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Supplementary Materials for

Negative Allosteric Modulation of the mGluR5 Receptor Reduces Repetitive Behaviors and Rescues Social Deficits in Mouse Models of Autism

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Supplementary Material

Material and Methods

Mice

C57BL/6J (B6) and BTBR T+ tf/J (BTBR) mice tested at NIMH were the offspring of breeding pairs purchased from The Jackson Laboratory (JAX). All mice were housed and bred in a conventional mouse vivarium at the National Institute of Mental Health (NIMH), Bethesda, Maryland, USA, using harem breeding trios. After two weeks with a male, females were separated into individual cages (Tecniplast) before delivery. Pups were kept with the dam until weaning at postnatal day 21. After weaning, juveniles were group housed by sex and strain in standard plastic cages in groups not exceeding four per cage. Cages were maintained in ventilated racks in temperature (20°C) and humidity (~55%) controlled colony rooms, on a 12 hour circadian cycle, lights on from 0700 to 1900 hr. Standard rodent chow and tap water were available *ad libitum*. In addition to standard bedding, a Nestlet square, shredded brown paper and a cardboard tube were provided in each cage.

Male B6, BTBR, and C58/J mice tested at Pfizer were purchased from JAX and delivered to the Pfizer vivarium at age 7-8 weeks. Mice were allowed two weeks to acclimate to the vivarium prior to behavioral testing, and were not tested less than three days after the last cage change. Mice were housed 4 per cage in ventilated caging racks in an environmentally controlled facility with food and water available *ad libitum*. They were maintained on a 12 h light/dark cycle with lights on from 0600 to 1800 hours. Light levels measured approximately 400-500 lux during the light phase. Background noise measured approximately 60-65 dB.

Ex vivo receptor binding

Male BTBR (n = 3), B6 (n=5) and C58 mice (n=3) weighing 18-22 g were injected systemically with GRN-529 (0.03, 0.1, 0.3, 1.0 or 3.0 mg/kg) or vehicle. The animals were sacrificed thirty

minutes after administration and the brains were hemisected. One hemisphere was frozen for measurement of GRN-529 concentration as described below. Forebrains were immediately dissected, weighed, and homogenized in 40 volumes of assay buffer (50 mM Tris, 0.9% NaCl, pH 7.5). Binding of [^{3}H]-MPEPy was determined using 1 nM radioligand and non-specific binding was defined with 10 μM MPEP. Incubations were initiated by the addition of tissue homogenate (1.25 mg wet weight/well) and proceeded at RT for 1 hour. The reaction was terminated by rapid vacuum filtration using ice-cold 50 mM Tris (pH 7.5) through pre-soaked (0.5% polyethyleneimine) GF/B filters. Radioactivity was measured using a TriCarb liquid scintillation counter.

Pharmacokinetics of GRN-529 in mouse plasma and brain

Approximately 1-2 ml of whole blood was collected from cardiac puncture into EDTA-containing tubes. Within an hour after collection, plasma was separated by spinning the samples at ~3000 rpm for 10 minutes at 4°C. Approximately 0.75 ml of plasma was collected into 96-well plates. Hemisections of brains were rinsed with saline, blotted dry, weighed and placed into preweighed 20 ml plastic scintillation vials in ice. Plasma and brain samples were stored at approximately -80°C until analysis. Tissue concentrations of GRN-529 were measured using liquid chromatography/mass spectrometry. To detect the 372.1 atomic mass unit fragment of GRN-529 formed from the parent ion at 392.0 atomic mass units, the declustering potential of the Sciex API 4000 mass spectrometer was set to 91 V, the collision energy was set to 71 eV, and the collision cell exit potential was set at 9 V.

Experimental Design for Behavioral Assays

Testing was conducted in dedicated behavioral testing rooms during the standard light phase, usually between 1000 and 1500 hr. Treatment groups consisted of 9-15 mice per drug dose or vehicle per strain at NIMH and 9-25 mice per drug dose and strain at Pfizer. Previous studies in our NIMH laboratory documented no sex difference in sociability or self-grooming in BTBR or B6 [64, 72, 82]. Therefore, male and female mice were used in all studies in approximately equal proportions at NIMH. Only males were employed at Pfizer. We used a between-subjects

design with a one week washout period, such that each mouse received a single acute dose of MPEP, MTEP, GRN-529, or vehicle, and was tested in a single behavioral task, one task per week. Drug doses, toe tattoo patterns, and digital videotapes were coded to ensure that the raters were blind to the treatment condition. All procedures were conducted in strict compliance with the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committees of the National Institute of Mental Health and Pfizer, Inc.

Drugs

In vivo receptor occupancy, plasma and brain pharmacokinetic data and/or pilot behavioral assays in B6, BTBR and C58 mice determined the doses and post-treatment interval for GRN-529, a novel and selective mGluR5 negative allosteric modulator developed by Pfizer's Chemical Screening Sciences group and characterized by Hughes et al [97]. Doses of MPEP and MTEP were based on previous publications [72, 99]. Compounds were administered acutely by intraperitoneal (i.p.) injection in a volume of 10 ml/kg. MPEP 10.0 mg/kg, and MTEP 0.3 mg/kg, 0.6 mg/kg, 1.0 mg/kg and 3.0 mg/kg (Sigma Aldrich), were dissolved in saline (0.9% NaCl). GRN-529 0.3, 1.0 mg/kg and 3.0 mg/kg (Pfizer, Groton, CT) was dissolved in 10% Tween-80 and saline (0.9% NaCl) [97]. Vehicle, MPEP, MTEP or GRN-529 was injected 30 minutes before the start of behavioral test sessions for each of the self-grooming, social approach, and open field behavioral tasks.

Self-grooming assay

Mice were scored for spontaneous repetitive self-grooming behavior at NIMH as previously described [22]. Each mouse was placed individually into a standard mouse cage, (46 cm length x 23.5 cm wide x 20 cm high). Cages were empty, to eliminate digging in the bedding, a potentially competing behavior. The room was illuminated at ~40 lux. A front mounted CCTV camera (Security Cameras Direct) was placed approximately 1 meter from the cages to record the sessions. After a 10-minute habituation period in the test cage, each mouse was scored with a stopwatch for 10 minutes for cumulative time spent grooming all body regions. A trained observer uninformed of the drug treatment scored the videos. Cumulative time spent self-

grooming was scored from the videos using a high-accuracy Traceable stopwatch (Thomas Scientific) with the auditory component silenced.

Repetitive behavior assays at Pfizer were conducted in a testing room with temperature, humidity, lighting (400-500 lux) and background noise conditions (60-65dB) similar to the housing room. Following the drug pretreatment period, each mouse was placed individually into an observation cage for a 10-minute habituation period, and a subsequent 10-minute observation period. The observation cages were empty standard mouse shoebox housing chambers (19.1 x 29.8 x 12.7 cm) with no bedding and a flat filter top lid. Two mice were scored simultaneously (one BTBR and one B6) by a trained observer blind to the dosing conditions. Cumulative grooming time was recorded using stopwatches (with the auditory device silenced) for a 10 minute period. A clean observation cage was used for each individual mouse. Mice were not returned to their home cages until all the mice within a cage had completed behavioral testing.

Stereotyped Jumping

Observations of stereotyped jumping behavior in C58 mice were conducted at Pfizer during the light cycle (0800 to 1600 hrs) in a testing room with identical lighting conditions as the housing room (~ 400 lux). Following pretreatment of GRN-529 (30 min, i.p.), mice were placed into individual Accuscan activity chambers (see open field methods below). The number of jumps was quantified for a period of 10 minutes by a trained observer blind to the dosing conditions. General exploratory activity in the open field was monitored simultaneously throughout a 30 minute period. Total distance, vertical activity, and center time were automatically collected using the Versamax activity monitor and analyzer software system. Test chambers were cleaned with 70% ethanol between test subjects. At least 20 minutes between cleaning and the start of the next session was allowed for ethanol evaporation and odor dissipation.

Sociability assay

Social approach was tested at NIMH in an automated three-chambered apparatus from a design originally developed by our group [86], using updated methods [22, 32, 62, 64, 72-74, 82, 87, 89]. The apparatus was a rectangular, three-chambered box made from clear polycarbonate. Retractable doorways within the two dividing walls allowed access to the side chambers. Number of entries and time spent in the chambers were automatically recorded from photocells embedded in the doorways. A top mounted CCTV camera (Security Cameras Direct) was placed over the boxes to record the session, for subsequent scoring of the videos for time spent sniffing the novel mouse and novel object. The apparatus was cleaned with 70% ethanol and water between subjects. At least five minutes elapsed between cleaning and the start of the next test session, to allow for ethanol evaporation and clearance of ethanol vapor odors. To increase throughput, four mice were run simultaneously, in four adjacent social approach chambers. External solid panels between chambers prevented transmission of visual cues and minimized transmission of olfactory and auditory cues (panels designed and installed by the NIMH/NINDS Section on Instrumentation). Computer controlled hardware and software were developed by George Dold (NIMH/NINDS Section on Instrumentation) using LabVIEW (National Instruments). Mice used as the novel stimulus target at NIMH were 129Sv/ImJ, aged 12–20 weeks old, bred and maintained in the NIMH vivarium from breeding pairs originally obtained from JAX, and matched to the subject mice by sex and age. At Pfizer, novel stimulus mice were DBA/2J, chosen as stimulus mice for social approach testing because of their low aggression (our unpublished finding). Several days before the start of experiments, stimulus mice were habituated to the apparatus and to the wire cup enclosure for 30 minutes per day during 2 habituation sessions per day for 2 days. The subject mouse was allowed to acclimate to the apparatus for 20 minutes before the sociability test, first for 10 minutes in the central chamber with the doors closed, followed by 10 minutes in the entire empty arena with the doors open. The subject was then briefly confined to the center chamber while a novel object (an inverted wire pencil cup, Galaxy Cup, Kitchen Plus) was placed in one of the side chambers and a novel mouse was placed inside an identical inverted wire cup in the other side chamber. The location (left or right) of the novel object and stranger mouse alternated across subjects. Novel mice were enclosed in a wire cup to ensure that all social approach was initiated by the subject, and to avoid complications of fighting and sexual activity, while allowing visual, olfactory, auditory,

and partial tactile contact through the widely spaced wire bars. A plastic cup (Solo Cup Company) with a lead weight was placed on the top of the inverted wire cups, to prevent the subject mouse from climbing and sitting on top of the inverted wire cup. Time spent in each chamber and number of entries into each chamber were calculated by the automated software, based on the movements of the subject mouse in sequentially breaking and unbreaking a series of photocell beams embedded in the openings between chambers [87]. Number of entries served as a within-task control for levels of general exploratory locomotion. Lack of innate side preference was confirmed in previous experiments and during the initial 10 minutes of habituation to the entire arena. After both stimuli were positioned, the doors were simultaneously re-opened and the subject was allowed access to all three chambers for 10 minutes. No innate side preference was found in B6 or BTBR during the task, as shown by similar amounts of time in the left and right side chambers during the 10 minute habituation session, before the start of social testing. An observer blind to the drug treatments scored the videos with a stopwatch for cumulative time in which the subject mouse sniffed the target mouse. At the end of each testing day, test chambers were thoroughly cleansed with Alconox detergent diluted with warm water, followed by extensive rinsing with hot water and air drying.

Adult Reciprocal Dyadic Social Interaction

Freely moving reciprocal social interaction tests were conducted between pairs of mice at 8 and 16 weeks of age. Investigative and soliciting social interaction parameters were assessed in B6 and BTBR subject mice. 129/SvImJ mice, bred at NIMH from breeders purchased from JAX, were employed as the stimulus partners, to evaluate the ability of adult B6 and BTBR subject mice to initiate social behavior in response to social cues from a uniform stimulus mouse. Subjects and partners were of the same sex, and each used only once. The reciprocal interaction test was conducted in the Noldus PhenoTyper 3000 chamber (25 cm x 25cm x 35 cm, Noldus) as previously described [62, 64, 82, 89, 91]. The floor of the arena was covered with a 0.5 cm layer of clean bedding. Subjects and stimulus partners were individually housed in a clean cage for an hour before the test. After this brief isolation period, two age- and sex-matched non-littermates were simultaneously placed in the arena and their interactions were videotaped for 10 minutes. The arena was cleaned with 70% ethanol between test subjects. At least five minutes between

cleaning and the start of the next session was allowed for ethanol evaporation and odor dissipation. Social interaction parameters were scored by well-trained observers, using Noldus Observer 8.0XT software, as previously described [62, 64, 82, 91, 100]. Parameters of social behaviors included nose to nose sniff (sniffing the nose and snout region of the partner), front approach (moving towards the partner from a distance, in a head-on manner), push-crawl (push = pushing the head underneath the partner's body and/or squeezing between the wall/floor and the partner; crawl = crawling over or under the partner's body), follow (walking straight behind the partner, keeping pace with the one ahead), total time spent in social contact (includes both direct active and indirect passive physical contact). These non-aggressive behaviors are evident in mice across post-weaning ages and in both sexes, making them suitable parameters for studying social traits that are not sex-dependent or age-specific [91, 101]. In addition to social behaviors, bouts of self-grooming and digging in the bedding that lined the floor of the arena (using nose and front paws to push bedding around the arena) were scored as measures of repetitive behaviors. In addition to social and repetitive behaviors, arena exploration (time spent exploring the arena, excluding all time spent interacting, engaging in repetitive behavior, and remaining still) and bouts of rearing (vertical upright posture against the wall or within the arena) were scored as measures of exploratory behavior. Aggressive attacks and allogrooming were rarely observed in these experiments and were not included in statistical analysis. All behaviors were analyzed for either frequency of occurrence or cumulative duration.

Open field locomotion

The open field test was conducted as an independent control for direct drug effects on physical activity that could confound the interpretation of results from the self-grooming and social approach tasks. General exploratory locomotion in a novel open field environment was assayed as previously described [22, 32, 64, 72, 89, 98]. Individual mice were placed in a standard Accuscan open field (AccuScan Instruments) at both NIMH and Pfizer. Illumination in the testing room measured ~ 30 lux at NIMH and ~ 400 lux at Pfizer. Test chambers consisted of clear Plexiglas sides and floor, approximately 40 x 40 x 30.5 cm. Mice were placed in the center of the open field at the initiation of the testing session. Photocells at standard heights for recording activity were aligned 8 to a side, dividing the chamber into 64 equal squares. Total

distance, vertical activity, and center time were automatically collected using the Versamax activity monitor and analyzer software system. Test chambers were cleaned with 70% ethanol between test subjects. At least five minutes between cleaning and the start of the next session was allowed for ethanol evaporation and odor dissipation.

Statistical analysis

Self-grooming and repetitive jumping were analyzed using a within strain One-Way ANOVA followed by a Tukey-Kramer highly significant different (HSD) posthoc test, using StatView statistical software (Citewise). Social approach was analyzed using a within groups Repeated Measures Analysis of Variance (ANOVA), using StatView software, to compare time spent in the side chambers in the sociability test, for each drug dose group and for the vehicle group, within each strain. Since times spent in each of the three chambers added to 10 minutes, and therefore were not independent, the test condition factor compared time spent only in the right versus left chambers. Center chamber times are shown in the graphs for illustrative purposes. Time spent sniffing the novel object versus the novel mouse was similarly analyzed for each dose within each strain. For entries during social approach, a between groups within strain drug by entries Repeated Measures ANOVA was performed. In cases where the overall ANOVA for entries was significant, the treatment factor for each strain was further analyzed with a Tukey's posthoc test to compare each drug dose group to its vehicle control group. Drug effects in the freely moving dyadic reciprocal social interaction task were compared between GRN-529 3.0 mg/kg and vehicle by Student's unpaired t-test for each strain, using Statview statistical software (Citewise). Open field locomotion was analyzed with a between groups within strain drug by total distance, center time or vertical activity Repeated Measures ANOVA, followed by Tukey's post hoc analysis, using SigmaPlot version 11.0 (Systat Inc.). At Pfizer, all data analyses were performed similarly, except that Prism GraphPad Version 5.01 software was used.

Results with full statistical analyses

Pharmacokinetics and ex vivo receptor binding

Displayed in Fig. 1 are (A) the chemical structure and mGluR5 binding properties of GRN-529 as reported by Hughes et al. [97] ; (B-D) the free plasma and brain concentrations thirty minutes after systemic administration in BTBR T+tf/J (BTBR), C57BL/6J (B6) and C58/J (C58) mice; (E) plasma and brain exposure levels over the 2 hours following systemic administration in B6 mice; (F) mGluR5 receptor occupancy levels thirty minutes after systemic administration of GRN-529 in BTBR, B6 and C58 mice; (G) the relationship of free, unbound brain levels of GRN-529 and mGluR5 occupancy. A thirty-minute pretreatment time was chosen for the single time-point dose-response exposure and mGluR5 occupancy experiments because the temporal profile of plasma and brain exposure observed in B6 mice revealed a peak in both compartments at thirty minutes.

The single time-point dose-response exposure and mGluR5 occupancy experiments revealed dose-dependent increases in exposure and occupancy in BTBR, B6 and C58 mice. Plasma and brain exposure levels achieved at any given dose were similar in B6 and C58 mouse strains, whereas exposure levels in BTBR mice were approximately 2-fold higher than in B6 or C58 mice. Higher exposure in BTBR mice may account for the leftward shift in the relationship between systemic dose of GRN-529 and mGluR5 occupancy in BTBR mice, as illustrated in Fig. 1F. However, further analysis of the exposure/occupancy relationship across strains revealed that the relationship between free, unbound brain levels of GRN-529 and mGluR5 occupancy is similar across all three mouse strains. Occupancy was ~30%, 60% and 85% at free, unbound concentrations of ~3 nM, 7 nM and 15 nM, respectively, in each strain.

Reductions in Repetitive Behaviors

Repetitive behavior experiments shown in Fig. 2 Panels A,B and Fig. S1 Panels A-F were conducted at NIMH. Experiments shown in Fig. 2 Panels C,D,E were conducted at Pfizer in Groton, CT. GRN-529 had no effect on the low self-grooming scores in C57BL/6J (B6) control mice (Panel A: $F_{3, 36} = 2.20$, $p > 0.05$). GRN-529 reduced the high repetitive self-grooming scores that characterize BTBR T+tf/J (BTBR) mice (Panel B: $F_{3, 36} = 3.60$, $p < 0.05$, significant at doses of 1.0 mg/kg ($p < 0.05$) and 3.0 mg/kg ($p < 0.05$) as compared to vehicle treatment. In a second independent cohort of B6 and BTBR mice tested at NIMH, GRN-529 again had no

significant effect on self-grooming in B6 (Fig. S1 Panel E: $F_{3, 43} = 2.47$, $p > 0.05$), and again reduced repetitive self-grooming scores in BTBR (Fig. S1 Panel F: $F_{3, 52} = 3.95$, $p < 0.05$), significant at the highest dose, 3.0 mg/kg ($p < 0.05$) as compared to vehicle. In a third independent cohort of B6 and BTBR mice tested at Pfizer, GRN-529 significantly reduced self-grooming in B6 (Fig. 2 Panel C: $F_{3, 71} = 5.87$, $p < 0.05$), which was high in this colony, relative to the NIMH colony. Higher self-grooming in B6 mice at Pfizer is likely due to differences between the vivarium conditions at Pfizer and NIMH, and to mice bred, reared and shipped from an external vendor to Pfizer versus internally bred and reared at NIMH. GRN-529 again reduced repetitive self-grooming scores in the third cohort of BTBR tested at Pfizer (Fig. 2 Panel D: $F_{3, 95} = 4.69$, $p < 0.05$), significant at 1.0 mg/kg ($p < 0.05$) and 3.0 mg/kg ($p < 0.05$), as compared to vehicle.

MTEP, a standard mGluR5 antagonist tested at NIMH using identical methods, showed an overall effect in B6 mice on self-grooming (Fig. S1 Panel C: $F_{4, 47} = 4.27$, $p < 0.05$), but did not produce a significant effect at any one dose of MTEP versus saline vehicle ($p > 0.05$). MTEP significantly reduced repetitive self-grooming in BTBR (Fig. S1 Panel D: $F_{4, 52} = 4.87$, $p < 0.05$), at 0.6 mg/kg and 1.0 mg/kg as compared to saline vehicle ($p < 0.05$). The highest dose of MTEP, 3.0 mg/kg, did not reduce self-grooming in BTBR. Replicating our previous report [72], 10 mg/kg of the prototypic mGluR5 antagonist MPEP significantly reduced repetitive self-grooming scores in BTBR (Fig. S1 Panel B: $t_{1, 21} = -2.56$, $p < 0.02$). MPEP had no significant effects on self-grooming in a separate cohort of B6 mice at NIMH (Fig. S1 Panel A: $t_{1, 19} = 0.13$, $p > 0.05$), replicating previously published results [72].

Repetitive behaviors, self-grooming and digging, scored during the freely moving dyadic reciprocal interaction behavioral task, illustrated in Fig. 4 Panels E,F,G,H were conducted at NIMH. GRN-529, 3.0 mg/kg, had no effect on the low self-grooming scores in B6 control mice (Fig. 4 Panel E: $t_{1,16} = -0.98$, $p > 0.05$). GRN-529, 3.0 mg/kg, reduced the high repetitive self-grooming scores in BTBR mice during a 10 minute session of freely moving reciprocal social interaction in the Noldus PhenoTyper open arena environment (Fig. 4 Panel F: $t_{1,20} = -2.09$, $p <$

0.05). Repetitive digging of bedding in the PhenoTyper arena was low in B6 treated with vehicle or GRN-529, 3.0 mg/kg (Fig. 4 Panel G: $t_{1,16} = -0.24$, $p > 0.05$). GRN-529, 3.0 mg/kg, also reduced the high repetitive digging scores in BTBR mice (Fig. 4 Panel H: $t_{1,20} = -2.84$, $p < 0.01$) in the PhenoTyper arena.

Rescue of sociability deficits

Sociability scores illustrated in Fig. 3 represent experiments conducted in the first cohort of B6 and BTBR mice tested at NIMH. Time spent sniffing the novel mouse versus time spent sniffing the novel object reflects a parameter that corroborates chamber time assessments, and is often a more sensitive read-out of intervention responses [87, 90]. B6 displayed more time spent sniffing the novel mouse than time spent sniffing the novel object after vehicle treatment and doses of GRN-529 (vehicle: $F_{1, 9} = 21.52$, $p < 0.05$; 0.3 mg/kg: $F_{1, 9} = 19.22$, $p < 0.05$; 1.0 mg/kg: $F_{1, 9} = 101.1$, $p < 0.05$; 3.0 mg/kg: $F_{1, 9} = 27.04$, $p < 0.05$). BTBR treated with vehicle did not show significant sociability on time spent sniffing ($F_{1, 7} = 5.48$, $p > 0.05$), as expected. GRN-529 restored sociability in BTBR on the sniffing parameter at each dose tested (0.3 mg/kg: $F_{1, 9} = 7.87$, $p < 0.05$; 1.0 mg/kg: $F_{1, 9} = 24.45$, $p < 0.05$; 3.0 mg/kg: $F_{1, 9} = 37.87$, $p < 0.05$).

On the chamber time parameter, B6 displayed significant overall sociability at all doses of GRN-529 and vehicle ($F_{3, 36} = 36.11$, $p < 0.001$). All B6 groups treated with vehicle or GRN-529 exhibited significantly greater time spent in the chamber with the novel mouse than in the chamber with the novel object (vehicle: $F_{1, 8} = 8.21$, $p < 0.05$; GRN-529 0.3 mg/kg: $F_{1, 9} = 11.25$, $p < 0.05$; GRN-529 1.0 mg/kg: $F_{1, 9} = 77.84$, $p < 0.05$ and GRN-529 3.0 mg/kg: $F_{1, 9} = 5.57$, $p < 0.05$). BTBR failed to display sociability on the chamber time parameter after vehicle treatment (vehicle: $F_{1, 9} = 0.004$, $p > 0.05$), consistent with previous findings [62, 64, 72, 74, 78, 82]. GRN-529 had a significant overall effect on sociability in BTBR on the chamber time parameter ($F_{3, 36} = 10.6$, $p < 0.05$). Time in the chamber with the novel mouse was not different from time in the chamber with the novel object for BTBR treated with vehicle ($F_{1, 9} = 0.004$, $p > 0.05$), or with low doses of GRN-529 (0.3 mg/kg: $F_{1, 9} = 3.50$, $p > 0.05$, 1.0 mg/kg: $F_{1, 9} = 1.84$,

$p > 0.05$). At the high dose, 3.0 mg/kg, GRN-529 reversed the lack of sociability in BTBR ($F_{1, 9} = 7.84$, $p < 0.05$).

Number of entries into the side chambers is an internal control measure for general exploratory activity during the social approach task, built into the automated social approach software. GRN-529 increased exploratory locomotion overall during the social approach test session in both B6 ($F_{3, 36} = 6.70$, $p < 0.05$) and BTBR ($F_{3, 36} = 9.85$, $p < 0.05$). Tukey's posthoc comparisons revealed that higher numbers of entries for both strains were significant only at the highest dose, 3.0 mg/kg, compared to vehicle ($p < 0.01$).

A second cohort tested at NIMH yielded similar results (Fig. S2). B6 displayed normal sociability on time spent sniffing (vehicle: $F_{1, 10} = 15.99$, $p < 0.05$; GRN-529 0.3 mg/kg: $F_{1, 11} = 53.8$, $p < 0.05$; GRN-529 1.0 mg/kg: $F_{1, 11} = 62.69$, $p < 0.05$; GRN-529 3.0 mg/kg: $F_{1, 13} = 30.79$, $p < 0.05$). For BTBR, absence of sociability on the sniffing parameter was again reversed by GRN-529 (vehicle: $F_{1, 13} = 1.07$, $p > 0.05$; GRN-529 0.3 mg/kg: $F_{1, 14} = 17.75$, $p < 0.05$; GRN-529 1.0 mg/kg: $F_{1, 14} = 31.45$, $p < 0.05$; GRN-529 3.0 mg/kg: $F_{1, 13} = 30.28$, $p < 0.05$). Sociability in B6 was significant for chamber time after treatment with vehicle and all doses of GRN-529 (vehicle: $F_{1, 10} = 14.8$, $p < 0.05$; GRN-529 0.3 mg/kg: $F_{1, 11} = 9.35$, $p < 0.05$; GRN-529 1.0 mg/kg: $F_{1, 10} = 7.04$, $p < 0.05$; GRN-529 3.0 mg/kg, $F_{1, 13} = 37.5$, $p < 0.05$). BTBR displayed lack of sociability on chamber time after treatment with vehicle and all doses of GRN-529 (vehicle: $F_{1, 12} = 0.01$, $p > 0.05$; GRN-529 0.3 mg/kg: $F_{1, 14} = 0.0001$, $p > 0.05$; GRN-529 1.0 mg/kg: $F_{1,14} = 0.23$, $p > 0.05$; GRN-529 3.0 mg/kg: $F_{1,13} = 1.70$, $p > 0.05$).

Cohort 2 tested at NIMH again showed an elevation in number of entries into the side chambers at the highest doses of GRN-529, indicating that the drug treatment increased exploratory locomotion during social approach in B6 ($F_{3, 44} = 9.1$, $p < 0.05$) and BTBR ($F_{3, 53} = 12.62$, $p < 0.05$), significant at the 1.0mg/kg ($p < 0.01$) and the 3.0 mg/kg ($p < 0.01$) doses for both strains.

Adult reciprocal social interaction behavioral parameters between freely moving dyads using B6 and BTBR subjects paired with a 129Sv/ImJ stimulus partners are illustrated in Fig. 4 A,B,C,D and Fig. S3 A,B,C,D,E,F. Control B6 mice treated with vehicle displayed significant higher levels of the nose to nose sniffing ($t_{1,18}$ = 3.06, $p < 0.01$) and more time spent in social contact parameters ($t_{1,18}$ = 3.73, $p < 0.005$) than BTBR treated with vehicle, consistent with earlier findings [60, 64, 82, 91]. B6 mice treated with GRN-529, 3.0 mg/kg, were not different than B6 mice treated with vehicle on nose to nose sniffing (Fig. 4 Panel A: $t_{1,16}$ = -0.036, $p > 0.05$) and time in social contact (Fig. 4 Panel C: $t_{1,16}$ = -0.278, $p > 0.05$) during the dyadic interaction task. BTBR mice treated with GRN-529, 3.0 mg/kg, exhibited increases in bouts of nose to nose sniffing (Fig. 4 Panel B: $t_{1,20}$ = 2.18, $p < 0.05$) and time in social contact (Fig. 4 Panel D: $t_{1,19}$ = 2.35, $p < 0.02$) compared to BTBR mice treated with vehicle during the dyadic interaction task. While GRN-529 improved social interaction on these two sensitive parameters, not all social measures were affected, consistent with previous studies of multi-parameter behavioral assays [78, 91, 102-103]. B6 mice administered GRN-529, 3.0 mg/kg, had no significant effect on the sociability parameters front approach (Fig. S3 Panel A: $t_{1,16}$ = 0.43, $p > 0.05$), follow (Fig. S3 Panel C: $t_{1,16}$ =1.1, $p > 0.05$) and push-crawl (Fig. S3 Panel E: $t_{1,16}$ = 1.40, $p > 0.05$) compared to vehicle treated B6 mice. BTBR mice administered GRN-529, 3.0 mg/kg, exhibited a trend toward significance on the sociability parameter of subject initiated front approach (Fig. S3 Panel B: $t_{1,18}$ = 1.43, $p = 0.1$) compared to vehicle treated BTBR. GRN-529, 3.0 mg/kg, had no significant effect on the sociability parameters follow (Fig. S3 Panel D: $t_{1,20}$ = 0.68, $p > 0.05$) or push-crawl (Fig. S3 Panel F: $t_{1,20}$ = 1.00, $p > 0.05$).

Absence of Sedation after GRN-529 Treatment

Accuscan open field locomotor activity in Cohort 1 (NIMH, Fig. 5) detected a statistically significant difference for strain ($F_{1, 72}$ = 28.9, $p < 0.0001$) and drug condition ($F_{3, 72}$ = 18.63, $p < 0.05$). Total distance in 5 minute time bins over the 30 minute session was highly significant, confirming normal habituation to the novel open field in both B6 ($F_{5, 360}$ = 90.2, $p < 0.0001$) and BTBR ($F_{5, 245}$ = 56.8, $p < 0.0001$). GRN-529 increased total distance traversed in B6 ($F_{3, 36}$ = 7.56, $p < 0.05$), significant at the 3.0 mg/kg dose ($q = 6.36$, $p < 0.01$). Center time ($F_{3, 36}$ = 0.86,

$p > 0.05$) and vertical activity ($F_{3, 36} = 1.39$, $p > 0.05$) were not altered by treatment with GRN-529 in B6. In BTBR, GRN-529 increased total distance traversed ($F_{3, 37} = 11.78$, $p < 0.05$), significant at doses 1.0 mg/kg ($q = 4.40$, $p < 0.01$) and 3.0 mg/kg ($q = 7.96$, $p < 0.01$) compared to vehicle). Center time ($F_{3, 37} = 0.75$, $p > 0.05$) and vertical activity ($F_{3, 37} = 1.58$, $p > 0.05$) were not altered by treatment with GRN-529 in BTBR.

Accuscan open field locomotor activity in Cohort 2 (NIMH, Fig. S4), again showed that GRN-529 increased total distance traversed in B6 ($F_{3, 41} = 7.80$, $p < 0.001$), significant at the 1.0 mg/kg dose ($q = 4.22$, $p < 0.05$) and the 3.0 mg/kg dose ($q = 6.72$, $p < 0.001$). Center time ($F_{3, 41} = 1.03$, $p > 0.05$) and vertical activity ($F_{3, 41} = 2.58$, $p > 0.05$) again were not altered by GRN-529 in B6. In BTBR, GRN-529 again increased total distance traversed ($F_{3, 53} = 23.36$, $p < 0.001$), significant at each dose tested (GRN-529 0.3 mg/kg, $q = 4.98$, $p < 0.01$; GRN-529 1.0 mg/kg, $q = 9.45$, $p < 0.001$; GRN-529 3.0 mg/kg, $q = 9.99$, $p < 0.001$) compared to vehicle. Center time ($F_{3, 53} = 1.42$, $p > 0.05$) and vertical activity ($F_{3, 53} = 1.75$, $p > 0.05$) again were not altered by GRN-529 in BTBR.

Accuscan open field locomotor activity in Cohort 3 (Pfizer, Fig. S6), similarly showed that GRN-529 increased total distance traversed in B6 ($F_{3, 23} = 106.2$, $p <0.0001$) significant at the 1.0 mg/kg dose ($p < 0.05$) and 3.0 mg/kg dose ($p < 0.05$). In B6 mice, center time was reduced at the 0.3 mg/kg dose ($F_{3, 23} = 15.45$, $p< 0.0001$). Vertical activity was not altered by treatment with GRN-529 ($F_{3, 23} = 13.63$, $p > 0.05$). In BTBR, GRN-529 again increased total distance traversed ($F_{(3, 23)} = 257.0$, $p < 0.0001$), significant at each dose tested (GRN-529 0.3 mg/kg, $p < 0.05$; GRN-529 1.0 mg/kg, $p < 0.05$; GRN-529 3.0 mg/kg, $p < 0.05$) compared to vehicle. Center time ($F_{3, 23} = 13.35$, $p < 0.002$) and vertical activity ($F_{3, 23} = 55.61$, $p < 0.0001$) were elevated at each dose of GRN-529 compared to vehicle in BTBR.

Exploratory activity was further scored as an internal control measure for general exploratory activity during the freely moving dyadic reciprocal interaction task (Fig. S3). GRN-529, 3.0 mg/kg, had no effect on the time spent exploring the arena overall during the social interaction

test session in B6 (Fig. S3 Panel G: $t_{1,16} = 0.01$, $p > 0.05$) or BTBR (Fig. S3 Panel H: $t_{1,20} = 1.47$, $p > 0.05$), compared to vehicle treated mice of the same strain. Bouts of rearing were unaffected by GRN-529, 3.0 mg/kg, in B6 (Fig. S3 Panel I: $t_{1,16} = 1.66$, $p > 0.05$) and BTBR (Fig. S3 Panel J: $t_{1,20} = 0.53$, $p > 0.05$), compared to vehicle mice of the same strain. During this social task, no significant increases in activity were detected after GRN-529 treatment, indicating absence of hyperactivity at the high dose that improved reciprocal social interactions.

C58 mice tested at Pfizer showed a small reduction in vertical activity after GRN-529, 3.0 mg/kg (Fig. S6 Panel A: $F_{3,23} = 3.87$, $p < 0.05$) and spent less time in the center of the arena after all three doses of GRN-529 (Fig. S6 Panel B: $F_{3,23} = 16.25$, $p < 0.001$).

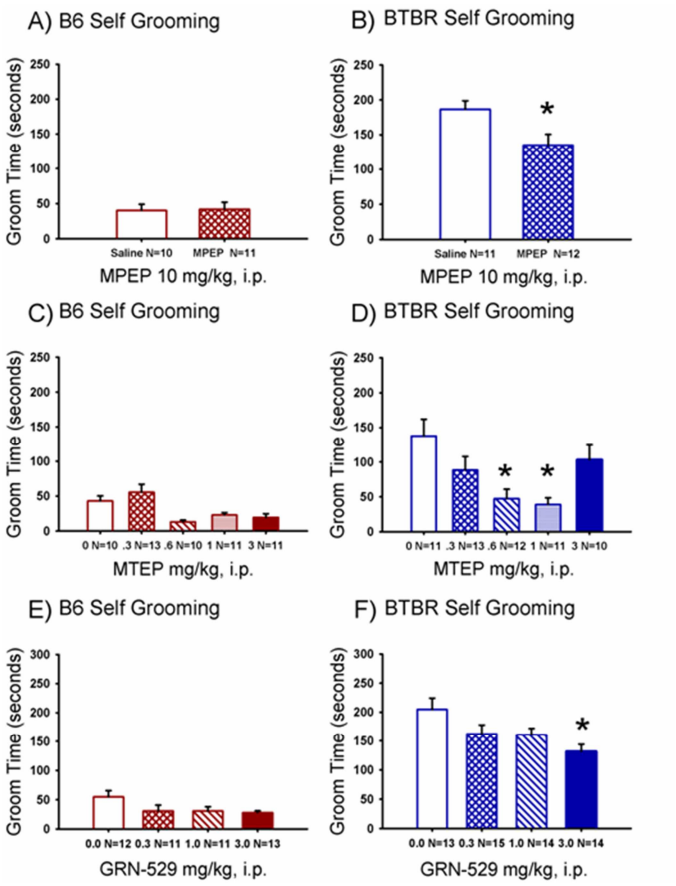

Fig. S1. mGluR5 antagonists reduced repetitive self-grooming in B6 and BTBR.

Cohort 2 tested at NIMH: (A) The prototypic mGluR5 antagonist MPEP had no significant effect on repetitive self-grooming in the control B6 strain. (B) MPEP (10 mg/kg) significantly reduced the high levels of repetitive self-grooming scores in BTBR (*$p < 0.05$), replicating our previous findings [72]. N = 10-12 per dose for each strain, tested at NIMH. (C) The more selective mGluR5 antagonist MTEP had no effect on self-grooming in B6 (0.3 mg/kg, 0.6 mg/kg, 1.0 mg/kg or 3.0 mg/kg, i.p.). (D) MTEP significantly reduced the high levels of repetitive self-grooming in BTBR, after treatment with 0.6 mg/kg and 1.0 mg/kg, i.p. (*$p < 0.05$) as compared to saline vehicle. N = 10-13 per dose for each strain. (E) B6 mice did not display any significant differences in the amount of time spent self-grooming after treatment with GRN-529. (F) BTBR displayed significant reductions in repetitive self-grooming after treatment with 3.0 mg/kg of GRN-529 (*$p < 0.05$) compared to vehicle. N = 10-15 per dose for each strain.

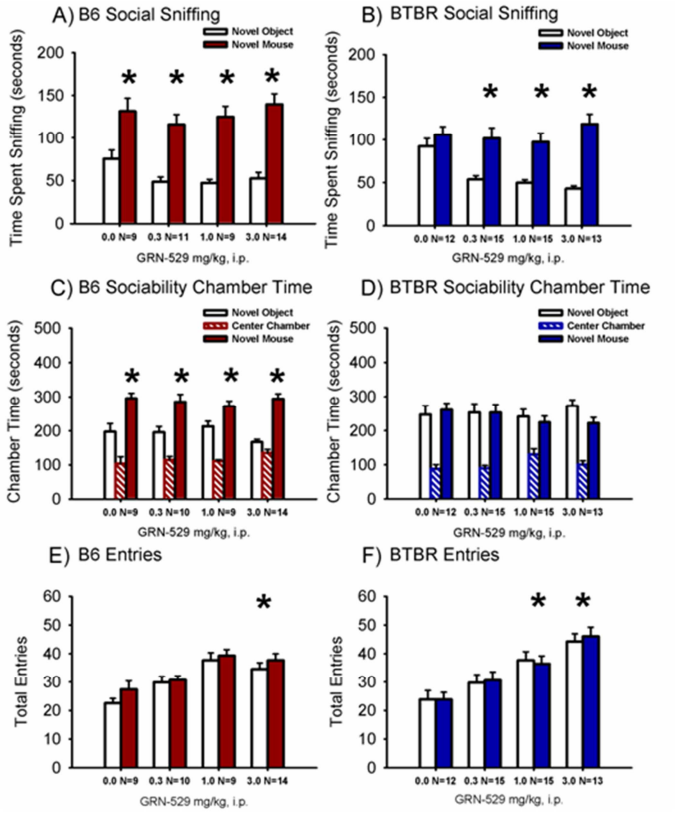

Fig. S2: GRN-529 partially ameliorated social approach deficits in BTBR.

Cohort 2 tested at NIMH: (A) B6 displayed normal sociability on the more specific and sensitive measure of time spent sniffing the novel mouse compared to the novel object at each dose of GRN-529 and vehicle. (B) BTBR treated with GRN-529 (0.3 mg/kg, 1.0 mg/kg and 3.0 mg/kg, i.p.) exhibited significant sociability on the sniff time parameter at all doses. (C) B6 displayed normal sociability, defined as spending more time in the chamber with the novel mouse than in the chamber containing the novel object, following a single i.p. dose of vehicle or GRN-529. (D) BTBR exhibited its characteristic lack of sociability, i.e. did not spend more time in the novel mouse chamber than in the novel object chamber, when treated with vehicle and GRN-529. *$p < 0.05$ novel mouse versus novel object in A-D. (E) B6 and (F) BTBR displayed a greater number of entries into the side chambers after GRN-529 treatment at doses of 1.0 mg/kg and 3.0 mg/kg,

i.p., indicating a general increase in exploratory activity during the social approach task at the higher doses tested. *p <0.05 compared to vehicle in E,F. N = 9-15 per dose for each strain.

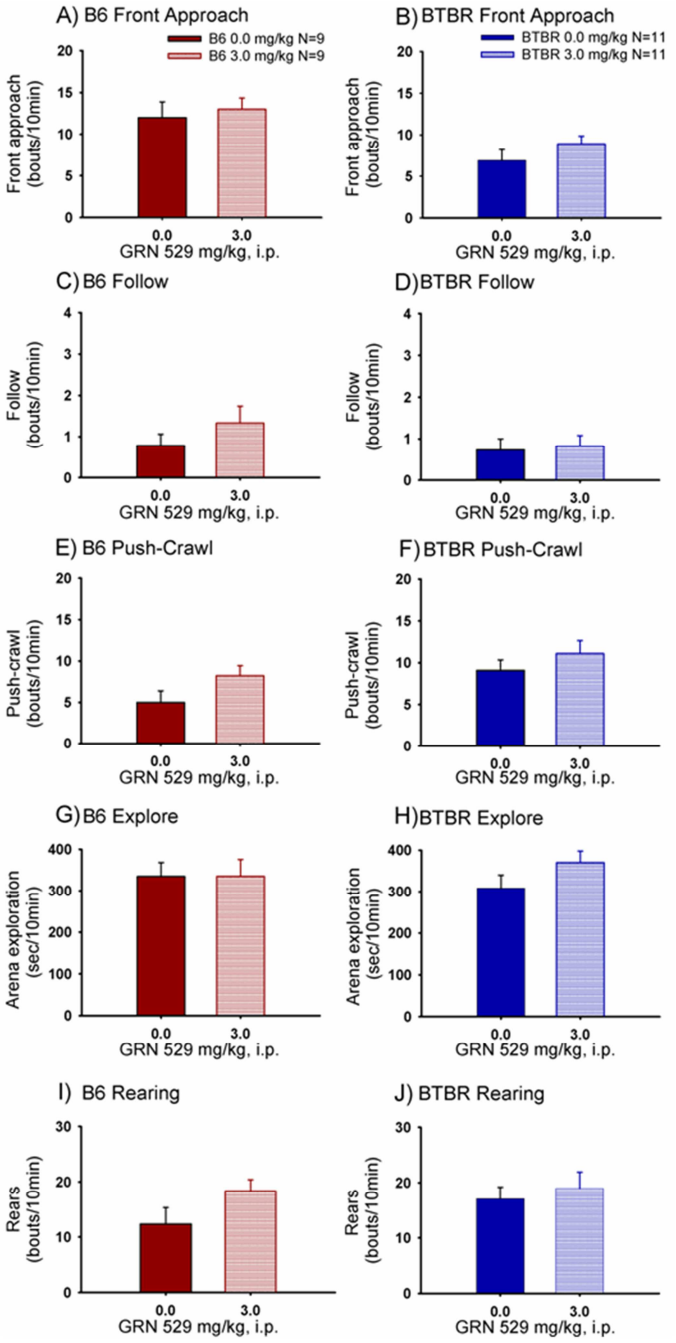

Fig. S3. GRN-529 partially ameliorated reciprocal interaction social deficits in BTBR without sedation or hyperactivation in BTBR.

Cohort tested at NIMH. Social interactions were assayed in the Noldus PhenoTyper 3000 arena by a blinded observer scoring with Noldus Observer 8.0XT software from recordings of a 10 minute test session. (A) The B6 control strain displayed normal sociability, as illustrated by initiating high levels of front approach to the 129Sv/ImJ partner stimulus mouse, following a single i.p. dose of vehicle or 3.0 mg/kg GRN-529. (B) BTBR exhibited its characteristic low sociability, by lower bouts of initiated front approach to the 129Sv/ImJ partner stimulus mouse. GRN-529, 3.0 mg/kg, in the BTBR, exhibited a trend but not statistically significant increase in bouts of initiated front approach compared to vehicle treated BTBR mice. (C) GRN-529, 3.0 mg/kg, had no effect on the number of bouts of the follow parameter in B6 and (D) BTBR compared to vehicle treated mice. (E) GRN-529, 3.0 mg/kg, had no effect on the number of bouts of push-crawl in B6 and (F) BTBR compared to vehicle treated mice. (G) GRN-529, 3.0 mg/kg, had no effect on the total time spent exploring the arena in B6 and (H) BTBR compared to vehicle treated mice. (I) GRN-529, 3.0 mg/kg, had no effect on number of rearing bouts in the arena in B6 and (J) BTBR compared to vehicle treated. N = 9-11 per treatment, GRN-529 3.0 mg/kg or vehicle for each strain.

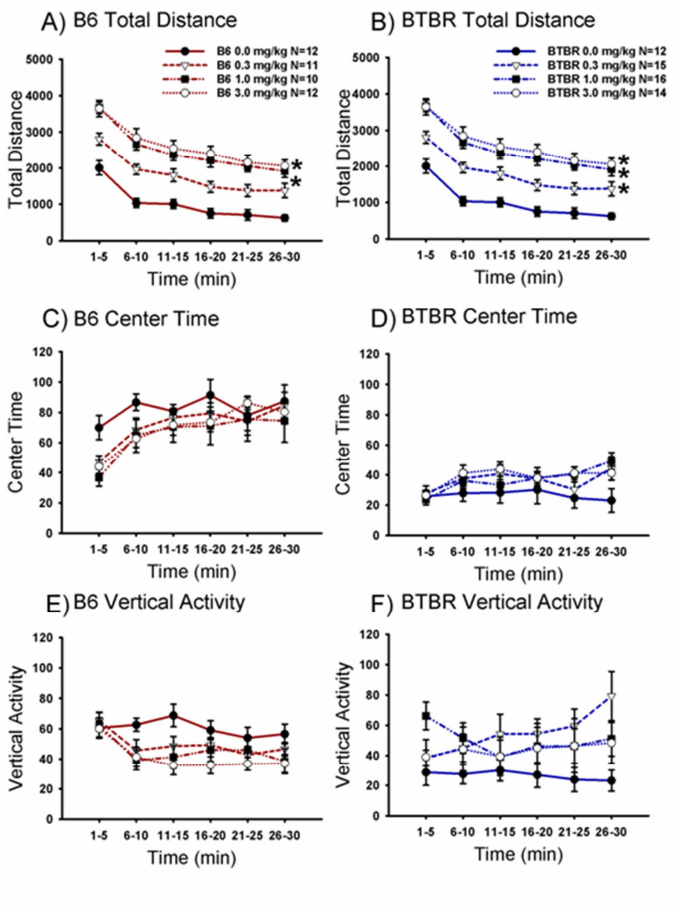

Fig. S4: GRN-529 did not induce sedation at doses that reversed repetitive and social deficits in cohort 2 B6 and BTBR mice tested at NIMH.

(A) B6 displayed a significant increase in total distance traversed following GRN-529 at the higher doses 1.0 mg/kg and 3.0 mg/kg, i.p. as compared to vehicle, indicating modestly increased exploratory activity. GRN-529 administration had no significant effect in B6 on time spent in (C) the center of the arena, or (E) vertical activity. N=10-12 per dose. (B) BTBR displayed significant increases in total distance traversed following GRN-529 at each dose tested compared to vehicle (*$p < 0.05$), indicating increased exploratory activity. GRN-529 administration had no significant effect on (D) time spent in the center of the arena or (F) vertical activity in BTBR. N=12-16 per dose. *$p < 0.05$ as compared to vehicle.

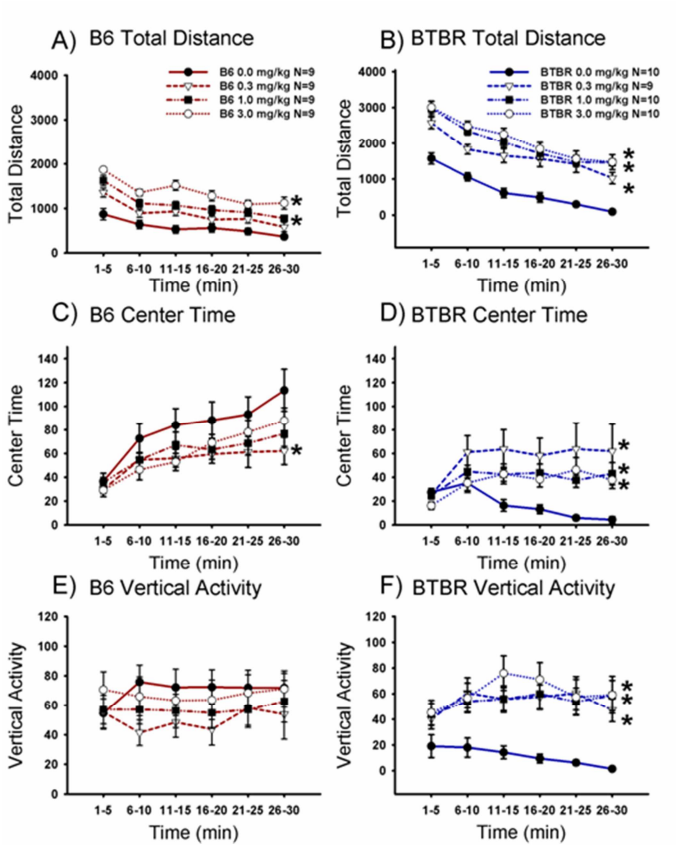

Fig. S5: GRN-529 did not induce sedation in cohort 3 B6 and BTBR mice at doses that reversed repetitive behaviors in cohort 3 at Pfizer.

(A) B6 displayed a significant increase in total distance traversed following GRN-529 at doses of 1.0 mg/kg and 3.0 mg/kg, i.p., as compared to vehicle, indicating increased exploratory activity. (C) GRN-529 0.3 mg/kg reduced time spent in the center of the arena, but had no effect on (E) vertical activity in B6 tested at Pfizer. N=9-10 per dose. (B) BTBR displayed significant increases in total distance traversed, (D) time spent in the center of the arena, and (F) vertical activity, following GRN-529 0.3mg/kg, 1.0 mg/kg and 3.0 mg/kg, i.p., again indicating moderately increased exploratory activity, independent of testing facility. *$p < 0.05$ as compared to vehicle. N=9-10 per dose.

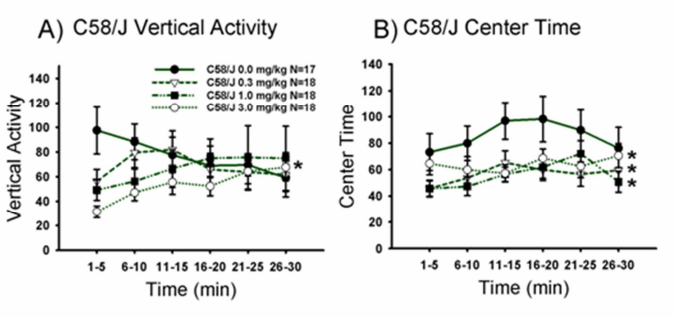

Fig. S6: GRN-529 did not induce sedation in C58 mice at Pfizer. (A) C58 displayed a significant reduction in vertical activity following GRN-529 at the highest dose, 3.0 mg/kg, i.p., as compared to vehicle, corroborating reductions in repetitive jumping. (B) GRN-529 administration reduced time spent in the center of the arena following GRN-529 0.3mg/kg, 1.0 mg/kg and 3.0 mg/kg. *$p < 0.05$ as compared to vehicle. N=17-18 per dose.