Mice with *GNAO1* R209H Movement Disorder Variant Display Hyperlocomotion Alleviated by Risperidone

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Highlights

- *Gnao1* $^{+/R209H}$ mice display hyperlocomotion and gait abnormalities.
- *Gnao1* $^{+/R209H}$ mice do not show enhanced seizure susceptibility as was observed in two other *Gnao1* mutant mouse models
- Similar to patients with *GNAO1*$^{+/R209H}$ mutations, the *Gnao1*$^{+/R209H}$ mutant mice show only a movement phenotype and no epilepsy
- Hyperlocomotion of *Gnao1* $^{+/R209H}$ mice was alleviated by risperidone

Abbreviations

AC: Adenylyl Cyclase

cAMP: Cyclic adenosine monophosphate

EIEE17: Early infantile epileptic encephalopathy 17

GOF: Gain-of-function

GPCR: G-protein coupled receptor

LOF: Loss-of-function

NEDIM: Neurodevelopment disorder with involuntary movements

RNP: Ribonucleoprotein

tracrRNA: Trans-activating crRNA

crRNA: CRISPR RNA

ssODN: Single-stranded oligodeoxynucleotide

DSB: Double strand DNA break

PAM: Protospacer-adjacent motif

Abstract

Neurodevelopmental delay with involuntary movements (NEDIM, OMIM: 617493) is a severe, early onset neurological condition characterized by a delay in psychomotor development, hypotonia, and hyperkinetic involuntary movements. Heterozygous *de novo* mutations in the *GNAO1* gene cause NEDIM. $G\alpha_o$, the gene product of *GNAO1,* is the alpha subunit of G_o, a member of the heterotrimeric $G_{i/o}$ family of G-proteins. G_o is found abundantly throughout the brain but the pathophysiological mechanisms linking $G\alpha_o$ functions to clinical manifestations of *GNAO1*-related disorders are still poorly understood. One of the most common mutant alleles among the *GNAO1* encephalopathies is the c.626G>A or p.Arg209His (R209H) mutation. We developed heterozygous knock-in *Gnao1*$^{+/R209H}$ mutant mice using CRISPR/Cas9 methodology to assess whether a mouse model could replicate aspects of the NEDIM clinical pattern. Mice carrying the R209H mutation exhibited increased locomotor activity and a modest gait abnormality at 8-12 weeks. In contrast to mice carrying other mutations in *Gnao1*, the *Gnao1*$^{+/R209H}$ mice did not show enhanced seizure susceptibility. The atypical neuroleptic risperidone has shown efficacy in a patient with the R209H mutation. It also alleviated the hyperlocomotion phenotype observed in our mouse model but suppressed locomotion in WT mice as well. In this study, we show that *Gnao1*$^{+/R209H}$ mice mirror elements of the patient phenotype and respond to an approved pharmacological agent.

Introduction

$G\alpha_o$ is the alpha subunit of the heterotrimeric G-protein and is the most abundant heterotrimeric G protein in the central nervous system comprising 1% of mammalian brain membrane protein. Mutations in *GNAO1*, which encodes $G\alpha_o$, have been linked to two distinct neurological conditions. In 2013, four children with early infantile epileptic encephalopathy were identified with mutations in *GNAO1* [1]. Since then, a growing number of patients presenting with epilepsy and/or hyperkinetic movement disorders have been found to exhibit *de novo* mutations in *GNAO1* [2, 3]. It was recognized in 2016 that some *GNAO1* mutations result in movement disorders without epilepsy [4, 5]. To date there are over 70 published cases of children with mutations in *GNAO1* presenting with early infantile epileptic encephalopathy (EIEE17: OMIM 615473) and/or neurodevelopmental disorder with involuntary movements (NEDIM: OMIM 617493) [1, 4-34].

More than forty pathological variants of *GNAO1* have been reported. Using a cell-based biochemical signaling assay, we classified many of those $G\alpha_o$ variants for their ability to support inhibition of cAMP production in transfected HEK293 cells [2]. Some mutant $G\alpha_o$ proteins were unable to support receptor-mediated inhibition of cAMP having a loss-of-function (LOF) mechanism. The LOF mutants were associated with epilepsy [2]. In contrast, mutations resulting in enhanced cAMP inhibition (gain-of-function, GOF) or those which supported normal cAMP regulation (NF) were generally associated with movement disorders, though some patients with these mutations also have a relatively mild seizure disorder [33].

To permit mechanistic and preclinical drug testing studies, we had previously created a mouse model with a *GNAO1* GOF mutation, G203R, that was identified in patients who showed both epilepsy and movement disorders [4]. As predicted, the *Gnao1*$^{+/G203R}$ (G203R) mutant model exhibited motor coordination and gait abnormalities as well as enhanced seizure susceptibility in pentylenetrazol (PTZ) kindling studies [35]. The R209H mutations is one of the most common pathogenic *GNAO1* mutations [9]. Patients with *de novo*, heterozygous R209H mutations in *GNAO1* display severe choreoathetosis and dystonia but do not present with seizures [5, 7, 12, 13, 17, 18]. Interestingly, the R209H mutation was found to have essentially normal function for

cAMP inhibition in HEK 293T cells. Despite this normal function in an *in vitro* assay, it causes a severe form of movement disorder in patients, often requiring intensive care unit admission [7, 13]. This discrepancy between the normal functionality *in vitro* and its clear clinical pathology made the *R209H* mutation of substantial interest for *in vivo* physiological studies to better understand its mechanism.

We used a battery of behavioral tests to measure motor skills in heterozygous *Gnao1*$^{+/R209H}$ mutant mice as well as performing PTZ kindling studies to assess seizure susceptibility. As expected *Gnao1*$^{+/R209H}$ mice did not show enhanced seizure susceptibility to PTZ kindling studies. Male and female *Gnao1*$^{+/R209H}$ mice both displayed significant hyperactivity in an open field assessment. This finding was surprising as our mice in our previous *GNAO1*-related movement disorder model, *Gnao1*$^{+/G203R}$ did not show significant differences on the open field test [35]. This difference in movement phenotype is consistent with the wide heterogeneity of movement patterns displayed by patients with GNAO1 mutations[36-42] .

Having a mouse model with a strong movement phenotype will facilitate mechanistic studies of *GNAO1* mutants and it allowed us to begin allele-specific preclinical drug testing. The neuroleptic risperidone was reportedly beneficial in a patient with R209H [7]. Here we show that risperidone attenuates hyperactivity in our animal model. This suggests that risperidone or related agents may be beneficial for *GNAO1* patients with the R209H mutation.

Experimental Procedures

Animals

Gnao1 $^{+/R209H}$ mice on a C57BL/6J background were generated in the MSU Transgenic and Gene Editing Facility (https://tgef.vprgs.msu.edu) as described below. Mice (8-12 weeks old) were housed on a 12-hour light/dark cycle, with *ad libitum* access to food and water. All experiments were performed in accordance with NIH guidelines; protocols were approved by the Michigan State University Institutional Animal Care and Use Committee.

Generation of Gnao1 R209H edited mice

Mutant *Gnao1*$^{+/R209H}$ mice were generated via CRISPR/Cas9 genome editing on a C57BL/6J genomic background. CRISPR gRNA selection and locus analysis were performed using the Benchling platform (Benchling, Inc. San Francisco, CA.). A gRNA targeting exon 6 of the *Gnao1* locus (ENSMUSG00000031748) was chosen to cause a double strand break (DSB) 3bp downstream of codon R209. A single stranded oligodeoxynucleotide (ssODN) carrying the R209H mutation CGC > CAC with short homology arms was used as a repair template (Figure 1 and Table 1). Ribonucleoprotein (RNP) complexes consisting of a synthetic crRNA/tracrRNA hybrid and Alt-R® S.p. Cas9 Nuclease V3 protein (Integrated DNA Technologies, Inc. Coralville, IA), were used to deliver CRISPR components along with the ssODN to mouse zygotes via electroporation as previously described [2, 35]. Edited embryos were implanted into pseudo-pregnant dams using standard techniques. Resulting litters were screened by PCR (Phire Green HSII PCR Mastermix, F126L, Thermo Fisher, Waltham, MA.), T7 Endonuclease I assay (M0302, New England Biolabs Inc.) and Sanger sequencing (GENEWIZ, Inc. Plainfield, NJ) for edits of the target site.

Genotyping and Breeding

Studies were done on N1 R209H heterozygotes with comparisons to littermate controls. To generate *Gnao1* $^{+/R209H}$ heterozygotes (N1 backcross), 2 founder *Gnao1* $^{+/R209H}$ mice, 1 male and 1 female, were crossed with WT C57BL/6J mice obtained directly from The Jackson Laboratory (Bar Harbor, ME).

DNA was extracted by an alkaline method (26) from ear clips done before weaning. PCR products were generated with primers flanking the mutation site (Fwd 5'

GGACAGGTGTCACAGGGGAT 3'; 5' ACTGGCCTCCCTTGGCAATA 3'). To produce a 375 base pair (bp) product, reaction conditions were: 0.8 µl template, 4 µl 5x Promega PCR buffer, 0.4 µl 10mM dNTPs, 1 µl 10 µ M Forward Primer, 1 µl 10 µ M Reverse Primer, 0.2 µl Promega GoTaq and 12.6 µ l DNase free water (Promega catalog # M3005, Madison WI). Samples were denatured for 4 minutes at 95° C then underwent 32 cycles of PCR (95° C for 30 seconds, 63° C for 30 seconds, and 72° C for 30 seconds) followed by a 7 minute final extension at 72° C. Ethanol precipitation was done on the PCR products and then samples were sent for Sanger sequencing (GENEWIZ, Inc. Plainfield, NJ).

Behavioral Assessment

Male and female *Gnao1* $^{+/R209H}$ mice (8-12 weeks of age) and their *Gnao1* $^{+/+}$ littermates underwent a battery of behavioral testing to assess motor phenotype as described previously [43]. Before each experiment, mice were acclimated for 10 minutes to the testing room. Experiments were performed by two female researchers. All behavioral studies were done by individuals who were blind to the genotype of the animals until completion of data collection.

Open Field

The open field test is frequently used to assess locomotion, exploration and anxiety [44, 45]. The test was conducted in Fusion VersaMax clear 42 cm x 42cm x 30cm arenas (Omnitech Electronics, Inc., Columbus, Ohio). *Gnao1* $^{+/R209H}$ mice of either sex and their littermates were placed in the arena for 30 minutes. Using the Fusion Software, we evaluated distance traveled (cm) in terms of novelty, sustained, and total movement corresponding to the first 10 minutes, 10-20 minutes and total of 30 minutes. As a potential measure of anxiety, the fraction of time spent in the center was assessed. The center area was defined as the 20.32cm x 20.32cm area within the middle of the arena.

Rotarod

To assess motor skills, we used the Economex accelerating RotaRod (Columbus Instruments, Columbus OH). The protocol occurred over a two-day period. On day 1, mice were

trained for three 2-min training sessions, with 10 minutes between each training trial. During the first two sessions, the rotarod maintained a constant rotational speed of 5 rpm. The third training trial started at 5 rpm and accelerated at 0.1 rpm/sec for 2 minutes. The following day, mice ran three more trials with a 10-min break in between: two more 2-min training trials and a final 5-min test trial. Each of these tests started at 5 rpm with constant acceleration of 0.1 rpm/sec. For all training and test trials, latency to fall off the spindle was recorded in seconds.

Grip Strength

To assess mouse grip strength, we used seven home-made weights (10, 18, 26, 34, 42, 49, 57 grams). The mouse was held by the middle/base of the tail and lowered to the weight. Once the mouse grasped the weighted ring with its forepaws, the mouse was lifted until the weight cleared the bench. For each weight, a mouse was given up to three trials to suspend the weight above the table for 3 seconds. If cleared, the next heaviest weight was tried. If not total time and maximum weight lifted was recorded and a grip strength score calculated from [46]. The calculated score was normalized to mouse body weight which was measured the day of the test.

DigiGait

Mouse gait analysis was performed on the DigiGait apparatus (Mouse Specifics, Inc, Framingham, MA). After acclimation, each mouse was placed on the treadmill at speeds of 18, 20, 22, 25, 28, 32, 36 cm/s. A 10-second clip was recorded with a video camera located below the belt. There was a 5-min rest between each speed. Recordings were analyzed with the DigiGait analysis program to assess the pre-specified parameters stride length and paw angle variability. Values for all four paws were averaged to give one value per mouse – the reported n values are the number of mice. In addition, the maximum speed at which each mouse was able to successfully complete a 10-second test after three attempts was recorded as described [35].

PTZ Kindling Study

A PTZ kindling protocol was performed as described [43, 47] to assess mouse susceptibility to seizure induction. Mice were injected with a sub-convulsive dose of PTZ

(40mg/kg, i.p.) every other day for up to 24 days mice and were observed for 30 minutes post-dose. Kindling was defined as tonic-clonic seizures on two consecutive injection days or death after which mice were euthanized. Kaplan-Meier survival analysis was done based on the number of injections to achieve kindling.

$G\alpha_o$ *protein expression*

Mice (6-8 weeks old) were sacrificed and their brains were dissected into different regions and flash-frozen in liquid nitrogen. For Western Blot analysis, tissues were thawed on ice and homogenized for 5 min with 0.5 mm zirconium beads in a Bullet Blender (Next Advance; Troy, NY) in RIPA buffer (20mM Tris-HCl, pH7.4, 150mM NaCl, 1mM EDTA, 1mM β-glycerophospate, 1% Triton X-100 and 0.1% SDS) with protease inhibitor (Roche/1 tablet in 10 mL RIPA). Sample homogenates were centrifuged for 5 min at 4°C at 13,000 G. Supernatants were collected and protein concentrations determined using the bicinchoninic acid method (BCA method; Pierce; Rockford, IL). Protein concentration was normalized for all tissues with RIPA buffer and 2x SDS sample buffer containing β-mercaptoethanol (Sigma-Aldrich) was added. Thirty µg of protein was loaded onto a 12% Bis-Tris SDS-PAGE gel and samples were separated for 1.5 hrs at 160V. Proteins were then transferred to an Immobilon-FL PVDF membrane (Millipore, Billerica, MA) on ice either for 2 h at 100 V, 400 mA or overnight at 30V, 50mA. Immediately after transfer, PDVF membranes were washed and blocked in Odyssey PBS blocking buffer (Li-Cor) for 40 min at RT. The membranes were then incubated with anti-Gαo (rabbit; 1:1,000; sc-387; Santa Cruz biotechnologies, Santa Cruz, CA) and anti-actin (goat; 1:1,000; sc-1615; Santa Cruz) antibodies diluted in Odyssey blocking buffer with 0.1% Tween-20 overnight at 4°C. Following four 5-min washes in phosphate-buffered saline with 0.1 % Tween-20 (PBS-T), the membrane was incubated for 1 hr at room temperature with secondary antibodies (both 1:10,000; IRDye® 800CW Donkey anti-rabbit; IRDye® 680RD Donkey anti-goat; LI-COR Biosciences) diluted in Odyssey blocking buffer with 0.1 % Tween-20. The membrane was subjected to four 5 min washes in PBS-T and a final rinse in PBS for 5-min. The membrane was kept in the dark and the infrared signals at 680 and 800nm were detected with an Odyssey Fc image system (LI-COR Biosciences). The $G\alpha_o$

polyclonal antibody recognizes an epitope located between positions 90-140 $G\alpha_o$ (Santa Cruz, personal communication) so there should be no interference from the R209H mutation.

Risperidone effects on motor behavior

Naïve 8-12 week old *Gnao1* $^{+/R209H}$ and *Gnao1* $^{+/+}$ littermates of either sex were tested for effects of risperidone on their activity in the open field arean. The study was run over 5 days: On day 1, mice underwent the open field protocol described above to establish a baseline. On day 3, mice were habituated in the experimental room for 10 min then given a single i.p. dose of either 2mg/kg or 0.5mg/kg risperidone (Cayman Chemical, Ann Arbor, MI) or vehicle control. Risperidone was dissolved in DMSO at a concentration of 5 mg/ml, Further dilutions were done in DI water. Thirty minutes following injection, mice were placed in the open field arena for a 30-minute testing time. On day 5, mice were retested in the same open field protocol without injection to assess drug washout.

Statistical Analysis

Data were analyzed with unpaired Students *t*-test or Mantel-Cox, *two-way* ANOVA with Bonferroni corrections as appropriate using Graphpad Prism 7.0 (GraphPad; La Jolla, CA). A $p < 0.05$ was considered the cut off for significance throughout. Detailed discussion of statistical analyses can be found within figure legends.

Results

Gnao1 $^{+/R209H}$ mice are produced at the expected frequency and have normal viability

Two founder *Gnao1 $^{+/R209H}$* mice, 1 male and 1 female, were crossed with C57BL/6J mice. Out of 98 offspring of a cross of *Gnao1$^{+/R209H}$* with WT mice, 51 heterozygotes and 47 WT were observed. *Gnao1$^{+/R209H}$* mice exhibit no overt postural or movement abnormalities or seizures under normal housing conditions. Adult mice showed no statistically significant differences in weight between WT and *Gnao1$^{+/R209H}$* genotypes of either sex.

Both male and female Gnao1 $^{+/R209H}$ are hyperactive in the Open Field arena

Patients with R209H mutation present with hyperkinetic movement disorders [5, 7, 12, 18]. To detect motor abnormalities, *Gnao1 $^{+/R209H}$* mice were subject to a battery of behavioral tests. The open field arena was used to test overall locomotor activity. We divided the test into two sections reflecting activity in a novel environment (0-10 minutes) then sustained activity (10-30 min). *Gnao1 $^{+/R209H}$* mice of both sexes showed markedly increased activity in both the novelty period and the sustained activity period compared to their wildtype littermates (Figure 2B). As a potential indicator of anxiety-like behavior. Male and female *Gnao1$^{+/R209H}$* mice also displayed reduced time in center (Figure 2B).

An accelerating rotarod test was used to assess motor coordination and balance. Neither male nor female *Gnao1$^{+/R209H}$ mice* display impaired performance (Figure 2C). Also grip strength, showed no differences between *Gnao1 $^{+/R209H}$* and wildtype littermates in either sexes (Figure 2D).

Male Gnao1$^{+/R209H}$ mice display a modestly reduced stride length

Gait patterns were assessed using DigiGait analysis. Male *Gnao1$^{+/R209H}$* mice showed a highly significant genotype effect with reduced stride length compared to wildtype littermates ($P<0.001$, 2-way ANOVA) but the magnitude of the effect was modest 5.6% decrease averaged across all speeds. In post-test analysis though, only the top speed (36 cm/sec) reached significance individually ($p<0.01$). Female *Gnao1$^{+/R209H}$* mice did not show significant differences

in stride length from WT (Figure 3C & 4D). However as previously reported for G203R mutant mice, the female *Gnao1*$^{+/R209H}$ showed a significantly reduced maximum run speed on the treadmill (Figure 3E $p<0.01$, t-test). This was not due to reduced body size as length, detected by the digigait system (WT 9.54 cm: vs R209H 10.17 cm) and weight were not significantly different. There was no significant difference in paw angle variability for either males or females.

Gnao1 $^{+/R209H}$ *mice are not sensitive to PTZ kindling*

Repeated application of a sub-threshold convulsive stimulus, leads to the generation of full-blown convulsions [48]. GNAO1 variants differ in their ability to cause epileptic seizures in patients. Those carrying the R209H mutant allele do not exhibit a seizure disorder [5, 7, 12, 18]. In accordance with the patients' pattern, *Gnao1*$^{+/R209H}$ mice did not show increased susceptibility to kindling-induced seizures (Figure 4 A&B). This contrasts with the previously reported increased kindling sensitivity in male G203R mutant mice [35].

Gnao1$^{+/R209H}$ *mice have normal* $G\alpha_o$ *protein expression in the brain*

To understand why R209H mutant mice do not show a phenotype difference in the kindling test while G203R mutants do, we assessed $G\alpha_o$ protein expression level. Cortex, hippocampus, striatum, cerebellum, brain stem and olfactory bulb were harvested and homogenized to measure the effect of the R209H mutation on $G\alpha_o$ protein expression. Western blots showed no difference in $G\alpha_o$ protein expression between WT and *Gnao1*$^{+/R209H}$ in any of the measured brain regions (Figure 5). This is consistent with our previous analysis of protein expression in HEK293T cells with transiently transfected $G\alpha_o$ R209H plasmid [49].

Risperidone treatment attenuated the hyperactivity of Gnao1$^{+/R209H}$ *mice*

Patients with *GNAO1* mutations were tried on multiple treatments to alleviate motor symptoms, (Table S1). Risperidone, an atypical antipsychotic drug showed beneficial effects in one of the patients. It has also been effective in drug-induced dyskinesia [50]. We show that *Gnao1*$^{+/R209H}$ mice exhibit complete abrogation of movement at 2 mg/kg risperidone, which has recovered after 2 days (Figure 6A&C). WT mice also show a significant decrease in locomotion

after 2 mg/kg risperidone treatment (Figure 6A). After a single 0.5mg/kg dose of risperidone both WT and *Gnao1*$^{+/R209H}$ mice exhibit a decrease in locomotion (Figure 6B). As expected, hyperactivity of mutant mice was observed during baseline testing on day 1 and the hyperactivity had returned on day 5 following the 2-day washout period after the risperidone doses (Figure 6C). Neither 2.0 mg/kg nor 0.5 mg/kg selectively affected *Gnao1*$^{+/R209H}$ as assessed by percent suppression (Supplement Figure 1).

Discussion

The goal of personalized medicine is to define treatments for an individual patient. Knowing which gene is involved is beneficial but different mutant alleles, even in the same gene, can produce quite distinct effects. Here we report a second mouse model of *Gnao1*-related neurological abnormalities. The *Gnao1 R209H* mutant mice display motor abnormalities but no seizures which differs from our previous *Gnao1* G203R mouse model [35]. The present results, however, are consistent with the clinical pattern of *GNAO1* R209H patients, who present with neurodevelopmental delay with involuntary movements (NEDIM) without seizures.

The phenotype of the *Gnao1* R209H mutant mice were somewhat unexpected as our previous *GNAO1* Mouse model, *Gnao1*$^{+/G203R}$, showed significant motor impairments in Rotarod and Digigait but had no changes in the open field test [43]. Patients with the G203R or R209H mutations both display movement disorder with choreathetosis and dystonia as the most common [3, 4]. The patients with R209H mutants, however, have a high frequency of hypotonia [36] while this is nearly absent in patients with the G203R mutation. Also, the R209H mutant patients have somewhat more choreoathetosis than do the G203R mutant patients but similar frequencies of dystonia [36]. The striking hyperactivity of the male R209H mutant mice points to increased dopamine signaling in the striatum as a possible mechanism. The suppression of that hyperactivity with risperidone is consistent with that model.

The two movement disorder-associated *GNAO1* mutations R209H and G203R also have different patterns in *in vitro* studies of cAMP regulation in HEK-293T cells [2]. Interestingly, the $G\alpha_o$ R209H mutants supports normal cAMP regulation and is expressed at normal levels both in HEK cells[49] and in the brain (this study). In contrast the G203R mutant is expressed at lower levels both in HEK cells[49] and in the mouse brain (data not shown). Despite lower expression, it signals more strongly (GOF) in the HEK cell assay.

The new *Gnao1* R209H mutant mice do have a readily detectable phenotype which will be valuable in assessing the biological function. It may help explain why children with GNAO1 R209H mutations have abnormalities despite the mutant protein having essentially normal function in cAMP assays in vitro [2]. It is possible that he pathogenicity of this mutant $G\alpha_o$ protein

arises from effects on the non-cAMP signal outputs from the G_o heterotrimeric protein (e.g. ion channels, synaptic vesicle release, or neurite outgrowth).

Effective treatments are a key goal as patients with the R209H mutation experience multiple incidents of hospitalizations [7, 9, 13]. Deep brain stimulation in the globus pallidus has proven effective in *GNAO1* patients in attenuating MD [5, 16, 21, 25]. However, that invasive treatment is reserved for refractory patients. Risperidone is one of the oral treatments that has proven to be beneficial, specifically in a patient with the R209H mutation [7]. Risperidone is an atypical neuroleptic, antagonizing D_2 and 5-HT receptors. $G\alpha_o$ couples to myriad G-protein coupled receptors including the dopamine D_2 receptor which is involved in movement control [51]. In our study, risperidone was able to significantly decrease the hyperlocomotion seen in our *Gnao1*$^{+/R209H}$ mouse model. At both the 0.5 mg/kg and 2.0 mg/kg dose of risperidone, hyperactivity was attenuated in our R209H mouse model. However, this response was not selective for the *Gnao1*$^{+/R209H}$ mutant mice as the WT mice also displayed a significant decrease in locomotion. This outcome suggests that risperidone treatment may be effective in repressing global movement, while not specifically targeting a Gnao1 R209H mechanism. However, as hyperkinetic movements in patients have been shown to be exacerbated under stress, illness or high temperature [7, 19], it would be interesting to see if the mouse movement phenotype were increased or induced by physical or pharmacological stress. It would be then interesting to see if risperidone treatment could target the induced movement abnormalities, potentially showing risperidone specific effects on the *Gnao1* R209H mutant model.

The new mouse model described here should provide a valuable tool for future mechanistic studies of *GNAO1* encephalopathies. The fact that the mouse has very distinct behavioral changes from our previous Gnao1 mutant mouse models (R209H and G184S), indicate that it is very important to consider the mutant allele as well as the mutant gene in considering genetic disorders and personalized therapies.

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Figure Legends

Figure 1. Targeting of the mouse *Gnao1* locus (A) Mouse *Gnao1* genomic locus (exon size not to scale), red outline is magnified in (B) showing exon 6 and relative location of codon 209, and PCR primers O586 and O587. (C) Location and exact sequence of gRNA target within exon 6, dotted red line denotes DSB, PAM is highlighted and sequence corresponding to gRNA protospacer is underlined (also in E). (D) Raw gel electrophoresis images showing PCR of the target region and T7 Endonuclease I (T7 Endo I) digestion analysis of founders 1324 – 1335 (n=12), with WT, H2O (-) and T7Endo I (+) controls. Founder 1324 (red number) was positive for the mutation on one allele and WT on the other, note that the single bp mismatch was not reliably detected by T7 Endo I assay. (E) Exact sequence of edited founder 1324 as aligned to WT reference genome, two peaks (G and A) are detected on the sequence chromatogram, indicating the presence of both WT and edited R209H allele.

Figure 2. *Gnao1* $^{+/R209H}$ mice show significant hyperactivity and reduced time in center in the open field arena (A) Representative heat maps of Gnao1 $^{+/R209H}$ mice and *Gnao1* $^{+/+}$ mice in the open field arena (B) Time spent in the open field arena was separated into 0-10 minutes (novelty) and 10-30 minutes (sustained). *Gnao1* $^{+/R209H}$ male and female mice exhibit increased locomotion in the novelty period. Hyperactivity was maintained throughout the sustained period as mice continued to show significant increase in distance traveled (2- way ANOVA; ****$p < 0.0001$, ***$p < 0.001$, * $p < 0.05$). *Gnao1* $^{+/R209H}$ mice of both sexes spend less time in center areas of the open field arena compared to wildtype littermates. (C) Neither male nor female *Gnao1* $^{+/R209H}$ mice show significant differences on the rotarod. (D) There is no significant difference between grip strength between wildtype and *Gnao1*$^{+/R209H}$ mice. Data are shown as mean ± SEM.

Figure 3. Male and female *Gnao1*$^{+/R209H}$ mice shows gait abnormalities in different tests on the DigiGait imaging system (A & B) Male *Gnao1* $^{+/R209H}$ mice showed reduced stride length compared to wildtype littermates (2-way ANOVA with Bonferroni multiple comparison post-test), while female *Gnao1* $^{+/R209H}$ mice show a normal stride length. (C & D) Neither male or female *Gnao1* $^{+/R209H}$ exhibited significant differences in paw angle variability compared to wildtype littermates.

(E) at speeds greater than 25 cm/s female *Gnao1* $^{+/R209H}$ shows reduced ability to run on a treadmill.

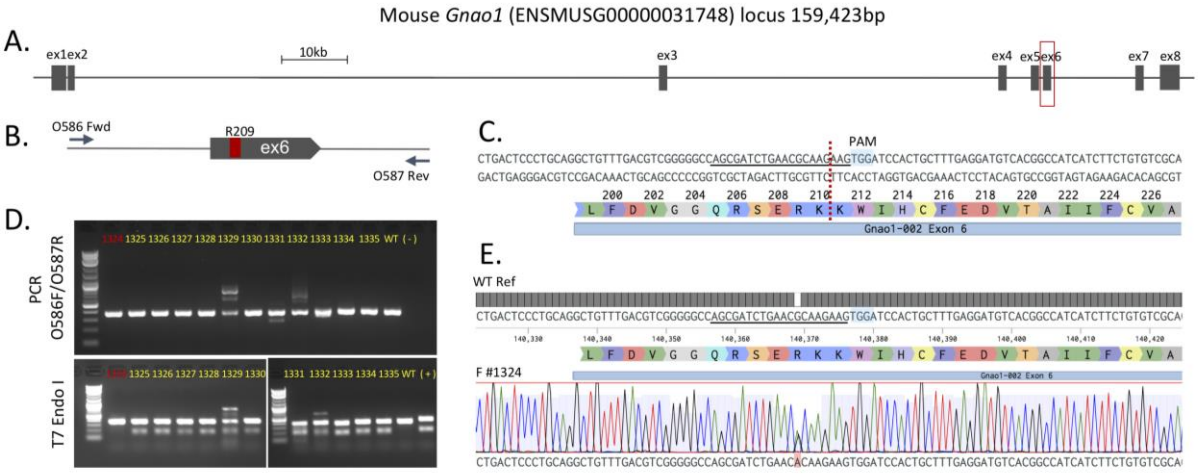
Figure 4. *Gnao1* $^{+/R209H}$ mice do not have an enhanced pentylenetetrazol (PTZ) kindling response (A&B) Neither male or female *Gnao1* $^{+/R209H}$ mice showed significant differences in sensitivity to PTZ injection compared to wildtype littermates (ns. Mantel-Cox test).

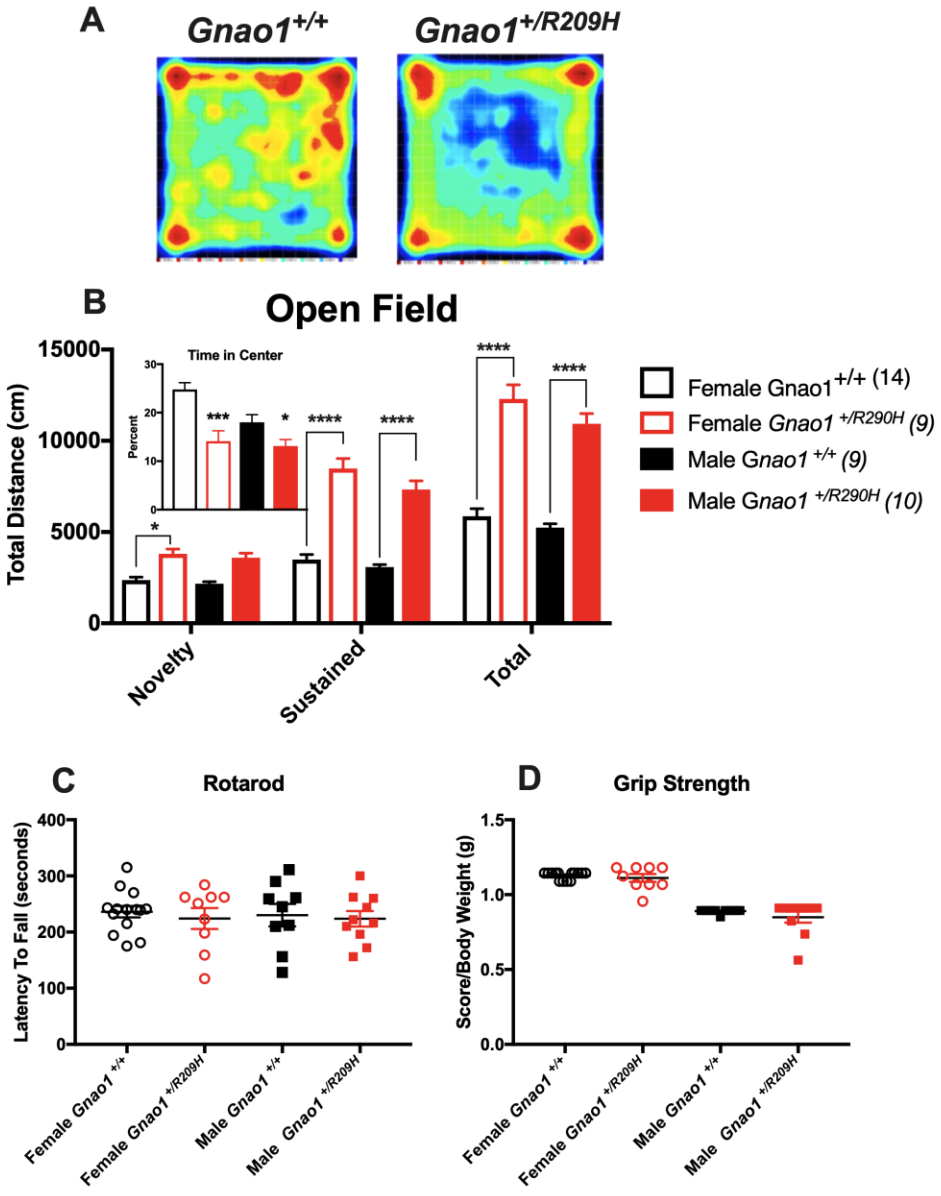
Figure 5. Western blot shows no difference in $G\alpha_o$ protein levels between *Gnao1* $^{+/R209H}$ and WT mice Brain regions (cortex, hippocampus, striatum, cerebellum, brain stem and olfactory bulb homogenates) from WT and *Gnao1*$^{+/R209H}$ mice were quantified for levels of $G\alpha_o$ protein. There was no significant difference in any of the regions between WT and mutant mice.

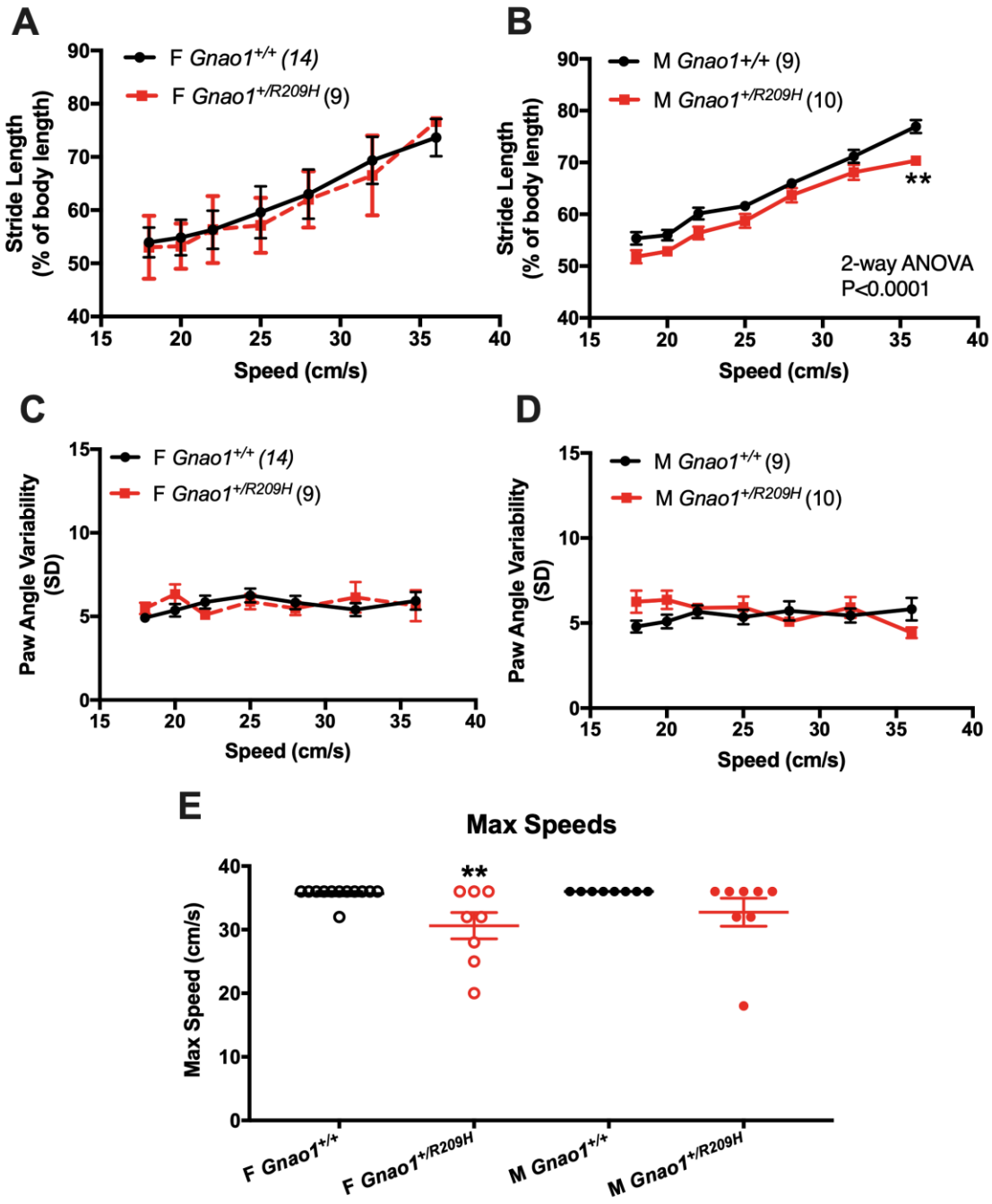
Figure 6. Risperidone treatments decreases hyperkinetic movements in *Gnao1* $^{+/R209H}$
(A) *Gnao1*$^{+/R209H}$ mice show complete abrogation of movement compared to vehicle treated *Gnao1*$^{+/R209H}$ following a 2.0 mg/kg dose of risperidone. Students unpaired T-test (B) At 0.5 mg/kg both WT and *Gnao1*$^{+/R209H}$ exhibit a significant decrease in locomotion. Students unpaired T-test. Wildtype mice also show a decrease in locomotion after 0.5 mg/kg risperidone treatment, (C) Comparison of 2.0 mg/kg and 0.5 mg/kg treatment in WT and *Gnao1*$^{+/R209H}$ mice. Hyperactivity of *Gnao1*$^{+/R209H}$ mice was observed during baseline testing and recovered following the 2-day risperidone washout.

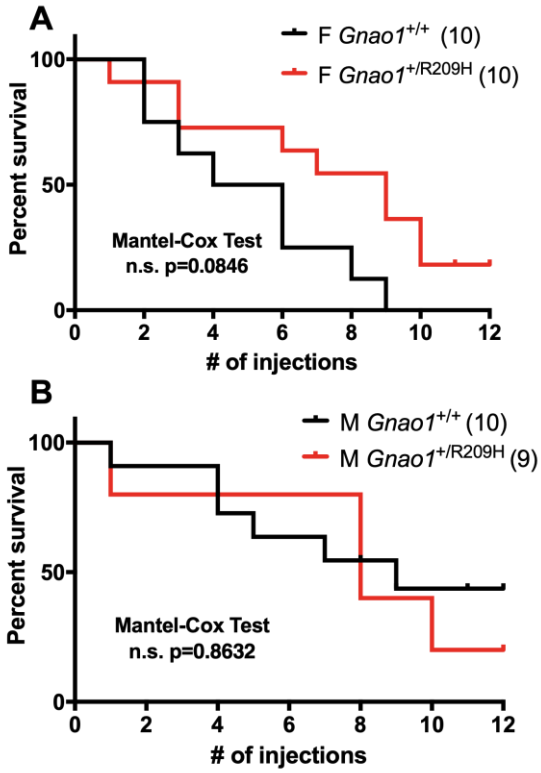
Table 1. Location and sequence of gRNA and ssODN template for CRISPR-Cas targeting Gnao1 locus; primers and genotyping method for *Gnao1*$^{+/R209H}$ mice

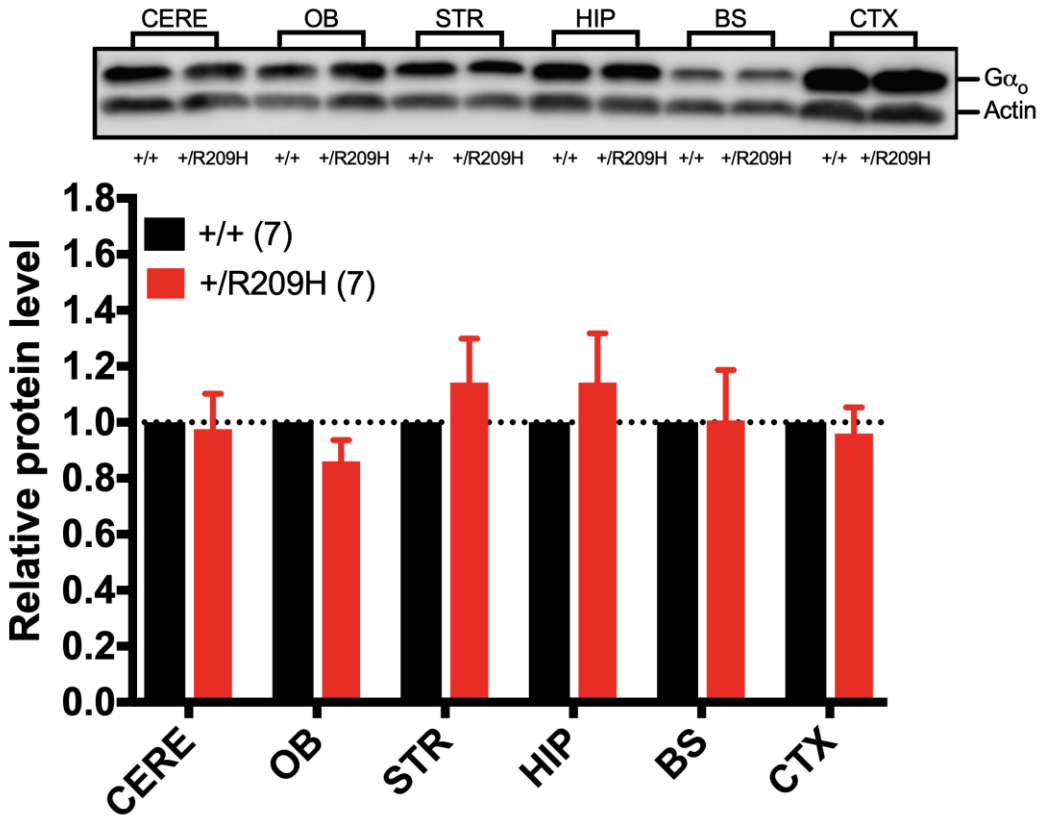
	Gnao1 R209H
Location	Chr 8: 93,950,334
gRNA target 5' N20-PAM -3'	5' AGCGATCTGAACGCAAGAAG TGG 3'
ssODN template (reverse complement)	GTTTCGTCCTCGTGGAGCACCTGGTCATAGCCGCTGAGTGCGAC ACAGAAGATGATGGCCGTGACATCCTCAAAGCAGTGGATCCACTTCTTG tGTTCAGATCGCTGGCCCCCGACGTCAAACAGCCTGCAGGGAGTCAGGG AAAGCTGTGAGGGCGGGGACGCCTA
PCR primers	O586 FWD: 5' GGACAGGTGTCACAGGGGAT 3' O587 REV: 5' ACTGGCCTCCCTTGGCAATA 3'
Genotyping	By Sanger Sequencing

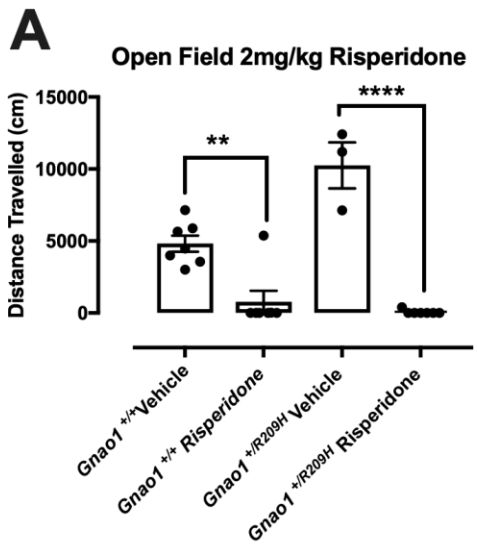


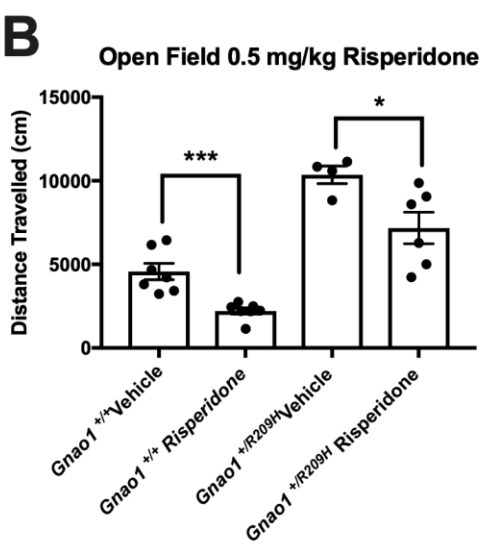












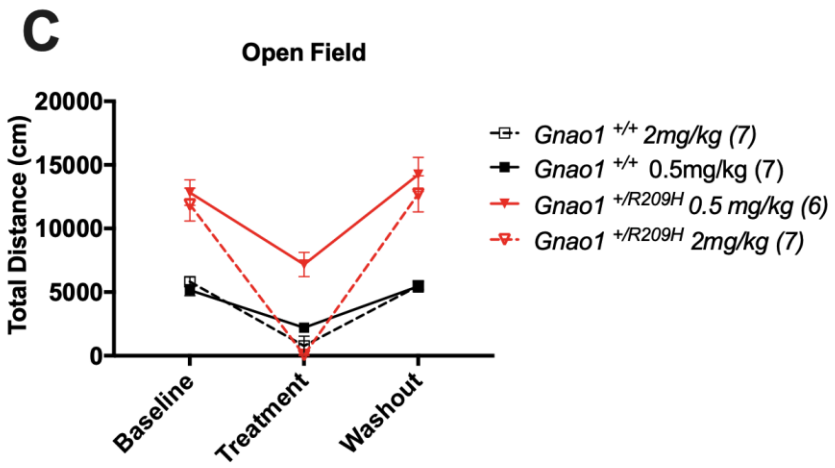
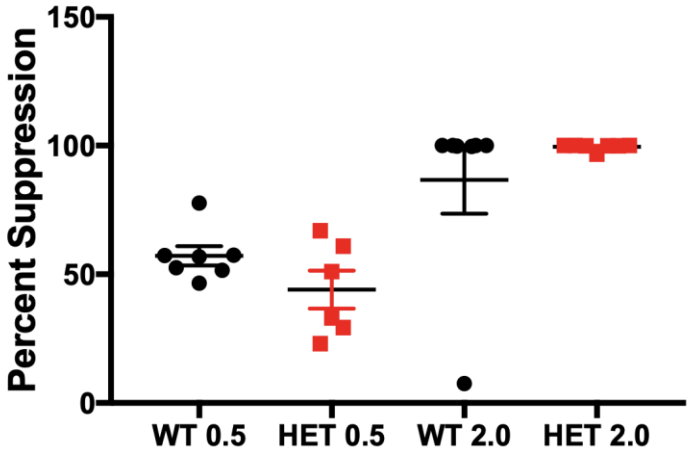


Table S1. *GNAO1* R209H patient classification

Patient No.	Sex	Amino Acid Change	Age of Onset	Presence of Epilepsy	Movement Disorder	Treatment	Motor Developmental Delay(MDD)/Intellectual Delay(ID)	Reference
1	M	R209H	17 mo	-	Chorea	DBS	MDD	Kulkarni et. al (2016)
2	M	R209H	2 y	-	Chorea	DBS	MDD	Kulkarni et. al (2016)
3	M	R209H	3 y	-	Chorea	Risperidone, BZD	MDD/ID	Anath et. al (2016)
4	M	R209H	1 y		Chorea	NA	MDD/ID	Menke et al (2016)
5	M	R209H	10 mo	-	Chorea Dystonia	TBZ, THP	MDD/NA	Dhamija et al (2016)
6	M	R209H	15 mo	-	Chorea, Dystonia	DBS	MDD/ID	Marecos et al (2018)
7	F	R209H	6 mo	-	Dystonia	NA	MDD/MID	Kelly et al (2018
-	F	R209C	NA	NA	Chorea	NA	MDD/ID	Saitsu et al (2016)
-	F	R209G	3 y	-	Chorea	None	MDD/ID	Anath et al (2016)

-	M	R209L	2 y	-	Chorea	NA	MDD/ID	Menke et al (2016)



Supplemental Figure S1. WT and *Gnao1*$^{+/R209H}$ mice show no difference in percent suppression of locomotion after oxotremorine treatment *Gnao1*$^{+/R209H}$ mice show similar sensitivity to risperidone treatment at 2.0 mg/kg or 0.5 mg/kg compared to WT treated mice, Student t-test.