Jabarin et al. *Translational Psychiatry* (2021)11:234
https://doi.org/10.1038/s41398-021-01347-1

Translational Psychiatry

ARTICLE Open Access

Pharmacological modulation of AMPA receptors rescues specific impairments in social behavior associated with the A350V *Iqsec2* mutation

Renad Jabarin[1], Nina Levy[2], Yasmin Abergel[1], Joshua H. Berman[1], Amir Zag[2], Shai Netser[1], Andrew P. Levy[2] and Shlomo Wagner[1]

Abstract

In this study we tested the hypothesis that pharmacological modulation of glutamatergic neurotransmission could rescue behavioral deficits exhibited by mice carrying a specific mutation in the *Iqsec2* gene. The IQSEC2 protein plays a key role in glutamatergic synapses and mutations in the *IQSEC2* gene are a frequent cause of neurodevelopmental disorders. We have recently reported on the molecular pathophysiology of one such mutation A350V and demonstrated that this mutation downregulates AMPA type glutamatergic receptors (AMPAR) in A350V mice. Here we sought to identify behavioral deficits in A350V mice and hypothesized that we could rescue these deficits by PF-4778574, a positive AMPAR modulator. Using a battery of social behavioral tasks, we found that A350V *Iqsec2* mice exhibit specific deficits in sex preference and emotional state preference behaviors as well as in vocalizations when encountering a female mouse. The social discrimination deficits, but not the impaired vocalization, were rescued with a single dose of PF-4778574. We conclude that social behavior deficits associated with the A350V *Iqsec2* mutation may be rescued by enhancing AMPAR mediated synaptic transmission.

Introduction

IQSEC2 is an X-linked gene that has been previously associated with ASD, ID, and drug-resistant epilepsy[1,2]. In this study we sought to demonstrate proof of concept for a pharmacological intervention aiming to rescue murine behavioral deficits associated with a specific missense mutation (replacement of alanine with valine at amino acid residue 350 of IQSEC2, denoted A350V IQSEC2) found in human *IQSEC2*[3]. A male child carrying this mutation was diagnosed with drug-resistant epilepsy, autism spectrum disorder and severe intellectual disability and is averbal[3]. We have recently reported that A350V *Iqsec2* CRISPR generated mice (A350V mice) exhibit several behavioral deficits, including increased locomotion in the open field test and impaired spatial learning in the Morris water maze test. However, these mice showed intact sociability and social novelty preference in the three-chamber test[4].

The IQSEC2 protein contains a catalytic domain (Sec7) characteristic of all GEFS (guanine exchange factors), which promotes GDP exchange for GTP on Arf6. It also contains an IQ like domain, which has been proposed to bind calmodulin, and thereby modulate the Sec7 GEF activity of IQSEC2[5–7]. We have previously demonstrated that the A350V *IQSEC2* mutation results in the inappropriate constitutive activation of the Sec7 domain of IQSEC2, which leads to a marked downregulation of hippocampal surface AMPAR in A350V mice. We have further demonstrated that the decrease in surface AMPAR, specifically in the surface GluA2 receptor, is associated with a marked decrease in basal synaptic transmission in the murine model[4]. These findings

Correspondence: Andrew P. Levy (alevy@technion.ac.il) or Shlomo Wagner (shlomow@research.haifa.ac.il)
[1]Sagol Department of Neurobiology, Faculty of Natural Sciences, University of Haifa, Haifa, Israel
[2]Technion Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel
These authors contributed equally: Renad Jabarin, Nina Levy

thereby identify AMPAR mediated transmission as a potential target for treating the deficits associated with the A350V mutation with precise medications.

In the current study, we analyzed the social behavior of A350V male mice in detail, using a battery of different social discrimination paradigms[8,9] as well as by examining several types of social vocalization. We then assessed the ability of PF-4778574, a positive allosteric modulator (PAM) of AMPAR, which serves to increase AMPAR mediated synaptic transmission[10,11] to rescue impairments in these behaviors exhibited by A350V mice.

Methods and materials

Study design

The initial objective of this study was to identify deficits in social behavior exhibited by A350V *Iqsec2* male mice, as compared to their WT littermates. Following the accomplishment of this objective, we defined the assessment of the short- (1–2 days) and long-term (7–11 days) effect of PF-4778574 treatment, as compared to vehicle injection, on these deficits as another objective. The initial sample size for behavioral experiments was determined to be 20 ± 5, based upon power calculations made in a previous study using C57BL/6 J mice[9]. For behavioral deficits in *Iqsec2* mice, we repeated the results in two independent cohorts, so overall sample size was double. The effect of vehicle treatment on sex preference (SxP) and emotional state preference (ESP) in both WT and A350V mice was examined at 1–2 days and 7–8 days following injection. The effect of PF-4778574 treatment on A350V mice was examined at 1–2 days (SxP + ESP) and again at 7–8 days (SxP) or at 11 days (ESP) following treatment. The results of PF-4778574 treatment were qualitatively replicated in two independent cohorts, with slightly smaller sample size due to animal availability. No animals were excluded from experiments and no outliers were defined. A350V and WT mice were always examined together on the same day in random order. All data collection was fully automated, with no involvement of an observer.

Animals

C57BL\6J subjects were commercially obtained (Envigo, Israel) naive males (8–12 weeks old, 3–5/cage). Social stimuli were in-house grown C57BL\6J juvenile (21–30 days old males (SP, SNP) or naïve adult male and female mice (SxP, ESP). Isolated stimuli for the ESP paradigm were each individually housed for 1–2 week.

A350V *Iqsec2* mice generation, maintenance, and genotyping were previously described[4]. All *Iqsec2* animal were bred and maintained in the animal facility of the faculty of medicine of the Technion Institute of Technology until age of 8–10 weeks and then transferred to the University of Haifa mice facility for at least one week before experiments. For all studies we compared the behavior of wild type (WT) and *Iqsec2* mutant littermates generated from the mating of a WT male crossed with a female heterozygous for the A350V mutation. As the *Iqsec2* gene is X-linked, this mating strategy results in half of the male mice being hemizygous for the WT *Iqsec2* allele and half of the male mice being hemizygous for the A350V mutant *Iqsec2* allele. All animals examined in this study were 2–4 months old males (WT and hemizygous littermates), except in the case of pup vocalizations, where both male and female (WT and heterozygous littermates) pups were examined (see below).

All animals were kept on a 12-h light\12-h dark cycle, light on at 1900 hours, with ad libitum access to food and water. Behavioral experiments took place during the dark phase under dim red light. All animal protocols and experiments were approved by the institutional animal care and use committees (IL0460416; IL1691117; IL1271118).

PF-4778574 preparation and injection

PF-4778574 [N- < (3 R,4 S)-3-[4-(5-cyano-2-thienyl)phenyl]tetrahydro-2H-pyran-4-yl>propane-2-sulfonamide] was obtained as a powder from Sigma (catalog #PZ0211) and solubilized (2.5 mg/ml) in dimethyl sulfoxide (sigma cat # D2650). Aliquots were stored at −20 °C and diluted immediately prior to injection in 5% dimethyl sulfoxide, 5% Kolliphor EL (Sigma cat #C5135), and 90% dH_20 as previously described[11]. The PF-4778574 (0.30 mg/kg) and vehicle solutions (200 ul) were administered subcutaneously and subject mice were returned to their home cage until the day of the experiment.

Behavioral assays

All social discrimination tasks were conducted using our published automated experimental system[8]. Social preference (SP) and social novelty preference (SNP) tests were conducted on the same day, as previously described[9].

The SxP test consisted of 15 min habituation to the arena with empty chambers, followed by exposing the subject for 5 min to both adult male and female social stimuli located in individual chambers at opposite corners of the arena.

The ESP test similarly consisted of a 15 min habituation, followed by exposing the subject for 5 min to both group-housed and socially isolated (1 wk) stimuli confined to individual chambers randomly located at opposite corners of the arena. Each isolated stimulus was used for two nonconsecutive tests. All stimuli used in all four tests (SP, SNP, SxP, and ESP) were C57BL\6J mice.

Vocalizations recording

Ultrasonic vocalizations were recorded using a condenser ultrasound microphone (Polaroid/CMPA, Avisoft) placed above the experimental arena and connected to an ultra-sound recording interface (UltraSoundGate 116Hme, Avisoft), which was plugged into a computer equipped with

the recording software Avi-soft Recorder USG (sampling frequency: 250 kHz; FFT-length: 1024points; 16-bit format). Vocalizations were recorded during a 10 min free interaction between 2 and 4 months old male and female, following a 10 min of habituation to the arena. For the recording of pup calls, 4–5 day-old pups were moved to a netted metal cup above which the microphone was placed and vocalizations were recorded for a period of 2 min. In these experiments, pups were recorded indiscriminately and their genotype determined later using PCR, as previously described[4]. Pup vocalizations were analyzed across three groups: (1) WT males and females, (2) A350 V heterozygous females, and (3) A350 V hemizygous males.

Data analysis

Video data analysis was performed by our published custom-made TrackRodent software, as previously described[8]. Audio data was analyzed using our TrackUSF custom-made software as described in https://www.biorxiv.org/content/10.1101/575191v1.

Statistical analysis

SPSS v21.0 (IBM) was used for statistical analysis. Following a Kolmogorov–Smirnov test for checking the normal distribution of the dependent variables and Levene's test for homogeneity, a 2-tailed paired *t*-test was used to compare between parameters within a group, and a 2-tailed independent *t*-test was used to compare a single parameter between distinct groups. Otherwise, the non-parametric Mann-Whitney *U* test was used to compare between parameters in the same group. For comparison between multiple groups and parameters a mixed analysis of variance (ANOVA) model was applied to the data. This model contains one random effect (ID), one within effect, one between effect, and the interaction between them. For comparison within a group using multiple parameters, a two-way repeated measures ANOVA model was applied to data. This model contains one random effect (ID), two within effects, one between effect and the interactions between them. All ANOVA tests were followed, if main effect or interaction were significant, by *post hoc* Student's t test. In case of violation of the normal distribution assumption a Mann–Whitney test was used for comparing between two groups while a Kruskal Wallis Test was used for comparing between multiple groups. Significance was set at 0.05. The parameters and results of all statistical tests are supplied in Dataset 1, while all results of the various experiments are supplied in Dataset 2.

Results

Social behavior of C57BL\6J adult male mice

For analyzing the social behavior of mice subjects in this study, we have used our previously described experimental system which allows automated and detailed analysis of discrimination behavior[8] across a battery of four social discrimination tests. First, we have used the social preference (SP) test to assess social motivation and the social novelty preference (SNP) test to assess social recognition[9]. We have also used the sex preference (SxP) test, in which the subject is exposed simultaneously to male and female conspecifics. Finally, we have used a novel test, termed the emotional state preference (ESP) test, in which the subject is exposed simultaneously to socially isolated and group-housed mice. Figure 1 shows the results of all these tests when conducted with C57BL/6J adult male mice. In the SP test (Fig. 1A) these mice showed a significant preference of the social stimulus over the object throughout the test (Fig. 1B, C; paired *t*-test - $t_{19} = 4.475$, $p < 0.001$). In the SNP test (Fig. 1D), the same subject mice showed a significant preference towards the novel mouse over the familiar one (Fig. 1E, F; paired *t*-test - $t_{19} = -2.66$, $p < 0.05$). In the SxP test (Fig. 1G), C57BL\6J mice spent significantly more time investigating a female over a male stimulus (Fig. 1H, I; paired *t*-test- $t_{24} = 5.137$, $p < 0.001$), indicating a preference for mice of the opposite sex. As for the ESP test (Fig. 1J), C57BL\6J subjects showed a preference to investigate the socially-isolated mouse over the group-housed mouse (Fig. 1K, L; paired *t*-test - $t_{13} = 3.03$, $p < 0.01$), suggesting a tendency to socialize more with mice which are emotionally manipulated[12,13]. As previously reported by us[9], in all tasks the subject's preference was expressed mainly by a strong bias in long (>6 s) investigation bouts, while we observed no preference between the stimuli when short (≤6 s) bouts were considered (Fig. 1M–P).

A350V Iqsec2 male mice exhibit specific deficits in social behavior

Next, we assessed the behavior of wild-type (WT) and A350V male littermates in the same battery of tests as described above for C57BL/6J mice. We found that both genotypes showed intact social preference in the SP test, with significantly greater investigation time for the social stimulus over the object (Fig. 2A–C, mixed-model ANOVA - within stimulus: $F_{(1,37)} = 10.06$, $p < 0.01$; between genotypes: $F_{(1,37)} = 0.259$, $p = 0.614$; stimulus × genotype: $F_{(1,37)} = 0.142$, $p = 0.709$; *post hoc* paired *t*-test - WT: $t_{18} = 2.178$, $p < 0.05$; A350V:$t_{19} = 2.357$, $p < 0.05$). There was also no difference between the two genotypes in the amount of time spent investigating each of the stimuli (t-test - social: $t_{36.9} = 0.557$, $p = 0.581$; object: $t_{37} = -0.007$, $p = 0.994$), suggesting that the A350V Iqsec2 mutation did not affect the motivation of the animals for social interactions. In the SNP test, both genotypes showed a similar preference for investigating the novel stimulus as observed in C57BL\6J mice (Fig. 2D–F; mixed-model ANOVA - within stimulus: $F_{(1,37)} = 23.3$, $p < 0.001$; between genotypes: $F_{(1,37)} = 0.002$, $p = 0.966$, stimulus × genotype:

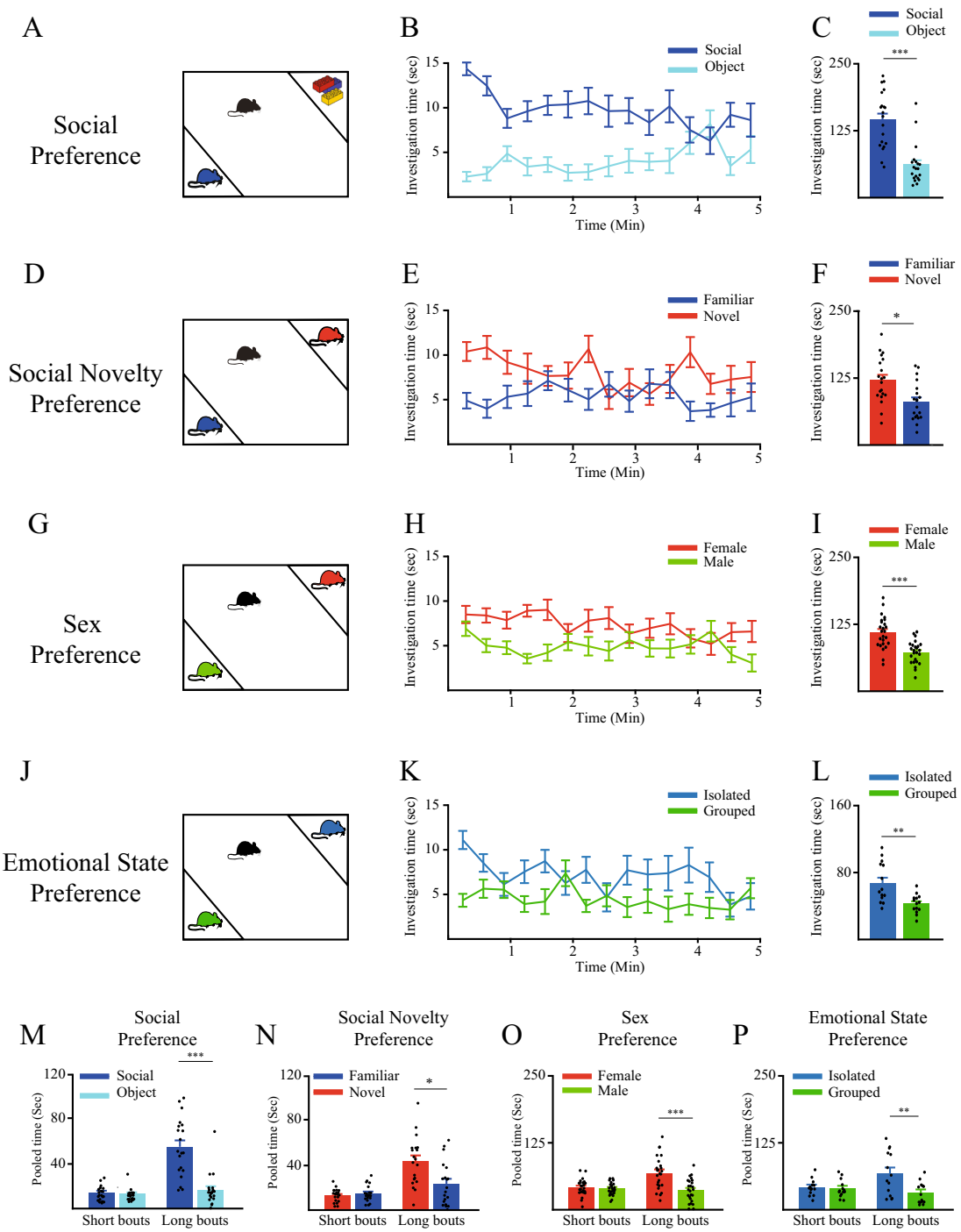

Fig. 1 Social discrimination tests conducted with adult C57BL\6J male mice. A Schematic depiction of the social preference (SP) test. **B** Mean investigation time measured separately for each stimulus (20-s bins) across the SP test ($n = 20$). **C** Mean investigation time summed separately for each stimulus throughout the SP test. **D**–**F** As in **A**–**C**, respectively, for the social novelty preference (SNP; $n = 20$) test. **G**–**I** As in **A**–**C**, respectively, for the sex preference (SxP; $n = 25$) test. **J**–**L** As in **A**–**C**, respectively, for the emotional state preference (ESP; $n = 14$) test. (**M**–**P**) Mean investigation time for each of the stimuli, pooled separately for short (<7 s) and long (≥7 s) investigation bouts, for the SP (**M**), SxP (**O**), and ESP (**P**) tests shown above (**A**–**L**). Note that the preference for a specific stimulus is reflected only by the long bouts in all of these tests. *Post hoc* paired t-tests following main effect in mixed-model ANOVA test; M- short bouts: $t_{19} = -0.576$, $p = 0.571$, long bouts: $t_{19} = -4.64$, $p < 0.001$; N- short bouts: $t_{19} = -0.93$, $p = 0.364$, long bouts: $t_{19} = 2.78$, $p < 0.05$; O-short bouts: $t_{24} = 0.973$, $p = 0.34$, long bouts: $t_{24} = 4.825$, $p < 0.001$; P-short bouts: $t_{13} = 0.708$, $p = 0.492$, long bouts: $t_{13} = 3.167$, $p < 0.01$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, paired *t*-test following the main effect in ANOVA test. All error bars represent SEM.

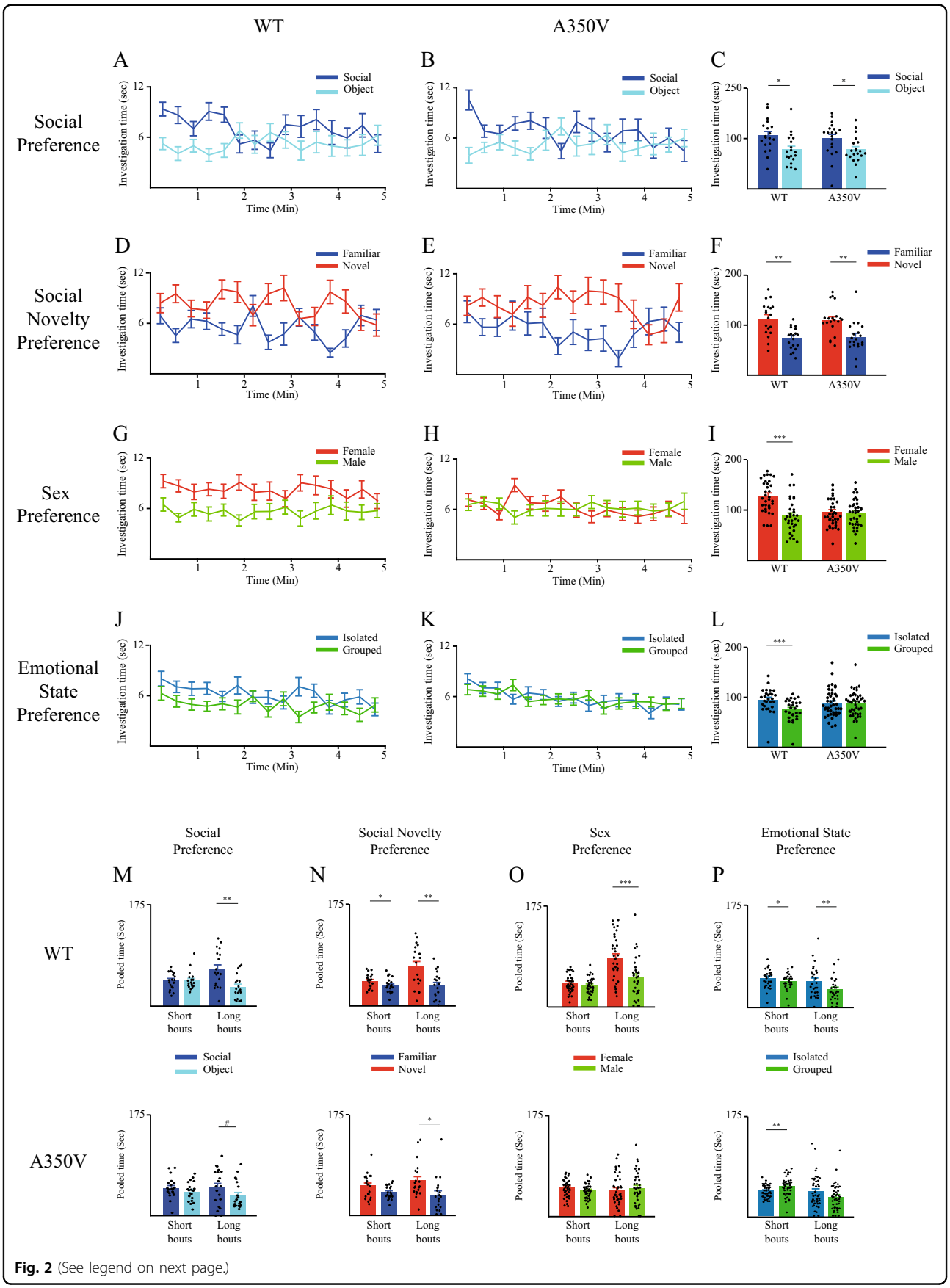

Fig. 2 (See legend on next page.)

(see figure on previous page)

Fig. 2 Deficits in social behavior of A350V *Iqsec2* mice. A, B Mean investigation time measured separately for each stimulus (20-s bins) across the SP test for WT (**A**, $n = 19$) and A350V (**B**, $n = 20$) mice. **C** Mean investigation time summed separately for each stimulus throughout the SP test for WT (left) and A350V (right) subjects. **D–F** Same in **A–C**, for the SNP test (WT - $n = 19$, A350V – $n = 20$). (**G–I**) Same in A-C, for the SxP test (WT - $n = 33$, A350V – $n = 36$). **J–L** Same in A-C, for the ESP test (WT - $n = 28$, A350V – $n = 43$). (**M-P**) Mean investigation time of WT (above) and A350V (below) mice for each of the stimuli, pooled separately for short (<7 s) and long (≥7 s) investigation bouts, for the SP (**M**), SNP (N), SxP (**O**), and ESP (**P**) tests shown above (**A–L**). Note that A350V mice showed an atypical significant preference for the group-housed stimulus when short bouts were considered. *Post hoc* paired t-tests following the main effect in mixed model ANOVA test; M- WT: short bouts: $t_{18} = -0.159$, $p = 0.876$, long bouts: $t_{18} = 3.109$, $p < 0.01$; A350V: short bouts: $t_{19} = 1.277$, $p = 0.217$, long bouts: $t_{19} = 6.7$, $p < 0.001$; N- WT: short bouts: $t_{18} = -2.286$, $p < 0.05$, long bouts: $t_{18} = -2.897$, $p < 0.01$; A350V: short bouts: $t_{19} = -1.954$, $p = 0.066$, long bouts: $t_{19} = -2.4$, $p < 0.05$; O- WT: short bouts: $t_{32} = 1.157$, $p = 0.256$, long bouts: $t_{32} = 3.65$, $p < 0.001$; A350V: short bouts: $t_{35} = -0.278$, $p = 0.776$, long bouts: $t_{35} = -0.243$, $p = 0.809$; P- WT: short bouts: $t_{27} = 2.11$, $p < 0.05$, long bouts: $t_{27} = 3.015$, $p < 0.01$; A350V: short bouts: $t_{42} = -3.23$, $p < 0.01$, long bouts: $t_{42} = 1.76$, $p = 0.086$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, paired *t*-test following the main effect in ANOVA test. All error bars represent SEM.

$F_{(1,37)} = 0.106$, $p = 0.746$; *post hoc* paired *t*-test - WT: $t_{18} = -3.721$, $p < 0.01$; A350V: $t_{19} = -3.13$ $p < 0.01$). Here too there was no difference between the WT and A350V littermates in the amount of time spent investigating each of the stimuli (t-test - Familiar: $t_{37} = -0.274$, $p = 0.785$, Novel: $t_{37} = 0.275$, $p = 0.785$).

In contrast, we found a significant deficit displayed by A350V mice compared to WT littermates in the SxP test. While WT male mice, similarly to C57BL\6 J male mice, showed a clear preference for females, A350V male mice showed no preference for either sex (Fig. 2G–I; mixed-model ANOVA—stimulus × genotype: $F_{(1,67)} = 11.18$, $p < 0.001$; *post hoc* paired *t*-test—WT: $t_{33} = 3.895$, $p < 0.001$; A350V: $t_{35} = -.416$, $p = 0.68$). There was no difference between the two genotypes when the total investigation time of both stimuli was considered (Mann-Whitney test —$U = -0.207$, $p = 0.836$), indicating that the deficit in sex preference behavior observed in A350V mice is not due to an overall decrease in social motivation. The difference in sex preference behavior between WT and A350V littermates was consistent across two cohorts of subjects (Fig. S1A–F). It should be noted that A350V males crossed with WT females generated on average 3.6 ± 3.1 litters (mean ± SEM; $n = 9$ males) as compared to WT males crossed with WT females which generated on average 4.8 ± 0.5 litters (mean ± SEM; $n = 19$ males). Thus, there does not seem to be any significant difference in the sexual activity of the A350V male mice as compared to the WT male mice ($U = 63.5$, $p = 0.365$, Mann–Whitney test).

A similar difference between WT and A350V littermates was observed in the ESP test. While WT mice showed a significant preference for investigating the isolated stimulus, similar to C57BL\6J mice, A350V mice showed no preference for either stimulus (Fig. 2J–L; mixed-model ANOVA - stimulus × genotype: $F_{(1,69)} = 4.329$, $p < 0.05$; *post hoc* paired *t*-test - WT: $t_{27} = 4.904$, $p < 0.001$; MT: $t_{42} = 0.171$, $p = 0.865$). Here too, there was no difference between WT and A350V mice in the total investigation time of both stimuli (Mann-Whitney test - $U = -0.353$, $p = 0.724$). This difference in ESP between WT and A350V mice was consistent across two cohorts of subjects (Fig. S1G–L). Interestingly, when analyzing the data separately for short and long bouts (Fig. 2M–P) we found that A350V mice showed an atypical significant preference for the grouped housed stimulus (Fig. 2P-lower; 2-way ANOVA- stimulus × bout duration: $F_{(1,42)} = 12.014$, $p < 0.001$; *post hoc* paired *t*-test-short bouts: $t_{42} = -3.23$, $p < 0.01$, long bouts- $t_{24} = 1.76$, $p = 0.086$).

We conclude that while A350V *Iqsec2* mice behave the same as their WT littermates in the SP and SNP tests, they exhibit specific deficits in the SxP and ESP tests.

A350V Iqsec2 male mice exhibit a specific deficit in mating calls

Male mice are known to emit ultrasonic vocalizations (USVs) when introduced to female mice (mating calls), but not to male mice[14]. We recorded mating calls of WT and A350V littermates and analyzed them using our novel analysis system (https://www.biorxiv.org/content/10.1101/575191v1) which enables separation of ultrasonic audio fragments (USFs) from noise signals and their clustering using a three-dimensional t-SNE analysis. We found that while a large number of WT animals emitted a reasonable amount of ultrasonic vocalizations, none of their A350V littermates exhibited such behavior (Fig. 3A–C) and this difference was statistically significant (Fig. 3D; Mann–Whitney test- $U = 399$, $p < 0.05$). In contrast, there was no apparent difference in the noise generated by the WT and A350V littermates (Fig. 3A), suggesting no distinction in viability and movement between the two groups. In order to demonstrate that these results were reproducible, we conducted a similar experiment with a new cohort of animals in a different laboratory by distinct research staff and obtained similar results (Fig. S2; Mann–Whitney test- $U = 39$, $p < 0.05$). In order to verify that A350V mice are capable of emitting ultrasonic vocalizations, we recorded pup separation calls from WT and MT littermates and found no significant difference

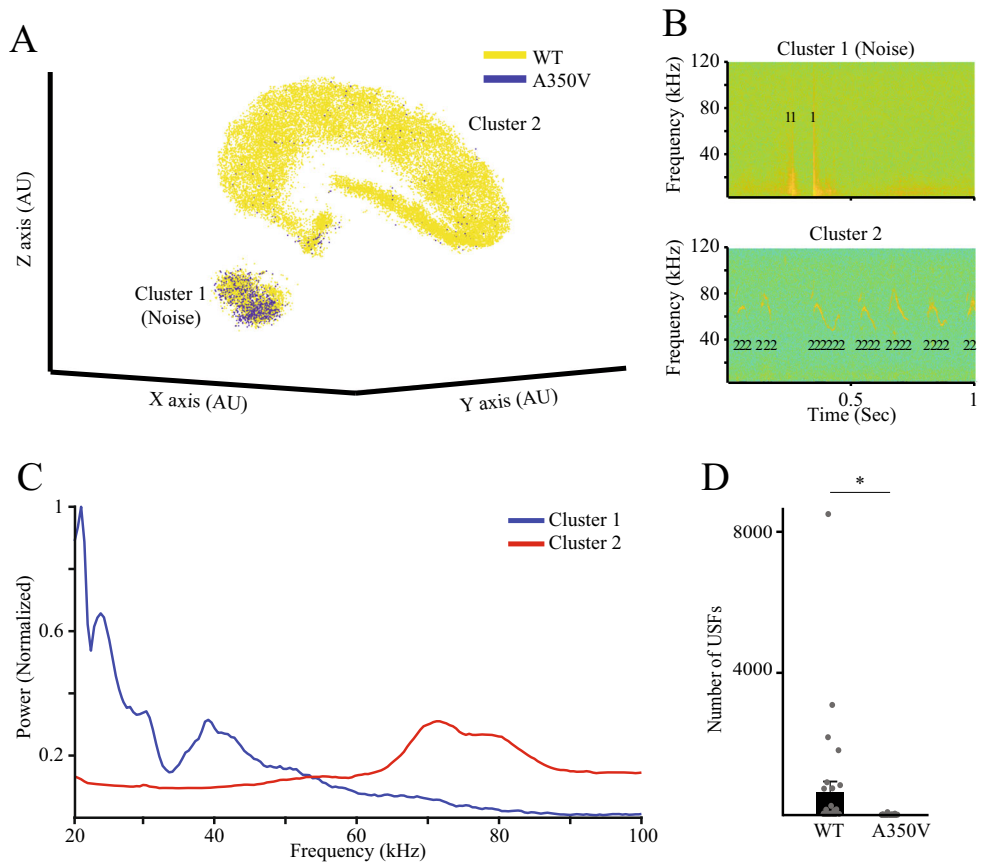

Fig. 3 A350V *Iqsec2* male mice do not emit mating calls. A 3D t-SNE analysis of all ultrasonic fragments emitted by WT (yellow; $n = 31$) and A350V (blue; $n = 36$) adult male mice during an encounter with a female C57BL/6J mouse. Note that while cluster 1 (noise) seem to contain similar number of fragments from both genotypes, cluster 2 (vocalizations) contains almost only fragments from WT mice. **B** Examples of USFs, denoted by their cluster numbers, superimposed on the spectrograms of their respective audio signals. Note that USFs of cluster 1 (upper example) represent non-vocal (noise) signals while USFs of cluster 2 (lower example) represent genuine vocalizations. **C** Power Spectral Density (PSD) analysis of all USFs recorded from all animals, calculated separately for each cluster. Note that in contrast to the PSD profile of cluster 1 which shows a wide range of frequencies, mainly at the lower range, the profile of cluster 2 shows a clear peak at the range of 60–90 kHz. **D** Comparison of the number of USFs from cluster 2 between the two genotypes. Note that A350V mice emitted significantly lower number of USFs as compared to WT animals (Mann–Whitney *U* test. U = 399.000, $^*p < 0.05$). All error bars represent SEM.

between them (Fig. S3; Kruskal Wallis test- chi = 0.469, $p = 0.791$). Altogether, these results suggest a specific deficit exhibited by A350V *Iqsec2* male mice in the emission of mating calls during interaction with a female mouse.

Rescuing social behavior deficits with PF-4778574

We previously revealed a specific deficit in surface expression of the GluA2 AMPAR subunit in the hippocampus of A350V *Iqsec2* mice, which was accompanied by a reduction in glutamatergic synaptic activity in this region[4]. Since the AMPAR modulator PF-4778574 was able to rescue social behavioral deficits in *Cntnap2*-knockout mice, which also display depressed glutamatergic transmission[15], we reasoned that enhancing AMPAR activity with PF-4778574 might alleviate the behavioral impairments exhibited by A350V mice. To examine this hypothesis, we assessed the behavior of A350V mice following vehicle administration as well as 1 day and 1–2 weeks following PF-4778574 administration.

In the SxP test, WT mice showed intact sex preference 1 or 2 days following vehicle injection (Fig. 4A; $t_{13} = 2.163$, $p < 0.05$, paired *t*-test). In contrast, no such preference was observed among either vehicle or drug injected A350V mice 1 or 2 days following vehicle injection (Fig. 4B; mixed-model ANOVA - within stimulus: $F_{(1,50)} = 0.051$, $p = 0.821$; between conditions: $F_{(1,50)} = 0.084$, $p = 0.773$, stimulus × condition: $F_{(1,50)} = 0.353$, $p = 0.550$). Nevertheless, 7 or 8 days after treatment drug injected A350V mice exhibited normal sex preference, in contrast to vehicle-injected animals (Fig. 4C; mixed-model ANOVA - within stimulus: $F_{(1,36)} = 8.382$, $p = 0.006$; between

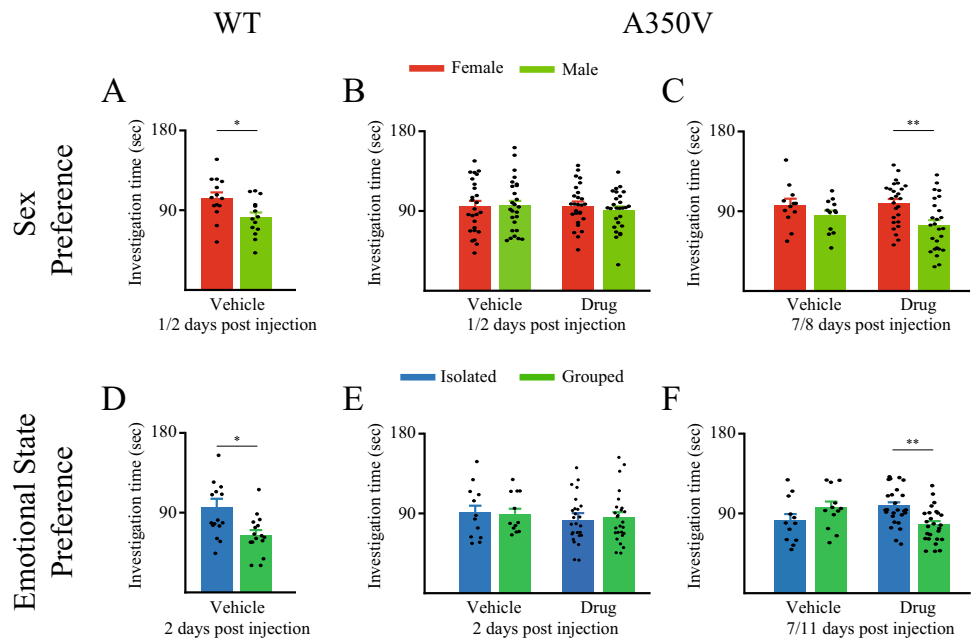

Fig. 4 PF-4778574 rescues sex social behavioral deficits in A350V *Iqsec2* mice. A Mean investigation time summed separately for the Female and Male stimuli throughout the SxP test, conducted by WT mice 2 days after vehicle injection. **B** Mean investigation time summed separately for the Female and Male stimuli throughout the SxP test conducted by A350V mice 1–2 days after either vehicle (left, $n = 26$) or PF-4778574 (right, n = 26) injection. **C** As in **B**, for the SxP test conducted by A350V mice 7–8 days after either vehicle (left, $n = 12$) or PF-4778574 (right, $n = 26$) injection. **D** Mean investigation time summed separately for the Isolated and Grouped stimuli in the ESP test conducted by WT mice 2 days after vehicle injection. **E** Mean investigation time summed separately for the Isolated and Grouped stimuli in the ESP test conducted by A350V mice 1–2 days after either vehicle (left, $n = 12$) or PF-4778574 (right, $n = 26$) injection. **F** As in **E**, for the ESP test conducted by A350V mice 7–11 days after either vehicle (left, $n = 12$) or PF-4778574 (right, $n = 26$) injection. $^*p < 0.05$, $^{**}p < 0.01$, paired *t*-test following the main effect in ANOVA test. All error bars represent SEM.

conditions: $F_{(1,36)} = 0.471$, $p = 0.497$, stimulus × condition: $F_{(1,36)} = 1.282$, $p = 0.265$; *post hoc* paired *t*-test: vehicle: $t_{11} = 1.974$, $p = 0.074$; drug: $t_{25} = 3.129$, $p < 0.01$).

A similar benefit of PF-4778574 in A350V mice was obtained in the ESP test. WT mice showed clear preference towards the isolated social stimulus 1 or 2 days following vehicle injection (Fig. 4D; $t_{15} = 2.65$, $p < 0.05$, paired *t*-test). In contrast, neither vehicle nor drug injected A350V mice showed preference between the two stimuli when tested 1 or 2 days following treatment (Fig. 4E; mixed-model ANOVA - within stimulus: $F_{(1,36)} = 0.01$, $p = 0.922$; between conditions: $F_{(1,36)} = 1.582$, $p = 0.217$; stimulus × condition: $F_{(1,36)} = 0.04$, $p = 0.842$). However, when tested 7 or 11 days after treatment, drug injected A350V mice exhibited a significant preference for the isolated stimulus, in contrast to vehicle injected A350V mice (Fig. 4F; mixed-model ANOVA - stimulus × condition: $F_{(1,36)} = 6.813$, $p = 0.013$; *post hoc* paired *t*-test - vehicle: $t_{11} = -1.28$, $p = 0.227$, drug: $t_{25} = 2.766$, $p < 0.01$).

To gain further support for the dynamics of the effect of the drug, we analyzed the distance traveled by the mice during each test. While no difference was found between the vehicle and the drug 1–2 days after the injection, a significant reduction in the traveled distance of drug-injected animals, as compared to vehicle, was observed at 7–11 days for both tests (Fig. S4; for SxP: 2 way ANOVA - between treatments: $F_{(1,87)} = 2.792$, $p = 0.098$; between times: $F_{(1,87)} = 6.524$, $p < 0.05$, treatment × time: $F_{(1,87)} = 6.524$, $p < 0.05$; *post hoc* independent *t*-test: 1–2 days: $t_{51} = -0.629$, $p = 1.0$; 7–8 days: $t_{14.79} = 2.916$, $p < 0.05$; for ESP: 2 way ANOVA - between treatments: $F_{(1,72)} = 15.869$, $p < 0.001$; between times: $F_{(1,72)} = 1.504$, $p = 0.224$, treatment × time: $F_{(1,72)} = 1.504$, $p = 0.224$; *post hoc* independent *t*-test: 1–2 days: $t_{36} = 1.642$, $p = 0.218$; 7–11 days: $t_{12.627} = 3.603$, $p < 0.01$).

Finally, we examined the effect of PF-4778574 administration on the lack of mating calls observed in A350V mice. We found no improvement in social vocalizations 1 hour and 7 days following drug administration (Fig. S5).

Discussion

In this study, we have employed a battery of social behavioral tests to characterize the deficits in social behavior exhibited by mice with a A350V mutation in the *Iqsec2* gene, a mutation that is associated with epilepsy, ID, and ASD in humans. We also demonstrated that a single administration of PF-4778574, a PAM modulator of the AMPAR, can rescue some of these deficits.

Numerous missense and nonsense mutations have been identified in the X-linked *IQSEC2* gene which has been associated with ID, ASD, and drug-resistant epilepsy[1]. A recent review summarizing 136 subjects and 70 different mutation types revealed the prevalence of ASD to be 25% in males and 30% in females[1]. No human female homozygous for IQSEC2 mutation was described so far. Specific genotype-phenotype correlations have not been identified to date. Ultimately for the purposes of precision medicine treatment of *IQSEC2* mutations it will be necessary to understand if the molecular pathophysiology of these mutations differs. The majority of human mutations are nonsense mutations and several recent studies in which IQSEC2 expression was manipulated in neurons[6,16] or the gene knocked out in mice models[17,18] may be informative in understanding the molecular pathophysiology and phenotypes of nonsense mutations as compared to the A350V *IQSEC2* missense mutation[4]. In vitro studies in neurons in which the catalytic activity of the Sec7 domain was inhibited or constitutively activated suggested that inhibition of the Sec7 activity of IQSEC2 would lead to an impairment in Arf6 mediated AMPAR recycling and consequently increased surface AMPAR expression[6,16] while mutations which appeared to increase Arf6 activation would result in downregulation of AMPAR expression as observed in A350V *Iqsec2* mice[4]. The phenotype of mouse knockout models of *Iqsec2*[17,18] appears to have several notable differences from the A350V *Iqsec2* mutation phenotype as investigated in this and prior studies. First, regarding epilepsy, both knockout models have reported seizures occurring at approximately day 90 while with the A350V mutation seizures are limited to days 14–21[5]. Second, both knockout models have reported an increase in anxiety-related behavior which we have not observed in A350V mice[4]. Differences between the knockout model and the A350V model are not surprising given that the A350V mutation appears to enhance the Sec7 activity of IQSEC2. Demonstration of such differences between loss of function and gain of function in IQSEC2 implies that treatments that may be beneficial for one mutation of *IQSEC2* may not be effective (or even harmful) for a different mutation in this gene. Indeed such a dichotomy has been demonstrated for another gene controlling AMPAR regulation, in which a knockout mutation in the *Thorase* gene resulted in an upregulation of surface AMPAR expression while a gain of function mutation in the same gene resulted in a downregulation in surface AMPAR[19–21].

In a previous study using the same mouse model used here, we demonstrated normal sociability and social novelty preference in the three-chamber test[4]. Here, we employed a novel experimental system designed for directly monitoring social investigation behavior, to examine the behavior of these mice in a battery of four social discrimination tests. In agreement with our previous paper, we found normal behavior of A350V mice in the SP test which assesses social motivation, as well as in the SNP test which assesses social cognition. We did find, however, that A350V mice did not discriminate between the stimuli in both the SxP and ESP tests. These results suggest an impairment in the preference of A350V mice towards specific social stimuli, such as opposite sex and emotionally manipulated stimuli, which may be caused by impaired ability to recognize such stimuli or by the reduced motivation to interact with them. Future studies will be needed to reveal which one of these possibilities is the right one.

Despite its apparent association with sexual behavior, recent works suggest that the preference exhibited by male and female mice to opposite-sex as compared to same-sex stimuli is actually reflecting emotional behavior. This type of socio-emotional behavior, examined by the SxP test, was found to be dependent on brain areas associated with emotional behavior, such as the prefrontal cortex (PFC)[22], nucleus accumbens[23] and medial amygdala[24], as well as on neuromodulators associated with emotion regulation such as serotonin and oxytocin[25]. Here we demonstrated, for the first time, that A350V male mice exhibit a clear deficit in the SxP test. Interestingly, several independent recent works reported atypical sexual orientation and attraction to members of the opposite sex, in association with ASD[26–28].

The ESP test is a novel social behavioral test, which is similar in principle to the affective state discrimination test recently described[12]. As this test examines whether the subject recognizes the emotional state of another individual, it seems to be highly relevant to ASD symptoms which involve impaired theory of mind[29,30]. This behavior was found to be specifically impaired in *Dys-1*$^{+/-}$ mice, a clinically relevant mouse model of cognitive and psychiatric liability[12]. Interestingly, similar to A350V *Iqsec2* mice, *Dys-1*$^{+/-}$ mice exhibit intact social preference and social novelty preference in the three-chamber test[12].

The results showing that both sex preference and emotional state preference behaviors depend on activity of PFC neurons[13,22] and on Oxt release in the amygdala[12,24] suggest that both share common brain mechanisms. Future studies should examine if similar mechanisms are compromised in *Iqsec2* A350V mice. Nevertheless, since *Iqsec2* A350V mice which have a strong construct validity as they carry the same mutation linked to ASD in a human patient, show deficits specifically in the SxP and ESP tests, these tests may be more sensitive to ASD-associated impairments than the sociability and social novelty preference which are commonly used in the field. Accordingly, we observed similar results with *Cntnap2*-knockout mice, a well-established ASD model (unpublished data).

Our observation that A350V *Iqsec2* mice had a dramatic reduction in mating calls during male-female interactions,

as opposed to their WT littermates, is in agreement with a previous study characterizing *Iqsec2*-knockout mice[18]. Such a strong deficit in mating calls was previously described for several genetic mouse models of ASD[31,32]. Nevertheless, PF-4778574 administration did not have any effect on this impairment, despite the rescue of sex preference behavior. This suggests that the lack of mating calls in A350V *Iqsec2* male mice is not due to their lack of motivation for social interactions with females, demonstrated by their impaired SxP behavior, but rather is likely due to a deficit in their social communication.

Benefits from PF-4778574 in the SxP and ESP tests were not observed immediately (1–2 days post administration) but rather 7–11 days following administration. At this point, our results differ from those previously described for *Cntnap2*-knockout mice, where the behavioral effects were observed already 30 min post drug administration[15]. Studies assessing the pharmacokinetics of PF-4778574 in mice[11] have shown that the drug has a very short life in both plasma and brain (less than one hour) similar to other AMPAR PAMs that have been described[33]. This short half-life corresponds with the acute electrophysiological effects of AMPAR PAMs in binding to AMPAR receptors and preventing their rapid deactivation[33,34]. However, AMPAR PAMs also have delayed downstream effects consequent to the AMPAR activation-dependent stimulation of a specific genetic program involving new transcription and protein synthesis[35]. For example, PF-4778574 and other functionally similar AMPAR PAMs stimulate the production and release of neurotrophins (i.e. BDNF), promote the proliferation of neuronal progenitor cells, long-term potentiation, and dendritic remodeling for several[1–10] days after treatment[34–37]. Notably, a recent study reported that a single administration of PF-4778574 in mice produced prolonged antidepressant actions that lasted for 7 days[38], a time frame similar to what we have observed regarding behavioral changes in A350V mice following a single treatment with PF-4778574.

More studies are needed in order to reveal what is the mechanism by which PF-4778574 induces its delayed and long-lasting effect. Moreover, our study was limited to a single protocol of PF-4778574 application, with the drug's dose and route of administration selected based on previous studies demonstrating its efficacy in rescuing AMPAR mediated memory impairments in wild type animals treated with ketamine[11] and social-behavioral defects in *Cntnap2*-knockout mice[15]. Thus, future studies are needed for the development of an optimal administration protocol that will allow maximal benefits from this drug or similar modulators of AMPAR activity.

To conclude, we have utilized a battery of social discrimination tasks to analyze modified social behavior in A350V *Iqsec2* mutant mice. We observed specific deficits in the social behavior of these mice, which suggest that these mice are impaired in socio-emotional behavior and social communication. Moreover, we found that a single administration of the AMPAR PAM PF-4778574 rescued the behavioral deficits, while having no effect on the deficits in social vocalization exhibited by A350V male mice. Decreased AMPAR mediated synaptic transmission appears to represent a convergent pathway for an increasing number of genetic syndromes, including Fragile X[39], Rett[40], Cdkl5[41], Rab39B[42], Shank[43], tuberous sclerosis[44], Atad1[19–21], and Cntnap2[15] as well as in non-genetic syndromes such as neonatal hypoxia[45], suggesting that it may be possible to extrapolate treatments from one of these disorders to the others. The findings of this study may therefore be broadly relevant for a large number of neurodevelopmental disorders of different etiologies and not just for this one mutation in *IQSEC2*.

Acknowledgements
This study was supported by The Human Frontier Science Program (HFSP grant RGP0019/2015 for SW), the Israel Science Foundation (ISF grants #1350/12, 1361/17 for S.W.), by a donation by the Milgrom family for SW and by the Ministry of Science, Technology and Space of Israel (Grant #3-12068 for S.W.).

Author contributions
Study conception and design—S.W., A.P.L.; Acquisition of data – R.J., N.L., Y.A., J.H.B., A.Z.; Analysis and interpretation of data—R.J., S.N.; Drafting of manuscript —S.W., A.P.L.

Code availability
All codes used for the current study are available from the corresponding author on reasonable request. The code used for the video analysis is publicly available at the following link: [https://github.com/shainetser/TrackRodent]. The code used for vocalization analysis is publicly available at the following link: [https://github.com/shainetser/TrackUSF].

Conflict of interest
The authors declare no competing interests.

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Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41398-021-01347-1.

Received: 14 October 2020 Revised: 19 March 2021 Accepted: 30 March 2021
Published online: 22 April 2021

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