooming
Mice were scored for spontaneous repetitive self-grooming behavior as previously described (Silverman et al., 2010a). Each mouse was placed individually into a standard mouse shoebox observation cage with no bedding and a flat filter top lid. After habituation for 10 min, animals were observed for another 10 min. Two mice were scored simultaneously by a trained observer, who was blind to mouse genotype. Cumulative grooming time in the observation period was recorded using stopwatches. Measurements were taken from animals aged 5–6 weeks.

Water T-Maze
To measure reversal learning, the water T-maze was performed as described (Bednar et al., 2002; Tsai et al., 2018). A transparent platform submerged about 1 cm below the surface of the water at one of the short arms of the T-maze and served as an escape for the animals. After 1 day consisting of a habituation swim trial (60 s) with no platform present, mice were given 15 trials a day for 3 consecutive days to learn the location of the platform. After 15 trials on day 4, the platform was changed to the other short arm of the maze. Mice were then tested for 15 additional trials (reversal day 1). Then for 2 subsequent days (reversal days 2 and 3), mice were given 15 trials per day. The number of correct trials and the number of trials required to achieve five consecutive correct trials were recorded. Measurements were taken from animals aged 8–9 weeks.
Statistical Analysis
Data are expressed as mean ± s.e.m., and statistical analysis was carried out using GraphPad Prism 8 software (GraphPad Software Inc., La Jolla, CA, USA). Statistical analyses included Student’s T-test (paired or unpaired), one-way ANOVA followed by Tukey’s post-hoc analysis, two-way repeated measures ANOVA followed by Bonferroni’s post-hoc analysis. P < 0.05 was considered statistically significant.

RESULTS

Abnormal Morphology of Scn8a Mutant Cerebellum
We generated Purkinje cell specific Scn8a knockout mice of genotype Scn8a<sup>flx/flx</sup>,<sup>Cre</sup> <sup>+</sup> (PC Scn8a mutant) mice according to the breeding scheme (Levin et al., 2006). As shown in Supplementary Figure S1, PC Scn8a mutant mice had reduced expression of Nav 1.6 in Purkinje cells compared with control mice, whereas expression remained high in granule cells and neurons (stellate cells and basket cells) of the molecular layer in PC mutant mice.

Consistent with earlier reports (Sprunger et al., 1999; Levin et al., 2006), cerebellar malformations were not detected in both 2 and 4-month-old PC Scn8a mutant mice. Calbindin staining indicated that the Purkinje cells in the mutant mice retained the regular orientation of dendrites present in control animals (Supplementary Figure S2). However, calbindin staining was noted in the granule cell layer of PC Scn8a mutant mice (Supplementary Figure S2, arrows). This pattern of labeling has previously been described in Scn8a<sup>med−/med</sup> (A1071T) mice (Dick et al., 1985) and Scn8a<sup>flx/flx,L7Cre</sup> mice (Levin et al., 2006) and may reflect axonal swelling. We revealed that PC density, size of soma, and thickness of the molecular layer were normal at both 2 and 4 months of age (Figure 1). However, by 5 months of age, there was a reduction in the density of Purkinje cells and the thickness of molecular cell (P < 0.01), but the size of Purkinje cells retained normal. At 9 months of age there was a significant reduction in the thicknesses of the molecular layer and both the size and the density of Purkinje cells was significantly reduced (P < 0.001. n > 3 per group, two-way ANOVA, Bonferroni’s post-hoc analysis). Overall brain volume was decreased at 9 months of age, as evident in the whole-mount brain (Figure 2A). MRI measurements revealed reduced area of the cerebellum of the mutant mice in both axial and coronal views (Figures 2B–D).

Increased Apoptosis in the Cerebellum of Scn8a PC Mutant Mice
To determine whether neurodegeneration could account for the observed cerebellar atrophy, we adopted TUNEL staining to quantify apoptosis. The number of TUNEL-positive cells was significantly increased in PC Scn8a mutant mice in both the molecular layer (Figures 2E,F) and cerebellar deep nuclei (Figures 2G,H) (P < 0.001; n = 4 for mutants and control, unpaired student’s t-test).

Reduced Excitability in Scn8a Mutant PCs
It was previously demonstrated that partial or complete loss of Nav1.6 from cerebellar Purkinje cells reduces excitability and repetitive firing (Raman et al., 1997; Khalilq et al., 2003; Levin et al., 2006). To examine excitability of PCs in our mice, evoked APs were recorded by whole-cell patch clamp. Repetitive firing spikes were evoked by current injection of 200 pA with 500 ms duration. The frequency of repetitive firing was inhibited in Scn8a mutant PCs compared with that in control (Supplementary Figures S3A,B). The mean frequency of repetitive firing at each current injection from 50 to 400 pA was significantly reduced in Scn8a mutant PCs (Supplementary Figure S3C). These results confirm the previous evidence that loss of Scn8a inhibits the excitability of PCs (Levin et al., 2006).

Impaired Motor Coordination in PC Scn8a Mutant Mice
By 6–8 weeks of age, PC Scn8a mutant mice developed a mildly ataxic, waddling gait that appeared to be caused by poor hindlimb coordination. However, at 6 weeks of age, mutant and control mice had comparable fore-base width and no difference in stride length and hind-base width (Figures 3A,B) (P > 0.05, Student’s unpaired t-test, control 6wk n = 10, mutant 6wk n = 10). However, at 12 weeks of age, PC Scn8a mutant mice displayed a narrower stride length (P = 0.022) and a wider hind-base width (P = 0.007) (control n = 6, mutant n = 9), demonstrating a deficit in motor coordination.

Consistent with previous work (Levin et al., 2006), mutant mice demonstrated impaired performance on the rotorod test (Supplementary Figure S4). At 5 weeks of age, the latency to fall was significantly different between wildtype and mutant mice [two-way repeated measures ANOVA F<sub>(1,18)</sub> = 27.99, Bonferroni post-hoc, P < 0.0001, n = 9 for control, n = 11 for mutant]. While control mice remained on the rod longer during each of 5 daily sessions of training, mutant mice had shorter latency to fall on day 1 (P < 0.001) and did not improve with time. These findings further demonstrate impaired motor coordination and poor motor learning in mice with ongoing PC loss.

Increased Anxiety-Like Behavior in PC Scn8a Mutant Mice
Patients harboring loss of function variants of Scn8a exhibit neuropsychological abnormalities including emotional instability, anxiety and attention deficit hyperactivity disorders (Trudeau et al., 2006; Wagnon et al., 2017). We therefore carried out behavioral testing of 6-week-old PC Scn8a mutant mice to assess their anxiety level.

The open field exploration test is a behavioral assay widely used to evaluate locomotor responses to novel environments in rodents. Representative tracks are shown in Figure 3C. During a 15-min test, the distance traveled by mutant mice in the open field during a 1 min time bin was similar to controls [Figure 3D, two-way ANOVA, Bonferroni’s post-hoc analysis, F<sub>(1,17)</sub> = 0.6760, P = 0.4223]. The total distance traveled...
FIGURE 1 | PC Scn8a mutant mice exhibit histological changes in cerebellum. (A,B) Abnormal PC morphology and gradual reduced PC number and molecular layer thickness in Scn8a mutant cerebellum (Scale bar upper row 200 µm, bottom row 100 µm). (C,D) The quantification of PC density and the quantification of PC soma area was performed in the IV–V. PC loss was not apparent until 5 months of age, and showed ongoing loss till 9 months (C). At 9 months, mutant Purkinje cell size decreased significantly (D). (E) The thickness of the molecular layer (ML) was normal in mutant cerebellum at 4 months of age, whereas it decreased afterward at 5 months (n ≥ 3 per group. Data shown are means ± SEM. **P < 0.01, ***P < 0.001, two-way ANOVA, Bonferroni’s post-hoc analysis).

FIGURE 2 | Reduced cerebellar volume and increased apoptosis in Scn8a PC mutant cerebellum. (A) Representative whole-mount images of control and PC Scn8a mutant brains at 9 months of age. The mutant cerebellum appears smaller than control. (B) Magnetic resonance imaging T2 axial and coronal images of the brain showing cerebellar volume loss in the mutant mice at 9–10 months of age (scale bar 10 mm). (C,D) Measurement of the cerebellar area of Scn8a mutant and control mice using T2-weighted 3.0 T MR imaging. Quantitative analysis revealed a significant reduction in cerebellar area in mutant animals in both axial (C) and coronal area (D) (Mean ± SEM; n = 5 per group; **P < 0.01, ***P < 0.001, Student’s unpaired t-test). (E,G) Sections of cerebellum of control and PC Scn8a mutant mice at 9–10 months of age labeled with TUNEL showing apoptosis in the molecular cell layer and cerebellar nuclei (Scale bar: 100 µm). (F,H) Percentage of TUNEL-positive cells per unit area of cerebellar molecular layer and deep cerebellar nuclei in the PC Scn8a mice compared with controls (n = 4 for each group, ***P < 0.001, Student’s unpaired t-test).
was also similar in controls and mutants [Figure 3E, Student unpaired t-test, $t_{(17)} = 0.822$, $P > 0.05$]. However, PC Scn8a mutants spent significantly less time in the center of the open field [Figure 3F, Student unpaired t-test, $t_{(17)} = 3.254$, $P = 0.0047$] and had fewer entries into the center [Figure 3G, Student unpaired t-test, $t_{(17)} = 3.982$, $P < 0.001$] (control $n = 8$, mutant $n = 11$).

As an independent test of anxiety, we used the light/dark box transition task (Bourin and Hascoet, 2003). The number of entries into the light compartment [$t_{(25)} = 5.297$, $P < 0.0001$] was significantly lower in mutant mice.
and the time spent in the bright area \([t_{(23)} = 4.129, P < 0.001]\) were significantly decreased in PC Scn8a mutant mice, indicating increased innate anxiety-like behavior (control \(n = 15\), mutant \(n = 13\) Student's unpaired \(t\)-test) (Figures 4A–C).

To further assess anxiety, we analyzed behavior on an elevated plus maze (Hogg, 1996) (Figures 4D–F). As in the open field, mutant mice demonstrated decreased total time spent in the open arms \([t_{(27)} = 4.191, P < 0.001]\), and a reduced number of entries into the open arms of the elevated plus maze \([t_{(27)} = 3.532, P = 0.0015]\) (control \(n = 13\) and mutant \(n = 16\), Student's unpaired \(t\)-test).

Taken together, these assays indicate that mutant mice display increased anxiety compared with control littermates.

**Altered Social and Repetitive Behaviors in PC Scn8a Mice**

A three-chambered apparatus was used to measure social approach and social novelty (Figure 5). Compared with control mice, PC Scn8a mutant mice spent less time in the side chamber with the novel mouse and more time with the novel object (Figure 5B, \(P > 0.05\)). Control mice spent significantly more time with the novel mouse than with the novel object \([P < 0.001,\)
Abnormal social behavior in PC Scn8a mutant mice. (A) Representative heat maps showing time spent by Control mice (upper panel) and PC Scn8a mutant mice (lower panel) at each location of the three-chambered apparatus during the test. (B,D) In the social approach test, WT mice spent more time in the chamber with the stranger animal than in the chamber with the inanimate object (B), and spent more time interacting with novel animal (D) in comparison with a novel object, whereas PC Scn8a mutant mice show no preference. (C,E) PC Scn8a mutant mice show no preference for the chamber with the novel animal in comparison with that with a familiar animal in an assay of social novelty (C), and spent similar time interacting with both the novel and familiar animals (E). This is in distinct contrast to control mice. Control: n = 16; Mutants: n = 17, ***P < 0.001, two-way ANOVA, Bonferroni’s post-hoc analysis.

We examined the amount of time each experimental mouse spent interacting with the novel mouse or the object through sniffing [Figure 5D]. Genotype×chamber interaction, $F_{(1,31)} = 7.420$, $P = 0.010$; Chamber effect $F_{(1,31)} = 11.17$, $P = 0.0022$; Genotype effect $F_{(1,31)} = 1.035$, $P = 0.3168$, two-way repeated measures ANOVA, Bonferroni’s post-hoc analysis]. While control mice showed more interest in interacting with the novel mouse ($P < 0.001$), mutant mice spent a comparable amount of time sniffing the novel mouse and the novel object ($P > 0.05$).

When the inanimate object was replaced with another novel mouse, control mice spent more time in the chamber containing a novel mouse than in the chamber with the familiar mouse.
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FIGURE 6 | Abnormal social and repetitive behaviors and impaired reversal learning in PC Scn8a mutant mice. (A) Mutant mice spent comparable time sniffing non-social stimuli whereas less time sniffing social odor cues ($n = 11$ per group). (B) Reduced sociability of KO mice is indicated by reduced time interacting with a female conspecific (Control: $n = 8$; Mutant: $n = 10$). (C) PC Scn8a mutant mice spent more time self-grooming (Control: $n = 10$; Mutant: $n = 11$). (D,E) On Day 1 to Day 3, mutants display normal acquisition learning of the escape platform location in the water T maze as indicated by total correct trials (D) and trials needed for 5 consecutive correct responses (E). However, on reversal day 1 and day 2, PC Scn8a mutant mice have significantly fewer correct trials and take more trials to achieve five consecutive correct responses (Control: $n = 9$, Mutant: $n = 12$). RD, Reversal Day. Data shown are means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Two-way ANOVA, Bonferroni post-hoc analysis in (A). Student’s unpaired t-test in (B–E).

(\textit{P} < 0.001), demonstrating normal sociability (genotype $\times$ chamber interaction $F_{(1,31)} = 6.596, P = 0.0153$, Chamber effect $F_{(1,31)} = 22.62, P < 0.0001$, Genotype effect $F_{(1,31)} = 3.479, P = 0.0717$, two-way repeated measures ANOVA, Bonferroni’s post-hoc analysis) (Figure 5C). In contrast, the PC Scn8a mutants failed to show any preference between the two social chambers ($P > 0.05$). Control mice also spent more time sniffing the novel animal over the familiar animal (Figure 5E, \textit{P} < 0.001), while mutant mice failed to show such preference ($P > 0.05$) (genotype $\times$ chamber interaction $F_{(1,31)} = 9.777, P = 0.0038$, chamber effect $F_{(1,31)} = 33.51, P < 0.0001$, genotype effect $F_{(1,31)} = 0.02699, P = 0.8706$, two-way repeated measures ANOVA, Bonferroni’s post-hoc analysis).

It is thought that impaired discrimination of social olfactory cues may contribute to social deficits in mice (Silverman et al., 2010b). We used a social olfaction assay to assess interaction with social olfactory cues (Figure 6A). When mice were first presented with three non-social odor cues (water, almond extract and banana extract), there were no significant differences between mutant and wildtype mice ($P > 0.05, n = 11$ Control and 11 mutants), demonstrating normal olfactory function. When social odors (social A and social B) were applied, PC Scn8a mutant mice spent less time sniffing the cotton tips than control mice, suggesting a lack of interest in social odor stimuli ($n = 11$ per group, two-way repeated measures ANOVA $F_{(2,20)} = 15.25$ Bonferroni post-hoc, $P < 0.0001$ for Social A1, $P = 0.001$ for Social A2, $P < 0.0001$ for Social B1, $P = 0.036$ for Social B2).

Social behavior was further tested by assessing social interaction with a female conspecific mouse. Compared with control mice, there was a significant reduction in the time spent sniffing, allogrooming, mounting, or following the female by male PC Scn8a mutant mice [Student’s $t$-test $t_{(18)} = 2.23, P = 0.038$; Control $n = 8$; Mutant $n = 12$] (Figure 6B).

One of the diagnostic behavioral symptoms of autism is repetitive behavior. We analyzed grooming as an index for stereotyped, repetitive behaviors. PC Scn8a mutant mice engaged in much longer bouts of self-grooming than control mice...
Perseveration (Behavioral Inflexibility) in PC Scn8a Mutant Mice

ASD patients often show inflexible and rigid behavior and thinking (Bralten et al., 2018). To evaluate behavioral flexibility, we used the reversal water-T maze assay (Figures 6D, E). During the first 3 days of training, while the platform was in the right arm of the maze, both groups of mice performed equally well and learned the location of submerged platform. On the first reversal day with the platform moved to the left arm of the maze, PC Scn8a mutant mice incorrectly visited the right arm more frequently than control mice. Mutant mice also needed more trials to achieve 5 consecutive correct responses. Comparable results were observed on Reversal day 2. [Reversal Day 1, number of correct trials: Student’s t-test $t_{(19)} = 5.097, P < 0.0001$; number of trials prior to five consecutive correct trials: Student’s t-test $t_{(19)} = 3.221, P = 0.0045$; Reversal Day 2: number of correct trials: Student’s t-test $t_{(19)} = 3.48, P = 0.0025$; number of trials prior to five consecutive correct trials: Student’s t-test $t_{(19)} = 3.467, P = 0.0026$. By the third reversal day, the mutant mice behaved similarly to the control mice in number of correct trials and number of trials prior to 5 consecutive correct responses.

DISCUSSION

The first link between human disease and SCN8A mutation was obtained in 2006 in a study of a small pedigree in which heterozygous carriers of a loss-of-function SCN8A mutation exhibited a range of phenotypes including ataxia, cognitive deficits, and emotional instability (Trudeau et al., 2006). Since the development of next generation sequencing, many pathogenic variants of SCN8A have been identified in patients with a spectrum of neurodevelopmental disorders, and some genotype-phenotype correlations have emerged. SCN8A missense variants with gain-of-function channel properties are associated with developmental epileptic encephalopathy with early onset of severe seizures, hypotonia, and paroxysmal dyskinesia. In contrast, loss of function mutations of SCN8A can cause autism or intellectual disability without seizures (Larsen et al., 2015; Gertler and Carvill, 2019; Liu et al., 2019; Meisler et al., 2021). In a recent study by Wong et al. (2021), they generated a novel Scn8a mouse model carrying the human R1620L mutation (with both gain- and loss-of-function effects) which exhibited a range of behavioral abnormalities, including hyperactivity, impaired learning and memory and social deficits. These findings suggest that SCN8A dysfunction may contribute to other neurological and neuropsychiatric disorders. Neuropsychiatric comorbidities were not previously studied in Purkinje cell specific Scn8a knockout model, and the role of reduced Nav1.6 in cerebellar function was incompletely characterized.

In this study, we provide evidence that SCN8A in cerebellar PCs has a key role in mechanisms involved in ASDs. We show that mice of C57BL/6J strain background with selective disruption of Scn8a in PCs display behavioral traits related to neuropsychiatric abnormalities such as ASDs and anxiety, associated with graded loss of PCs and progressive cerebellar atrophy.

One of the main symptoms of cerebellar dysfunction in humans is ataxia (Schniepp et al., 2017). Our results revealed that PC Scn8a mutant mice exhibit deficits in motor coordination and motor learning in the rotarod test. Gait analysis demonstrated wide base and ataxic gait. Similar features are also seen in patients with loss of function mutation of SCN8A (Trudeau et al., 2006), and in patients with ASD and other murine models of ASD (Fatemi et al., 2012; Tsai et al., 2012; Reith et al., 2013; Cupolillo et al., 2016).

PC Scn8a mutant mice were previously shown to be impaired in delay eyeblink conditioning (Woodruff-Pak et al., 2006), an additional connection to cerebellar dysfunction and autism. Eyeblink conditioning is affected in the general ASD patient population, and is viewed as a biomarker for ASD (Oristaglio et al., 2013; Welsh and Oristaglio, 2016). Eyeblink-conditioning defects appear more often in mouse autism models than in non-autism-like phenotypes (Kloth et al., 2015). Given the similarity of the PC Scn8a mutant mice to other autism models with regards to motor deficit, delay eye-blink conditioning impairment and electrophysiological changes in Purkinje cells, it is not surprising that PC Scn8a mutant mice also exhibit ASD-relevant social deficits.

Purkinje cell loss is the most consistent presentation in postmortem studies of ASD patients, with 35–95% fewer cerebellar Purkinje cells in ASD brains than controls (Whitney et al., 2009; Wegiel et al., 2014; Mosconi et al., 2015). Purkinje cells are recognized as key cells mediating autism-like phenotypes in mice (Fatemi et al., 2012; Tsai et al., 2012; Reith et al., 2013; Cupolillo et al., 2016). PC Scn8a mutant mice also displayed abnormalities in the composition of the cerebellum. The thickness of molecular layer as well as the density of Purkinje cells were comparable at early age. However, Purkinje cell beginning to lose between 4 and 5 months of age, with an ongoing and significantly decreased in both Purkinje cell density and thickness of molecular layer in PC Scn8a mutant mice by 9 months of age (Figure 1). Progressive PC loss is accompanied by cerebellar atrophy indicated by both gross anatomy and MR imaging of cerebellum.

Previous studies demonstrated a relationship between cerebellar function and behavior including sociability (novel mouse vs. novel object), social preference (familiar mouse vs. novel mouse), social odor preference (conspecific urine vs. water), and male-female interaction (Tsai et al., 2012; Cupolillo et al., 2016). Our demonstration of social deficits in three-chambered tests were highly consistent with earlier studies in other cerebellar genetic mouse models of autism (Tsai et al., 2012; Reith et al., 2013). PC Scn8a mutant mice also displayed reduced responses to female social cues in a male-female reciprocal social interaction context. Mutant mice spent significantly less time sniffing the social odors than control mice in the context of olfaction test.

Autism and anxiety disorders are frequently comorbid with each other (Adams et al., 2020; Baribeau et al., 2020, 2021), and autistic and anxious traits are highly correlated (Ha et al., 2016; Tatsukawa et al., 2019). Likewise, co-existing anxiety-like

(Figure 6C) [n = 14 per group, Student’s t-test $t_{(26)} = 2.48, P = 0.019$].
behaviors were observed in the Scn8a PC mutant mice. Anxiety-like behaviors in the mutant mice included reduced exploration behavior in the center in the open field test and reduced non-social anxiety in the elevated plus maze and light-dark transition test. Similar anxiety-like behaviors also presented in autistic mice with Purkinje cell-specific deficiency of Shank2 (Ha et al., 2016) and Pia/p mice (Winkler et al., 2020). McKinney et al. (2008) investigated mice heterozygous for a null mutation of Scn8a (Scn8a+/−) and demonstrated avoidance of well-lit, open environments as well as pronounced stress-induced coping behavior. Impaired Purkinje cell firing was also demonstrated in heterozygous Scn8a+/− null mice (Raman et al., 1997; Khaliq et al., 2003). Our data on the PC-specific KO of Scn8a suggest that the enhanced anxiety-like behaviors in Scn8a heterozygous null mice may be mediated by altered output signaling from the cerebellum.

It has been observed that cognitive inflexibility and cerebellar pathology co-occur in psychiatric disorders (e.g., autism, schizophrenia, addiction). Recent studies using lurcher mutant mouse, which lose 100% of their Purkinje cells postnatally or lurcher mouse chimeras, which lose varying numbers of Purkinje cells, suggested impairment in behavioral flexibility, reversal learning and increased repetitive behaviors, as well as higher level cognitive processes (Dickson et al., 2010, 2017; Martin et al., 2010). Inflexibility to change in routine can be viewed as a form of perseverative behavior, and assessments of reversal learning are used as a behavioral endpoint in studies of rodent models. In water T-maze tests, the initial acquisition of the behavior was normal but PC Scn8a mutant mice exhibited impaired reversal learning. Autistic patients often exhibit repetitive behaviors that refuse to change (Hollander et al., 2003). This feature can be recapitulated by rodent grooming behaviors (Crawley, 2004). PC Scn8a mutant mice displayed increased repetitive behavior, including self-grooming. All these results, together with previous findings in lurcher mutant mouse and lurcher chimeras, indicated that cerebellar pathology may play a causal role in the generation of repetitive behaviors and cognitive inflexibility.

Previous research found the levels of Bcl-2 and P53 protein, two important markers of apoptosis, were increased in cerebellum of autistic subjects compared with controls (Araghi-Niknam and Fatemi, 2003). Dong et al. (2018) demonstrated elevation of ER stress signals, oxidative stress, and apoptosis in the molecular layer of the autistic cerebellum. Deep cerebellar nuclear cells to which Purkinje cells project are abnormal in ASD, showing enlargement during childhood followed by reduction in size and number during adolescence and adulthood (Bauman, 1991). Using the TUNEL assay, we found increased cell apoptosis in molecular layer of PC Scn8a mutant mice. Interestingly, we also noted obvious cell apoptosis in neurons of the deep cerebellar nuclei.

In Purkinje cells lacking Nav1.6 channels, both spontaneous firing activity and high frequency discharge are impaired (Raman et al., 1997; Khaliq et al., 2003; Levin et al., 2006). We also confirmed the reduced excitability in Scn8a mutant PCs. Since PC provide the only output from the cerebellar cortex, the reduced firing of Purkinje cells observed in many ASD-like mouse models (Tsai et al., 2012; Cupolillo et al., 2016; Yang et al., 2020) may disinhibit the downstream deep cerebellar nuclei, which gate the outgoing information to the thalamus, basal ganglia, and neocortex (Sunberg and Sahin, 2015), potentially influencing integrative networks (Bostan and Strick, 2018). Neurons of the deep cerebellar nuclei receive major basal and driven inhibition from PC, but they are also spontaneously active, producing action potentials even without excitation (Zheng and Raman, 2011). Therefore, we suggest that loss of Scn8a in Purkinje cells might disrupt Purkinje-mediated inhibition and increase firing in the deep cerebellar nuclei. The resulting elevated spontaneous activity of cerebellar nuclei could result in oxidative stress and induced apoptosis. Further research is needed to elucidate the mechanism connecting Purkinje cell loss to cerebellar apoptosis. However, it should be noted that our study was performed on a single inbred strain, C57BL/6J. Therefore, our understanding of these effects is limited to this single genome type. However, recent studies showed that results from studies that utilize diverse genetic backgrounds are a better model of complex disease across individuals and are more likely to generalize across patient populations (Neuner et al., 2019). Therefore, further research is needed to validate the effects of loss of Scn8a expression in Purkinje cells on cognitive and autistic behavior changes using different inbred strains.

**CONCLUSION**

We have demonstrated that mice with loss of Scn8a in Purkinje cells provide a new model of features of ASD. Mice of C57BL/6J strain with homozygous loss-of-function of Nav1.6 channels in Purkinje cells exhibit motor deficits and autistic traits such as deficits in social interaction, stereotyped behaviors and anxiety-like behaviors, demonstrating an ASD-like phenotype with strong resemblance to other Purkinje cell-dependent models of ASD (Fatemi et al., 2012; Tsai et al., 2012; Reith et al., 2013; Cupolillo et al., 2016). Together with previously reports of reduced spontaneous and repetitive firing (Raman et al., 1997; Levin et al., 2006), learning deficits in the rotarod test (Levin et al., 2006) and delay eyelink conditioning impairment (Woodruff-Pak et al., 2006), these studies support PC Scn8a mutant mice as a model that reproduces many symptoms of patients with CN8A loss of function mutations.

Our study has demonstrated the specific contribution of loss of Scn8a in cerebellar Purkinje cells to behavioral deficits characteristic of ASD. These results provide novel insights into mechanisms contributing to the pathogenesis of ASDs. Future research to unravel the pathogenesis underlying molecular and cellular alterations will improve our understanding of the still enigmatic fields of ASD and anxiety, and open new avenues for molecular diagnosis and therapy.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author/s.
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2022.822129/full#supplementary-material

**SUPPLEMENTARY FIGURE S1** Sagittal cryosection of cerebellar Nav1.6 staining (green) and calbindin staining (red) showing the deficiency of Nav1.6 expression in PC of the Scn8a mutant mice. Nav1.6 staining in control Purkinje cells are indicated by yellow arrows. Scale bar 50 µm.

**SUPPLEMENTARY FIGURE S2** Scattered calbindin staining in the granule cell layer along the proximal segment of PC axons of Purkinje Scn8a mutant mice (arrows). Scale bar 100 µm.

**SUPPLEMENTARY FIGURE S3** Reduced excitability of Scn8a mutant PCs. *(A&B)* The evoked action potential in control and Scn8a mutant PCs in response to depolarization of 200 pA. *(C)* Comparison of the mean frequency of repetitive firing at command potentials *(n = 10 for both control and Scn8a mutant groups). All data were presented as mean ± SEM. **“P < 0.001.”**

**SUPPLEMENTARY FIGURE S4** Motor coordination and motor learning assessed on an accelerating rotarod. PC Scn8a mutants displayed decreased time on an accelerating rotarod and no improvement with training *(Control: n = 9, Mutant: n = 11, P < 0.001, two-way ANOVA, Bonferroni’s post-hoc analysis)*.
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