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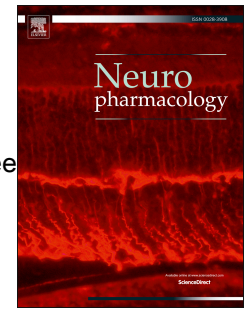

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PII: S0028-3908(24)00056-X

DOI: https://doi.org/10.1016/j.neuropharm.2024.109889

Reference: NP 109889

To appear in: *Neuropharmacology*

Received Date: 18 September 2023

Revised Date: 26 January 2024

Accepted Date: 20 February 2024

Please cite this article as: Fonteneau, M., Brugoux, A., Jaccaz, Dé., Donello, J.E., Banerjee, P., Le Merrer, J., Becker, Jéô.AJ., The NMDA receptor modulator zelquistinel durably relieves behavioral deficits in three mouse models of autism spectrum disorder, *Neuropharmacology* (2024), doi: https://doi.org/10.1016/j.neuropharm.2024.109889.

The NMDA receptor modulator zelquistinel durably relieves behavioral deficits in three mouse models of autism spectrum disorder

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Running title: Zelquistinel relieves autism in 3 mouse models

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Number of words in abstract: 236

Number of words in main text: 5473

Number of figures: 5

Number of tables: 1

Supplementary material: 1 file

ABSTRACT

Autism spectrum disorders (ASD) are complex neurodevelopmental disorders characterized by deficient social communication and interaction together with restricted, stereotyped behaviors. Currently approved treatments relieve comorbidities rather than core symptoms. Since excitation/inhibition balance and synaptic plasticity are disrupted in ASD, molecules targeting excitatory synaptic transmission appear as highly promising candidates to treat this pathology. Among glutamatergic receptors, the NMDA receptor has received particular attention through the last decade to develop novel allosteric modulators. Here, we show that positive NMDA receptor modulation by zelquistinel, a spirocyclic β-lactam platform chemical, relieves core symptoms in two genetic and one environmental mouse models of ASD. A single oral dose of zelquistinel rescued, in a dose-response manner, social deficits and stereotypic behavior in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice while chronic intraperitoneal administration promoted a long-lasting relief of such autistic-like features in these mice. Subchronic oral mid-dose zelquistinel treatment demonstrated durable effects in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$, *Fmr1*$^{-/-}$ and *in utero* valproate-exposed mice. Carry-over effects were best maintained in the *Fmr1* null mouse model, with social parameters being still fully recovered two weeks after treatment withdrawal. Among recently developed NMDA receptor subunit modulators, zelquistinel displays a promising therapeutic potential to relieve core symptoms in ASD patients, with oral bioavailability and long-lasting effects boding well for clinical applications. Efficacy in three mouse models with different etiologies supports high translational value. Further, this compound represents an innovative pharmacological tool to investigate plasticity mechanisms underlying behavioral deficits in animal models of ASD.

Keywords : Autism Spectrum Disorder; Social behavior; Stereotypies; Genetic mouse models; Valproic Acid; N-Methyl-D-Aspartate receptor

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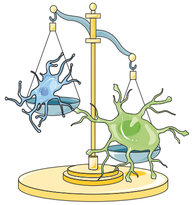
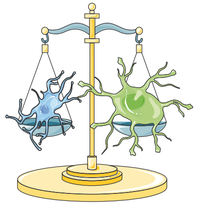
ASD core symptoms

E/I imbalance



Physiological behavior

Restored E/I balance



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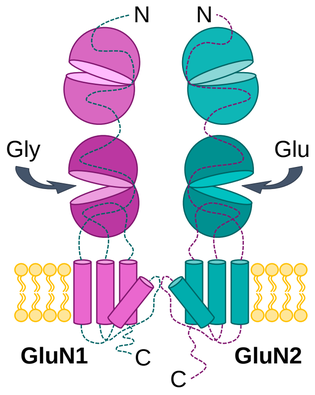
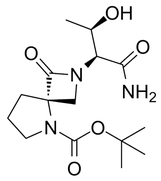
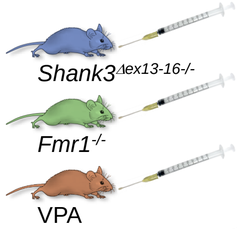
Allosteric modulation of NMDA receptor

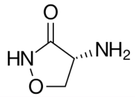


Zelquistinel





D-cycloserine

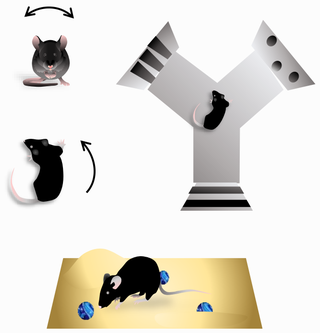


Vehicle

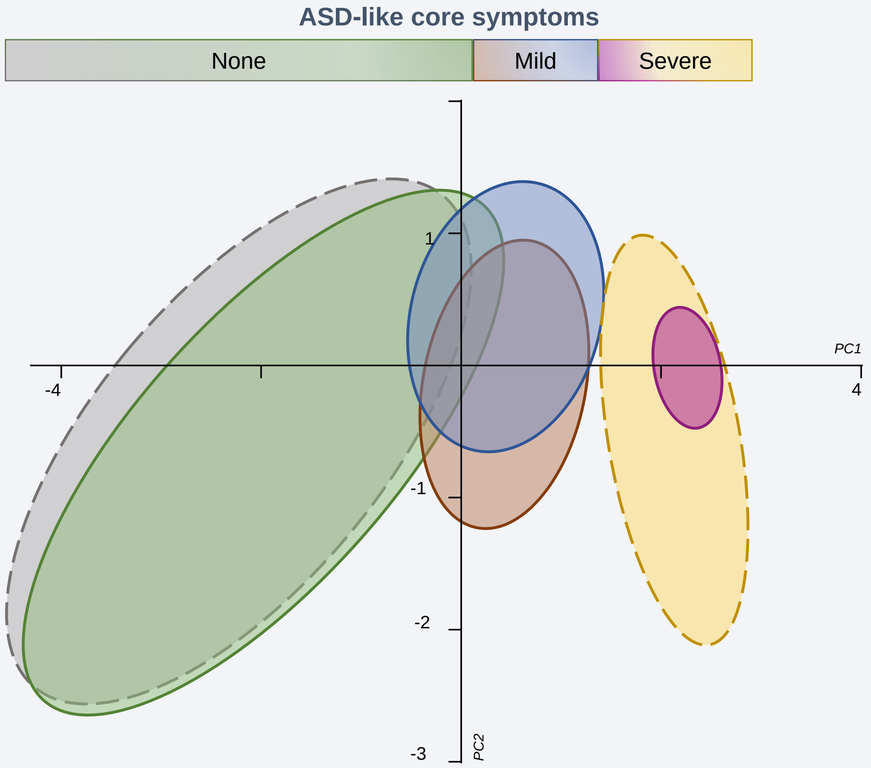
Behavioral phenotyping

Social behavior

Stereotypies



One week after the end of zelquistinel treatment, behavior still remains normalized in *Fmr1* KO mice, and partially relieved in two other models.



1. INTRODUCTION

Autism spectrum disorders (ASD) are complex neurodevelopmental disorders with high heterogeneity and heritability. Their diagnostic is reached in presence of impaired social communication and interaction together with a restricted, repetitive repertoire of behaviors, interests and activities (American Psychiatric Association, 2013). Besides these core symptoms, ASD are often associated with neurobehavioral comorbidities, such as high anxiety, cognitive and motor deficits, aggressive behavior, sleep disorders and epilepsy (Johnson et al., 2007; Mazurek et al., 2013; Pouw et al., 2013; Robinson, 2012; Veenstra-VanderWeele and Blakely, 2012; White et al., 2012; Whyatt and Craig, 2013). Approved pharmacological treatments for ASD mostly target associated symptoms (Dove et al., 2012; Goel et al., 2018). Notably, atypical antipsychotics risperidone and aripiprazole are prescribed to alleviate irritability and aggression associated with ASD but did not prove consistent efficacy in treating core symptoms (Goel et al., 2018; Persico et al., 2019).

Genetic, imaging and preclinical data from mouse models of ASD have pinpointed abnormal brain wiring, altered synaptic function, notably excitatory, and disrupted excitation-inhibition (E/I) balance in ASD (Bagni and Zukin, 2019; Courchesne et al., 2020; Lee et al., 2017; Trobiani et al., 2020; Varghese et al., 2017; Volk et al., 2015). Strikingly, genes coding for proteins related to the glutamatergic synapse or function appear among the most robust candidates for ASD etiopathology (Bagni and Zukin, 2019; Rojas, 2014). Among glutamatergic receptors, NMDA receptors (NMDAR) are hetero-tetramers comprised of two GluN1 and two GluN2 subunits, encoded by *Grin1* and *Grin2a-d* genes, respectively. Genetic studies have evidenced *de novo* mutations and rare variants of *Grin* genes in patients with ASD (O'Roak et al., 2012; Pan et al., 2015; Tarabeux et al., 2011); consistent with this, genetically modified mice bearing

similar mutations display autistic-like behavior and/or comorbidities (Mielnik et al., 2021; Saunders et al., 2013; Shin et al., 2020; Teng et al., 2016). Moreover, mouse models of ASD with different etiologies including genetic ones, as *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ and *Fmr1*$^{-/-}$ models, or environmental ones as mice exposed *in utero* to valproic acid (VPA), share similar decreases in NMDAR expression and/or signaling during adulthood (Chau et al., 2017; Krueger et al., 2011; Martin et al., 2016; Martin and Manzoni, 2014; Peça et al., 2011; Schütt et al., 2009). In this context, molecules that can positively modulate NMDAR neurotransmission have received particular attention as potential treatments for ASD.

NMDAR is unique among ligand-gated ion channels in requiring the occupation of two distinct ligand sites, one by glutamate, and one by glycine/D-serine, for activation. Pharmacologists have taken advantage of this property in developing NMDAR glycine-site agonists, i.e. D-cycloserine, or allosteric modulators devoid of the neurotoxic effects of full NMDAR agonists. The partial agonist D-cycloserine showed promising therapeutic effects, with some improvement of social withdrawal and repetitive behaviors in ASD patients (Aye et al., 2021; Deutsch and Burket, 2023; Minshawi et al., 2016; Posey et al., 2004; Urbano et al., 2015; Wink et al., 2017) and in several mouse models of this pathology (Burket et al., 2013; Um et al., 2018; Won et al., 2012). Rapastinel (formerly GLYX-13), a NMDAR positive allosteric modulator derived from the monoclonal antibody B6B21, was shown to rescue the deficit in play-induced prosocial ultrasonic vocalizations in a rat line selected for this feature (Moskal et al., 2011). Rapastinel also displayed long-lasting NMDAR mediated plasticity and antidepressant effects (Donello et al., 2018; Pothula et al., 2021a).

Zelquistinel (AGN-241751, GATE-251) is a novel rapastinel derivative that exhibits high oral bioavailability and potent, positive allosteric modulation of NDMAR

independent of the glycine/D-serine site. As rapastinel, zelquistinel has showed rapid and sustained antidepressant effects by enhancement of NMDAR function and persistent synaptic plasticity (Banerjee et al., 2019; Burgdorf et al., 2022; Pothula et al., 2021b). The aim of the present study was to explore the therapeutic potential of zelquistinel, compared to the reference molecule D-cycloserine, in relieving multiple ASD-like behavioral features in the well validated genetic *Shank3*$^{\Delta ex13\text{-}16}$ knockout model, and to assess how these effects would generalize to two additional models sharing with the former an altered E/I balance and NMDAR dysfunction: the genetic *Fmr1* null model and the environmental *in utero* VPA exposure model (Davis and Broadie, 2017; Lee et al., 2017; Peça et al., 2011; Tartaglione et al., 2019).

2. MATERIAL AND METHODS

2.1. Animals, breeding procedures and housing conditions

The mutant *Shank3*$^{\Delta ex13\text{-}16}$ (B6.129-Shank3$^{tm2Gfng/J}$, so called *Shank3B*$^{-/-}$, lacking the PDZ domain) (Peça et al., 2011) mouse line was acquired from Jackson Laboratories (Farmington, USA) and bred on a hybrid 50% 129SVPas - 50% C57BL/6J background. *Fmr1*-KO2 mice (Mientjes et al., 2006) were generously provided by R. Willemsen (Erasmus University Medical Center, Rotterdam, The Netherlands) and bred on a C57BL/6J background. In these two mouse lines, equivalent numbers of male and female mice were used for experiments; they were bred in-house from homozygous parents, which were bred from heterozygous animals, to prevent genetic derivation. Mice in the same cage were of the same genotype: this breeding scheme likely favored social deficits in mutant animals by maintaining them together during early post-natal development (Becker et al., 2014; Pujol et al., 2018). *In utero* VPA-exposed mice were obtained by administering VPA intraperitoneally to pregnant time-mated C57BL/6J females on gestational day E=12.5 at a single dose of 400 mg/kg; control dams received a saline (NaCl 0.9%) injection. Equivalent numbers of male and female VPA/saline-exposed offspring were used for the experiments. VPA and saline-exposed mice were housed separately. All animals were aged 8-12 weeks at the beginning of experiments. Except otherwise stated, mice were group-housed (2-4) and maintained on a 12hr light/dark cycle (lights on at 7:00 AM) at controlled temperature (21±1°C); food and water were available *ad libitum*. Cages of control and experimental mice were assigned randomly to a treatment group by the staff of the animal facility (blind to experimenters), provided that gender ratio was equivalent between groups, and that mice from different litters would meet during the direct social interaction test. All experimental procedures were conducted in accordance with the European

Communities Council Directive 2010/63/EU and approved by the Comité d'Éthique en Expérimentation Animale Val de Loire (C2EA-19). Routine veterinary care and animals' maintenance was provided by dedicated and trained personnel.

2.2. Drugs

Zelquistinel (AGN-241751, GATE-251) was provided by Allergan (Madison, USA; batch#1751/01) and administered either by oral gavage or intraperitoneally (i.p.) at doses of 30, 100, 300 or 1000 µg/kg diluted in saline solution (NaCl 0.9%). D-cycloserine was purchased from Sigma Aldrich (Saint-Quentin Fallavier, France) and diluted in saline solution at a dose of 40 mg/kg. The dose of D-cycloserine was chosen based on previous studies not to induce hyperlocomotion (Rhine et al., 2019). When treatment was given subchronically (5 days, *per os*), behavioral testing started 4 days after beginning of daily administration; when treatment was given chronically (i.p.), behavioral testing started 8 days after beginning of daily administration and treatment was maintained for 18 consecutive days (see timelines in related figures). On testing days, or when treatment was given acutely, zelquistinel and D-cycloserine were administered respectively 60 min and 30 min before behavioral assays. Valproic acid was purchased from Sigma Aldrich (Saint-Quentin Fallavier, France) and diluted in saline solution at a dose of 400 mg/kg.

2.3. Behavioral experiments

Experiments were conducted and analyzed blind to genotype and experimental condition. Testing was performed during the light phase. In our experiments, no tendency for sex-dependent effects on behavior were detected. In the acute zelquistinel treatment experiment, three different cohorts of *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice were used: one for direct social interaction tests and two for other behavioral tests. To

assess effects of chronic administration, experiments were performed successively in the same cohort of *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice (Becker et al., 2014; Derieux et al., 2022; Pujol et al., 2018). Testing order was chosen to minimize the incidence of anxiety on subsequent assays. The minimal duration of chronic treatment was set at one week before starting behavioral assessment, based on previous studies using different compounds (Becker et al., 2020, 2014; Derieux et al., 2022; Le Merrer et al., 2024). Treatment was maintained until all behavioral tests were performed. As concerns subchronic administration experiments, four cohorts were used: one cohort of *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice for each pharmacological treatment (zelquistinel and D-cycloserine), one cohort of *Fmr1*$^{-/-}$ mice, and one cohort of *in utero* VPA-exposed mice. Oral gavage was chosen for subchronic administration for best translational value, and 5-day duration was set to ally prolonged effects of zelquistinel with best tolerability of daily gavage in mice. Detailed protocols for assessing social abilities (direct social interaction test, three-chamber social preference test), stereotyped behavior (marble burying test, scoring of spontaneous motor stereotypies and alternation in the Y-maze) and anxiety-like behavior (novelty-suppressed feeding test) can be found in Supplement 1.

2.4. Principal component analysis

A standard principal component analysis (PCA) was performed on behavioral data from zelquistinel and D-cycloserine subchronic *per os* experiments in the three mouse models used for this study to reduce the dimensionality of our data set and allow better visualization and identification of meaningful underlying variables. Among social interaction parameters, we selected time in nose or paw contact and number of following episodes as markers of prosocial behavior and grooming after social contact as a marker of social discomfort/avoidance. We considered the two first extracted

principal components (PC1 and PC2) as accounting for the most relevant variance in the data set and used them for schematic representation.

2.5. Statistics

Statistical analyses were performed using Prism 9.0.0 (GraphPad Software, USA). We defined sample size (GPower 3.1) to ensure enough statistical power using ANOVA or Kruskal-Wallis analysis of variance to detect significant effect on our parameters (effect size f=1.80, α=0.05, σ=5, n=8, power=0.96). For all comparisons, values of p<0.05 were considered as significant. If normality of residuals was respected, statistical significance in behavioral experiments was assessed using two-way or three-way analysis of variance followed by Newman-Keuls post-hoc test. In case of non-normality, non-parametric statistical tests were applied (i.e. Kruskal-Wallis test followed by Dunn's post-hoc test); consequently genotype (or *in utero* valproate exposition) and treatment effects could not be discriminated. Statistical tests and values are available in Tables S1-S4.

3. RESULTS

3.1. A single per os administration of zelquistinel relieved social deficits and stereotypic behavior in *Shank3*$^{\Delta ex13\text{-}16}$ knockout mice

We first evaluated the dose-response effects of a single administration of vehicle or zelquistinel (30, 100, 300 or 1000 µg/kg) administered *per os* to *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice and their wild-type counterparts. Mice were successively tested for direct social interaction one hour (D1), 24 hours (D2), 48 hours (D3) and one week (D8) after acute zelquistinel treatment (Figure 1A). In this test, *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice displayed a severe decrease in prosocial behaviors, namely nose and paw contacts as well as followings, while grooming after social contact was increased, a sign of social discomfort. 60

minutes after acute administration (D1, Figures 1B and S1A), zelquistinel dose-dependently restored social interaction parameters in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice, by increasing the time spent in nose contact *(genotype [G] x treatment [T]: $F_{4,78}$=47.0, p<0.0001)*, as well as the number of following episodes for the highest dose *(H_9=36.4, p<0.0001)*. Conversely, zelquistinel suppressed grooming episodes occurring immediately after a social contact *(H_9=48.4, p<0.0001)*. Rearing activity was used in this test to monitor general activity. On D1, zelquistinel modulated this parameter following an inverted U-shaped curve in wild-type mice; an increase in vertical activity was detected in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice for the highest dose *(G x T: $F_{4,78}$=9.8, p<0.0001)*. Of note, the highest (nose contacts) or the two highest (paw contacts) doses of zelquistinel decreased social interaction in wild-type controls.

On D2 (Figures 1C and S1B), the beneficial effects of zelquistinel were fully maintained, as illustrated by persistent restoration of the time spent in nose contact in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice *(H_9=53.6, p<0.0001)*. From D3 (Figures 1D and S1C), these effects progressively faded away; the efficiency of zelquistinel in restoring time in nose contact in mutant mice was decreased *(G x T: $F_{4,78}$=20.3, p<0.0001*) and became undetectable at D8 (Figures 1E and S1D) *(G x T: $F_{4,78}$=13.5, p<0.0001).* Detrimental effects of zelquistinel at 1000 µg/kg were still observed in wild-type mice. A single *per os* administration of zelquistinel thus restored social interaction in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice, and these effects were fully maintained for 24 hours.

Then, we assessed the acute effects (single administration, one hour before testing) of zelquistinel at the dose of 100 µg/kg, considered as optimal (significant effects in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice up to three days and no detrimental effect in wild-type controls), on repetitive/perseverative behavior and social preference in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice and their wild-type counterparts (Figures 1F and S2A). *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice display

spontaneous, stereotyped, circling episodes and head shakes (Figures 1G and S2B) normalized by oral zelquistinel *(G x T: $F_{1,28}$=28.7, p<0.0001* and *$F_{1,28}$=26.1, p<0.0001,* respectively*)* without modifying rearing and grooming. In the Y-maze (Figure 1H), vehicle-treated *Shank3$^{\Delta ex13\text{-}16\text{-}/\text{-}}$* mice made more arm entries *(H_3=8.2, p<0.05)*, an index of locomotor activity, less alternate arm returns *(G x T: $F_{1,28}$=4.5, p<0.05)* and more frequent perseverative same arm returns *(H_3=15.5, p<0.01)* than vehicle-treated wild-type mice; zelquistinel normalized their behavior. After two weeks, mice tested for motor stereotypies were evaluated in the three-chamber test following a single administration of zelquistinel at 100 µg/kg or vehicle, so that mice previously injected with vehicle received zelquistinel, and vice versa (Figure 1F). *Shank3$^{\Delta ex13\text{-}16\text{-}/\text{-}}$* mice displayed a severe deficit in social preference (Figures 1I and S2C); zelquistinel attenuated this deficit by increasing the time spent exploring a congener versus a mouse *(S x G x T: $F_{1,28}$=6.0, p<0.05)* and suppressing their preference for making longer lasting nose contacts with the toy *(S x G x T: $F_{1,28}$=111.7, p<0.0001),* thus increasing their interaction ratio *(H_3=26.8, p<0.0001)* without modifying their number of entries in both chambers. Therefore, a single acute *per os* administration of zelquistinel relieved stereotypic/perseverative behavior and deficient social preference in *Shank3$^{\Delta ex13\text{-}16\text{-}/\text{-}}$* mice.

3.2. Chronic intraperitoneal zelquistinel treatment relieved multiple autistic-like features in *Shank3$^{\Delta ex13\text{-}16}$* knockout mice

We next performed a chronic administration design to explore more comprehensively the effects of zelquistinel on ASD-like behavioral features in *Shank3$^{\Delta ex13\text{-}16\text{-}/\text{-}}$* mice. To avoid irritation due to prolonged daily oral gavage, we preferred to use the intraperitoneal route. Knockout mice and their *Shank3$^{\Delta ex13\text{-}16+/+}$*

counterparts received a daily i.p. injection of vehicle versus zelquistinel (100 µg/kg) for 18 days (Figure 2A and S3A). They were successively tested for social, stereotyped and anxiety-like behaviors. Social interaction was reassessed 1 week (D25) and 2 weeks (D32) after cessation of treatment.

As regards social behavior, after 9 days of i.p. administration, zelquistinel treatment fully restored social interaction in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice (Figures 2B and S3B). Notably, zelquistinel rescued their time spent in nose contact *(G x T: $F_{1,28}$=67.8, p<0.0001)* and paw contact *(G x T: $F_{1,28}$=34.5, p<0.0001)*, as well as their number of following episodes *(G x T: $F_{1,28}$=4.3, p<0.05)* and suppressed excessive grooming after social contact *(H_3=24.5, p<0.0001)*. No significant effect could be detected on vertical activity. One week after cessation of treatment (D25, Figures 2C and S3C), zelquistinel effects were preserved on time spent in nose contact *(H_3=19.2, p<0.001)* and paw contact *(H_3=17.9, p<0.001)*, number of following episodes *(H_3=18.3, p<0.001)* and grooming after social contact *(H_3=28.1, p<0.0001)*. *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice previously treated with zelquistinel displayed a reduction of rearing activity *(G x T: $F_{1,28}$=6.2, p<0.05)*. After two weeks treatment-free (D32, Figures 2D and S3D), beneficial effects on time in nose contact (*H_3=26.2, p<0.0001*) and paw contact (*H_3=27.1, p<0.0001*) were not anymore significant while excessive grooming after social contact remained abolished *(H_3=28.0, p<0.0001)*.

After 15 days of chronic dosing, i.p. zelquistinel completely rescued social preference in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice (Figures 2E and S3E). Indeed, in the 3-chamber test, chronic zelquistinel restored a preference for spending more time in nose contact *(S x G x T: $F_{1,28}$=62.9, p<0.0001)* with a congener versus a toy in mutant animals, resulting in a complete normalization of their interaction ratio *(G x T: $F_{1,28}$=232.3, p<0.0001)* without modification of the number of chamber entries.

As regards stereotyped/repetitive behavior, prolonged i.p. administration of zelquistinel suppressed excessive marble burying (*G x T: $F_{1,28}$=15.5, p<0.001)* in *Shank3$^{\Delta ex13\text{-}16\text{-}/\text{-}}$* mice (Figure 2F) and tended to decrease stereotyped circling behavior *(H_3=8.0, p<0.05)* and head shakes *(H_3=8.4, p<0.05)* (Figures 2G and S3F). Neither vertical activity nor grooming behavior were modified. When exploring a Y-maze (Figure 2H), *Shank3$^{\Delta ex13\text{-}16\text{-}/\text{-}}$* mice displayed a decreased number of arm entries that was rescued by chronic zelquistinel *(G x T: $F_{1,28}$=4.9, p<0.05)*; the latter similarly restored spontaneous alternation in mutant mice *(G x T: $F_{1,28}$=16.9, p<0.001)*, likely by suppressing perseverative same arm entries *(H_3=26.1, p<0.0001)* in these animals.

Finally, chronic zelquistinel normalized increased latency to feed *(G x T: $F_{1,28}$=24.0, p<0.0001)* and restored food intake *(G x T: $F_{1,28}$=14.7, p<0.001)* of mutant mice in the novelty-suppressed feeding anxiety test (Figure 2I).

Together, these results indicate that chronic zelquistinel treatment relieved core symptoms of social deficit and increased stereotyped behaviors, as well as comorbid exacerbated anxiety, in the *Shank3$^{\Delta ex13\text{-}16}$* knockout mouse model of ASD.

3.3. Subchronic *per os* administration of zelquistinel and D-cycloserine reduced motor stereotypies in two genetic mouse models of autism spectrum disorder

Promising effects of chronic zelquistinel treatment in *Shank3$^{\Delta ex13\text{-}16\text{-}/\text{-}}$* mice raised the question of potential generalization to other mouse models of ASD, involving a different etiology. We first questioned whether repeated oral administration would also produce prolonged significant benefits on stereotyped and social behaviors in *Shank3$^{\Delta ex13\text{-}16\text{-}/\text{-}}$* mice. To ensure the best tolerability to repeated oral gavage, we administered zelquistinel (100 µg/kg) or vehicle *per os*, once daily for 5 days and

assessed motor stereotypies (D4) and social interaction (D5, D12, D19) in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice (Figures 3A, 4A and S4A). We then performed the same experiments in the *Fmr1* knockout model of Fragile X syndrome (Figures 3A, 4A and S5A), and in mice exposed to VPA *in utero*. Due to Covid19 crisis, stereotypic behavior and social interaction two weeks after end of treatment were not evaluated in the latter (Figures 4D and S6A). Then, we evaluated the effects of a reference molecule (i.e. D-cycloserine; 40 mg/kg) in the most extensively characterized model in this study (i.e. *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice) using the same experimental paradigm (Figures 3A, 4A and S7A).

On D4 (Figure 3B), 60 min after oral gavage, zelquistinel reduced the number of circling episodes in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice *(H_3=9.7, p<0.05)*, but failed to diminish excessive grooming *(H_3=20.9, p=0.0001)* and head shakes *(G: $F_{1,30}$=24.7, p<0.0001)* or to restore the number of burying episodes *(G: $F_{1,30}$=57.4, p<0.0001)* and time spent burying *(H_3=20.6, p=0.0001)* in these animals. Zelquistinel increased the number of rearing episodes in all mice *(T: $F_{1,30}$=6.7, p<0.05).* Subchronic zelquistinel treatment thus partially decreased stereotyped behavior in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice.

In *Fmr1*$^{-/-}$ mice (Figure 3C), subchronic zelquistinel administration tended to reduce excessive circling *(G x T: $F_{1,30}$=3.6, p=0.0689; G: $F_{1,30}$=5.7, p<0.05)* while reducing the number of head shakes *(G: $F_{1,30}$=8.4, p<0.01; T: $F_{1,30}$=16.7, p<0.001)* in both *Fmr1*$^{-/-}$ mice and their wild-type controls; conversely, zelquistinel increased grooming episodes (*T: $F_{1,30}$=7.3, p<0.05)*. No effects were detected on rearing or burying activity. In conclusion, subchronic *per os* administration of zelquistinel decreased stereotyped behavior in *Fmr1* null mice.

Under the same conditions (Figure 3D), D-cycloserine reduced spontaneous circling in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice *(G x T: $F_{1,28}$=5.9, p<0.05)*, without affecting other parameters.

Therefore, subchronic oral D-cycloserine decreased stereotyped behavior in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice.

3.4. Subchronic *per os* administration of zelquistinel long-lastingly rescued social behavior in three mouse models of autism spectrum disorder

As regards social interaction, on D5 of repeated administration (Figures 4B and S4B), zelquistinel restored the time spent in of nose contact *(G x T: $F_{1,28}$=61.0, p<0.0001)* and suppressed grooming after social contact *(H_3=26.1, p<0.0001)* in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice. On D12 (Figures 4B and S4C), the effects of zelquistinel on time spent in nose contact were not significant anymore *(H_3=24.8, p<0.0001)* but reduction of grooming after social contact *(G x T: $F_{1,28}$=20.8, p<0.0001)* was maintained. Also, the number and duration of nose contacts were still restored while paw contacts were lost (Figure S4C). Finally, on D19 (Figures 4B and S4D), no effect of zelquistinel administration was detectable anymore, neither on time spent in nose contact *(G: $F_{1,28}$=177.9, p<0.0001)* nor on grooming after social contact *(H_3=19.2, p<0.001)*. Thus, repeated oral zelquistinel efficiently and long-lastingly relieved social interaction deficit in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice.

In *Fmr1*$^{-/-}$ mice (Figures 4C and S5B), subchronic zelquistinel treatment rescued social deficit by normalizing the time spent in nose contact *(G x T: $F_{1,32}$=22.5, p<0.0001)* and reducing grooming after social contact *(H_3=22.5, p<0.0001)*. On D12 (Figures 4C and S5C), the effects of zelquistinel on time spent in nose contact *(G x T: $F_{1,32}$=17.1, p<0.001)* and grooming after social contact *(H_3=22.9, p<0.0001)* in *Fmr1*$^{-/-}$ mice were fully maintained, together with other parameters (Figure S5C). Zelquistinel-induced rescue of social deficits in *Fmr1* null mice persisted up to 2 weeks after the end of treatment (D19, Figures 4C and S5D), especially on time spent in nose contact *(G x T: $F_{1,32}$=25.9, p<0.0001)* and grooming after social contact *(G x T: $F_{1,32}$=32.34,*

p<0.0001). In conclusion, subchronic *per os* administration of zelquistinel long-lastingly relieved social interaction deficit in *Fmr1* null mice.

Five days of repeated *per os* zelquistinel administration completely rescued the social interaction deficit observed in prenatally VPA-exposed mice (D5, Figures 4D and S6B), restoring their time spent in nose contact *(H_3=19.8, p<0.001)* and normalizing their number of grooming episodes after social contact *(H_3=24.3, p<0.0001).* On D12 (Figures 4D and S6C), the effects of zelquistinel on time spent in nose contact (*H_3=29.3, p<0.0001)* and grooming after social contact *(VPA: $F_{1,32}$=45.0, p<0.0001; T: $F_{1,32}$=11.4, p<0.01)* were no longer significant; the duration of nose contacts and number of following episodes remained restored but paw contacts were lost (Figure S6C). Therefore repeated oral administration of zelquistinel efficiently and long-lastingly rescued social interaction behavior in mice exposed to VPA *in utero*.

In *Shank3$^{\Delta ex13\text{-}16\text{-}/\text{-}}$* mice, on D5 of repeated administration (Figures 4E and S7B), D-cycloserine increased the mean duration of nose contacts *(G x T: $F_{1,28}$=52.9, p<0.0001)* and suppressed grooming after social contact *(H_3= 30.3, p<0.0001)*. This treatment increased the time spent in nose contact in both *Shank3$^{\Delta ex13\text{-}16\text{-}/\text{-}}$* mice and wild-type controls *(G: $F_{1,28}$=1179.0, p<0.0001; T: $F_{1,28}$=7.8, p<0.01)*. On D12 (Figures 4E and S7C), beneficial effects of D-cycloserine administration on the mean duration of nose contacts *(G: $F_{1,28}$=414.1, p<0.0001)* and number of grooming episodes after social contact *(H_3=26.2, p<0.0001)* had vanished. Subchronic oral D-cycloserine thus improved social interaction in *Shank3$^{\Delta ex13\text{-}16\text{-}/\text{-}}$* mice, but less efficiently and durably than zelquistinel.

3.5. Carry-over effects of subchronic oral zelquistinel were more prolonged in *Fmr1* knockout mice

Finally, we performed a PCA including social interaction parameters collected from subchronic *per os* zelquistinel and D-cycloserine experiments in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$, *Fmr1*$^{-/-}$ and VPA-exposed mice (Figure 5) either under treatment or a week after cessation of treatment. We selected 4 parameters, time in nose or paw contact and number of following episodes as markers of prosocial behavior and grooming after social contact as a marker of social discomfort/avoidance. Better behavioral improvements under zelquistinel treatment compared to D-cycloserine administration were evidenced by the clustering of mutant and VPA-exposed mice under zelquistinel treatment with control subjects. One week after cessation of treatment, treated *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ and VPA-exposed individuals shifted towards the ASD-like individuals, showing a weakening of zelquistinel effects. In contrast, treated *Fmr1*$^{-/-}$ mice were still found among the control cluster, showing longer-lasting sensitivity to beneficial zelquistinel effects.

In summary, zelquistinel relieved social interaction deficit and stereotyped behavior, more efficiently than D-cycloserine, in three mouse models of ASD. These beneficial effects were more pronounced under chronic i.p. administration in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice and under subchronic oral administration in *Fmr1*$^{-/-}$ mice (Table 1).

4. DISCUSSION

In the present study, we evaluated whether zelquistinel, a novel compound that positively modulates NMDAR activity, may relieve autistic-like behavioral deficits in mouse models with either a genetic or environmental cause.

In the *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mouse model of ASD, we compared three paradigms of zelquistinel administration. After a single oral gavage, zelquistinel dose-dependently relieved social interaction deficits, with a maximum effect reached at the dose of 300

µg/kg. In comparison, in wild-type C57BL6/J mice, dose-dependent antidepressant-like effects of zelquistinel were detected at low doses only (10 and 50 µg/kg) (Pothula et al., 2021b). We also observed a carry-over effect of zelquistinel (3 days), consistent with durable antidepressant effects in wild-type mice and rats after a single dose (Banerjee et al., 2019; Burgdorf et al., 2022; Pothula et al., 2021b). Also, deleterious effects were observed in wild-type controls since the dose of 300 µg/kg for paw contacts. At the highest dose, a deregulation of NMDAR-dependent plasticity may have caused persistent detrimental effects of zelquistinel in wild-type mice. When zelquistinel daily exposure at the intermediate dose of 100 µg/kg was repeated (subchronic oral or chronic i.p. administration), beneficial effects on social behavior in mutant mice were increased, with a complete restoration of paw contacts, and maintained up to a week, suppression of grooming after social contact being still detectable after 2 weeks (chronic i.p. administration). Deleterious effects in wild-type counterparts were not seen under these conditions, likely because they were negligeable at the dose of 100 µg/kg, even after repeated dosing. Furthermore, chronic treatment allowed a complete normalization of social preference in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice compared to partial improvements after acute gavage; benefits on stereotyped and perseverative behaviors, in contrast, were maximal after a single administration. Beneficial effects of zelquistinel on behavior were not predicted by its locomotor effects. Indeed, unlike high doses of D-cycloserine (320 mg/kg), zelquistinel did not increase general activity (Rhine et al., 2019).

Globally, repeated zelquistinel dosing revealed no desensitization of its beneficial effects, which were instead increased under chronic administration. The effects of zelquistinel on social interaction and motor stereotypies were compared across mouse models of ASD under subchronic (5 days) oral gavage conditions (100 µg/kg). The

positive NMDAR modulator demonstrated therapeutic effects in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$, *Fmr1*$^{\text{-}/\text{-}}$ and VPA-exposed mice. However, we detected more prolonged carry-over effects of zelquistinel in the *Fmr1*$^{-/-}$ mice. Notably, paw contacts during social interaction, the most difficult parameter to rescue in our experiments, were still fully restored one week after cessation of treatment in these mice, while they had vanished in the other models. Moreover, significant effects on other social parameters were still detectable two weeks after treatment withdrawal in this mouse model. The significant behavioral effects were unlikely to be due to persistent drug exposure, as brain drug levels fell below the limit of detection within two hours after 100 µg/kg dosing in mice (Table S5) in contrast with rats (Banerjee et al., 2019; Burgdorf et al., 2022). In the latter, despite such short half-life, zelquistinel-induced synaptic plasticity was still maintained one week later (Banerjee et al., 2019; Burgdorf et al., 2022). Thus, carry-over effects indicate that plastic events have developed in the brain of treated mice. Our behavioral results suggest that zelquistinel-induced plasticity was more long-lasting in *Fmr1*$^{-/-}$ mice compared to *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ and VPA-exposed mice.

Zelquistinel has been shown to positively modulate the activity of the NMDA glutamate receptor, a key player in glutamatergic synapses (Burgdorf et al., 2022). Remarkably, *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$, *Fmr1*$^{-/-}$ and VPA-exposed mice share synaptic function deficits, with altered excitatory dendritic spines and disrupted E/I balance (Booker et al., 2019; Mychasiuk et al., 2012; Peça et al., 2011; Tartaglione et al., 2019). SHANK3 is a scaffolding protein anchored at the bottom layer of the postsynaptic density (PSD) of excitatory dendritic spines and connects upper layers to the cytoskeleton machinery (Wilkinson and Coba, 2020). *Shank3* haploinsufficiency or mutations profoundly disorganize the structure of the glutamatergic synapse, with a loss of spines and decreased PSD thickness (Jiang and Ehlers, 2013). In *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice, the levels

of postsynaptic GluN2A and GluN2B, but not GluN1, subunits are decreased in the striatum (Peça et al., 2011). Furthermore, knocking down *Shank3* in the ventral tegmental area results in an increase in the AMPA/NMDA ratio in this region (Bariselli et al., 2016). The Fragile X Messenger Ribonucleoprotein (FMRP), encoded by *Fmr1*, regulates the expression of numerous key proteins of the glutamatergic synapse, including ASD-associated proteins SHANK3, PSD-95 and NMDAR subunits GluN1, GluN2A and GluN2B (Krueger et al., 2011; Schütt et al., 2009). FMRP loss or deactivation induces an increase in PSD-95 expression and in the insertion of AMPA receptors at the postsynaptic membrane, associated with abnormal dendritic spine formation (Coley and Gao, 2018; Colledge et al., 2003; Tsai et al., 2012). In the mPFC and dentate gyrus of *Fmr1*$^{-/-}$ mice, the expression of GluN2A, GluN2B and GluN1 is decreased (Bostrom et al., 2015; Krueger et al., 2011). In the mPFC and nucleus accumbens of these animals, the AMPA/NMDA ratio is increased (Martin et al., 2016; Neuhofer et al., 2015). Finally, in VPA-exposed rodents, multiple proteins of the postsynaptic densities such as SHANK3, PSD-95, CAMKIIα, GluN2A and GluN2B subunits show dysregulated expression, whose direction depends on species, sex, age, and brain region (Alò et al., 2021; Chau et al., 2017; Kim et al., 2016, 2013; Rinaldi et al., 2007). The AMPA/NMDA ratio in VPA-exposed rats also depends on the age, with no alteration in amygdala and mPFC of young animals but an increase in the mPFC of adults (Lin et al., 2013; Martin and Manzoni, 2014). Thus, shared alterations in glutamatergic synapse function/plasticity and increased AMPA/NMDA ratio in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$, *Fmr1*$^{-/-}$ and VPA-exposed mice may represent the neurobiological substrate of zelquistinel therapeutic effects. More prolonged effects of zelquistinel in the *Fmr1*$^{-/-}$ mice compared to *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ and VPA-exposed mice suggest that

glutamatergic transmission is better preserved in this model, making it more responsive to plasticity.

Targeting NMDAR, which tightly interact with PSD proteins such as CAMKIIα, SHANK3 or PSD-95 to regulate synaptic plasticity and structural changes of excitatory dendritic spines, appears highly relevant to restore dysregulated E/I balance in ASD (Cai et al., 2021; Horder et al., 2018). Interestingly, endogenous co-agonists glycine and D-serine, that target preferentially extrasynaptic GluN1-2B heterodimers or synaptic GluN1-2A(-2B) di-(tri-)heteromers, respectively, both normalize synaptic plasticity in *Fmr1* knockout mice (Bostrom et al., 2015; Papouin et al., 2012). D-cycloserine, a partial agonist binding to the glycine/D-serine site, not only reverses social interaction deficits in *Grid1*, *Lrcc4* and *Shank2* knockout mice but also improves NMDAR-induced synaptic plasticity deficiency and abnormal dendritic spine formation (Gupta et al., 2015; Um et al., 2018; Won et al., 2012). However, D-cycloserine was shown to have weak effects on social interaction deficits in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice (Rhine et al., 2019), as also evidenced in the present study by partial and short-lasting improvements in social behavior and stereotypies after subchronic administration. These results are consistent with the results of clinical trials showing modest social improvements in subjects with ASD under D-cycloserine treatment (Aye et al., 2021; Deutsch and Burket, 2023; Minshawi et al., 2016; Posey et al., 2004; Urbano et al., 2015; Wink et al., 2017). Initially described as another partial agonist at glycine/D-serine site, the tetrapeptide rapastinel rather acts as a NMDAR positive allosteric modulator known to induce sustained synaptic potentiation and rapid long-lasting antidepressant effects (Burgdorf et al., 2013; Donello et al., 2018; Pothula et al., 2021a; Zhang et al., 2008). Interestingly, rapastinel was also shown to have potential to relieve autistic-like symptoms (Moskal et al., 2011).

Zelquistinel is a spirocyclic β-lactam platform-based derivative of rapastinel. As rapastinel and NYX-2925, another spirocyclic β-lactam platform-based compound, zelquistinel modulates NMDA-inward currents following an inverted U-shaped dose-response curve. Orally administered in rodents in a similar range of doses than in our study (i.e. 10 to 300 µg/kg), this compound was shown to induce sustained NMDAR-induced long-term plasticity and long-lasting emotional and behavioral positive outputs preferentially via GluN2B-containing NMDAR, just as rapastinel and NYX-2925 (Banerjee et al., 2019; Bowers et al., 2020; Burgdorf et al., 2022; Donello et al., 2018; Khan et al., 2018; Pothula et al., 2021a, 2021b). Because of its greater inward Ca^{2+} conductance, GluN2B is a NMDAR subunit especially regulated during postnatal development to be progressively restricted to extrasynaptic localization in adult forebrain. Such neurodevelopmental regulation is correlated with an increase in PSD-95 and GluN2A subunits at postsynaptic membrane (Bellone and Nicoll, 2007; Coley and Gao, 2018; Gray et al., 2006; Sheng et al., 1994). However, GluN2B expression remains high in some brain regions, particularly within striatum and striatal-like structures as central amygdala (Goebel and Poosch, 1999; Lopez de Armentia and Sah, 2003). Rapastinel preferentially targets extrasynaptic GluN2B-containing NMDAR and increases GluN2B and AMPA GluA1 subunits cell surface expression (Burgdorf et al., 2013; Zhang et al., 2008). In the same way, NYX-2925 increases GluN2B synaptic level and its colocalization with PSD-95, and promotes the insertion of AMPA GluA1 subunits to synaptic membrane at picomolar range concentration through NMDAR-dependent non-ionotropic signaling (Bowers et al., 2020). Since zelquistinel was recently developed, *in vivo* pharmacological and synaptic mechanisms of its action are not fully elucidated. However, a single oral dose of 50 µg/kg in mice was shown to both induce behavioral effects via GluN2B-containing

NMDAR and to increase synaptic expression of PSD-95 and AMPA GluA1 receptor expression (Pothula et al., 2021b). Furthermore, it was recently demonstrated that SHANK3 and GluN2B reciprocally bind to CAMKIIα to respectively induce inactive CAMKIIα form or active GluN2B/PSD-95/CAMKIIα complex leading to structural long-term potentiation, the tardive and durable form of synaptic plasticity (Cai et al., 2021). Considering the high similarity between the structures and plasticity-inducing properties of rapastinel, NYX-2925 and zelquistinel, one could expect that the latter also increases the expression of synaptic GluN2B and long-lastingly normalizes dysregulated AMPA/NMDA ratios, even at very low doses. Such mechanisms may have underlined the rapid but sustained effects of zelquistinel in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$, *Fmr1*$^{\text{-}/\text{-}}$ and VPA-exposed mice. Future functional investigations will aim at challenging this hypothesis, as well as better understanding the pharmacological properties of zelquistinel (i.e. binding site, NMDAR subunit selectivity, mechanism and type of allosteric modulation) to identify the molecular underpinnings of its long-lasting benefits in different mouse models of ASD (Geoffroy et al., 2022; Hackos and Hanson, 2017).

Some limitations can be highlighted in this study. In VPA-exposed mice, we could not assess motor stereotypies after 4 days of gavage and social interaction two weeks after cessation of treatment. The effects of zelquistinel on social interaction in this model, however, look very similar as in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice, suggesting comparable behavioral effects. Also, in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice, the different routes of administration used between subchronic (oral) and chronic (i.p.) zelquistinel treatment paradigms made their comparison delicate. However, zelquistinel has a weak hepatic first-pass metabolism in rodents and easily crosses blood brain barrier, making differences in pharmacokinetics and pharmacodynamics likely negligeable between oral and i.p. administration (Burgdorf et al., 2022). Thus, greater relieving effects after chronic

treatment were most probably a consequence of prolonged exposure. Finally, future studies will aim at exploring the potential benefit of administering this compound at young age, when neurodevelopmental alterations are not fully set.

In conclusion, we evidenced that zelquistinel, a positive NMDAR modulator, relieves core symptoms in three mouse models of ASD, with either a genetic or environmental cause, using two different routes of administration. Robust efficacy in several models with different etiologies supports a high translational potential for clinical applications. Moreover, oral bioavailability and long-lasting effects, particularly in the *Fmr1* knockout model of Fragile X syndrome, make zelquistinel a promising therapeutic candidate for ASD treatment. Further, zelquistinel represents an exciting innovative tool to investigate plasticity mechanisms underlying behavioral deficits in animal models of ASD, as likely targeting GluN2B, a key molecular substrate in this pathology (O'Roak et al., 2012; Stessman et al., 2017).

5. AUTHOR CONTRIBUTIONS

Mathieu Fonteneau: Conceptualization, Investigation, Formal Analysis, Visualization, Writing – Original Draft, Writing – Review and Editing; **Agathe Brugoux:** Investigation, Formal Analysis; **Déborah Jaccaz:** Investigation, Formal Analysis; **John E Donello:** Conceptualization, Resources, Writing – Review and Editing; **Pradeep Banerjee:** Conceptualization, Resources, Writing – Review and Editing; **Julie Le Merrer:** Conceptualization, Methodology, Investigation, Formal Analysis, Visualization, Writing – Original Draft, Writing – Review and Editing, Supervision, Project administration, Funding acquisition; **Jérôme AJ Becker:** Conceptualization, Methodology, Investigation, Formal Analysis, Visualization, Writing – Review and Editing, Supervision, Project administration, Funding acquisition.

6. DECLARATION OF INTEREST

This work was supported by Allergan Laboratories, Gate Neurosciences, the Centre National de la Recherche Scientifique (CNRS), Institut National de la Santé et de la Recherche Médicale (Inserm), Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), and Université de Tours. Allergan Laboratories and Gate Neurosciences provided fundings and had a role in the study design. Other institutions had a role in funding salaries for permanent staff and providing optimal technical and scientific environment for research. Pradeep Banerjee is an employee of Allergan (now part of AbbVie). John E. Donello was an employee of Allergan and is now an advisor of Gate Neurosciences. All the other authors declare that they have no conflict of interest.

7. ACKNOWLEDGMENTS

We thank the Experimental Unit PAO-1297 (EU0028, Animal Physiology Experimental Facility, DOI: 10.15454/1.5573896321728955E12) from the INRAE-Val de Loire Centre for animal breeding and care. We acknowledge Audrey Léauté for her technical assistance in performing behavioral experiments.

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9. LEGEND TO FIGURES

Figure 1. Dose-dependent effects of a single *per os* zelquistinel administration on social interaction, motor stereotypies and perseverative behavior in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ and wild-type controls

(A) In a first series of experiments, *Shank3*$^{\Delta ex13\text{-}16+/+}$ and *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice received a single *per os* injection of vehicle (n=12 per genotype) or zelquistinel (30, 100, 300 or 1000 µg/kg; n=8 per genotype and per dose) and were successively tested for direct social interaction (10 min) **(B)** one hour, **(C)** 24 hours, **(D)** 48 hours and **(E)** one week after treatment. **(F)** In a second series of experiments, a first cohort of animals (blue) received vehicle (n=8 per genotype) or zelquistinel (100 µg/kg; n=8 per genotype) *per os* at day 1 and day 15 and were tested after 60 min **(G)** for motor stereotypies during 10 min and **(I)** in the 3-chamber test during 10 min; a second cohort (black) of *Shank3*$^{\Delta ex13\text{-}16+/+}$ and *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice received a single *per os* injection of vehicle (n=8 per genotype) or zelquistinel (100 µg/kg; n=8 per genotype) and was tested after 60 min **(H)** for Y-maze exploration during 5 min. Data are presented as scattered plots with mean ± SEM. Asterisks: zelquistinel-treated wild-type mice and vehicle-treated knockout mice compared to vehicle-treated wild-type mice; daggers: zelquistinel-treated knockout mice compared to vehicle-treated knockout mice; hashes: stimulus x genotype x treatment interaction, mouse versus object comparison; one symbol $p<0.05$, two symbols $p<0.01$, three symbols $p<0.001$. 3-Ch: 3-chamber social preference test; AAR: alternate arm returns; D: day; M: mouse; MS: motor stereotypies; p.o.: *per os*; SAR: same arm returns; SI: social interaction test; SPA: spontaneous alternation; T: toy. More parameters available in Fig. S1 and S2 (Supplement 1).

Figure 2. Effects of chronic intraperitoneal administration of zelquistinel on autistic-like symptoms in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ and wild-type controls

(A) *Shank3*$^{\Delta ex13\text{-}16+/+}$ and *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice received vehicle (n=8 per genotype) or zelquistinel (100 µg/kg; n=8 per genotype), i.p. daily for 18 days; behavioral assays were conducted from day 8 to day 32 to assess multiple core and comorbid autistic-like symptoms. Social interaction (10 min) was tested **(B)** on day 9, **(C)** one week after end of treatment and **(D)** two weeks after end of treatment. **(E)** Social preference (10 min) was assessed on days 15 and 16. As regards stereotypic behavior, **(F)** stereotyped marble burying (15 min) was measured on day 8, **(G)** motor stereotypies (10 min) on days 10 and 11 and **(H)** Y-maze exploration pattern (5 min) on day 12. Finally, **(I)** conflict between novelty and anxiety in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ mice, was assessed in the novelty-suppressed feeding test (15 min) on days 17 and 18. Data are presented as scattered plots with mean ± SEM. Asterisks: zelquistinel-treated wild-type mice and vehicle-treated knockout mice compared to vehicle-treated wild-type mice; daggers: zelquistinel-treated knockout mice compared to vehicle-treated knockout mice; hashes: stimulus x genotype x treatment interaction, mouse versus object comparison; one symbol $p<0.05$, two symbols $p<0.01$, three symbols $p<0.001$; (t): treatment effect. 3-Ch: 3-chamber test; AAR: alternate arm returns; D: day; i.p.: intraperitoneal; M: mouse; MB: marble burying test; MS: motor stereotypies; NSF: novelty-suppressed feeding test; SAR: same arm returns; SI: social interaction test; SPA: spontaneous alternation; T: toy. More parameters available in Fig. S3 (Supplement 1).

Figure 3. Effects of subchronic *per os* administration of zelquistinel and D-cycloserine on motor stereotypies in *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ and *Fmr1*$^{-/-}$ ASD mouse models

(A) Mice received vehicle (n=8 per genotype), zelquistinel (100 µg/kg; n=8 in *Shank3*$^{\Delta ex13\text{-}16+/+}$ condition, n=10 in *Shank3*$^{\Delta ex13\text{-}16-/-}$ condition, n=10 to *Fmr1*$^{+/+}$ mice, n=8 to *Fmr1*$^{-/-}$ mice) or D-cycloserine (40 mg/kg; n=8 per genotype) *per os* daily for 5 days. Effects of zelquistinel on motor stereotypies (10 min) were assessed on day 4 **(B)** in *Shank3*$^{\Delta ex13\text{-}16-/-}$ mice and **(C)** *Fmr1*$^{-/-}$ mice and their both respective wild-type controls. **(D)** Effects of D-cycloserine in *Shank3*$^{\Delta ex13\text{-}16-/-}$ mice were similarly assessed. Data are presented as scattered plots with mean ± SEM. Asterisks: zelquistinel/D-cycloserine-treated wild-type mice and vehicle-treated knockout mice compared to vehicle-treated wild-type mice; daggers: zelquistinel/D-cycloserine-treated knockout mice compared to vehicle-treated knockout mice; one symbol $p<0.05$, two symbols $p<0.01$, three symbols $p<0.001$; (g): genotype effect; (t): treatment effect. D: day; MS: motor stereotypies; p.o.: *per os*; SI: social interaction.

Figure 4. Effects of subchronic *per os* administration of zelquistinel and D-cycloserine on social interaction in *Shank3*$^{\Delta ex13\text{-}16-/-}$, *Fmr1*$^{-/-}$ and *in utero*-exposed valproate ASD mouse models

(A) Mice received vehicle (n=8 per genotype or *in utero* treatment), zelquistinel (100 µg/kg, n=8 both in *Shank3*$^{\Delta ex13\text{-}16+/+}$ and *Shank3*$^{\Delta ex13\text{-}16-/-}$ mice, n=10 both in *Fmr1*$^{+/+}$ and *Fmr1*$^{-/-}$, n=10 both in *in utero* saline- and VPA-exposed mice) or D-cycloserine (40 mg/kg; n=8 per genotype) *per os* daily for 5 days. Prosocial effects of zelquistinel on social interaction (10 min) were assessed on days 5, 12 and 19 in **(B)** *Shank3*$^{\Delta ex13\text{-}16-/-}$, **(C)** *Fmr1*$^{-/-}$ and **(D)** *in utero* VPA-exposed mice, and their respective controls. **(E)** Effects of D-cycloserine in *Shank3*$^{\Delta ex13\text{-}16-/-}$ mice were similarly assessed. Data are presented as scattered plots with mean ± SEM. Asterisks: zelquistinel/D-cycloserine-treated wild-type/saline-exposed mice and vehicle-treated knockout/VPA-exposed

mice compared to vehicle-treated wild-type/saline-exposed mice; daggers: zelquistinel/D-cycloserine-treated knockout/VPA-exposed mice compared to vehicle-treated knockout/VPA-exposed mice; two symbols $p<0.01$, three symbols $p<0.001$; (g): genotype effect; (t): treatment effect; (v): *in utero* VPA exposition effect. D: day; MS: motor stereotypies; p.o.: *per os*; SI: social interaction. More parameters available in Fig. S4 to S7 (Supplement 1).

Figure 5. Long-lasting effects of subchronic *per os* administration of zelquistinel and D-cycloserine over the different ASD mouse models

A PCA was performed on social interaction parameters across the 3 mouse models either during (left panels) or a week after end of subchronic *per os* zelquistinel or D-cycloserine administration (right panels). Prosocial parameters (time in nose contact, time in paw contact, number of following episodes) were opposed to grooming after social contact, a social avoidance parameter, along PC1 at the two time points (top panels). At both time points and across treatments, projection in the subjects' space (bottom panels) dissociated individuals with social interaction deficit (positively correlated with PC1) from subjects showing canonical social interaction (negatively correlated with PC1). Percentage of variance explained is quoted for each PC. D: day; Foll: number of following episodes; GASC: grooming after social contact; PC: principal component; TINC: time in nose contact; TIPC: time in paw contact.

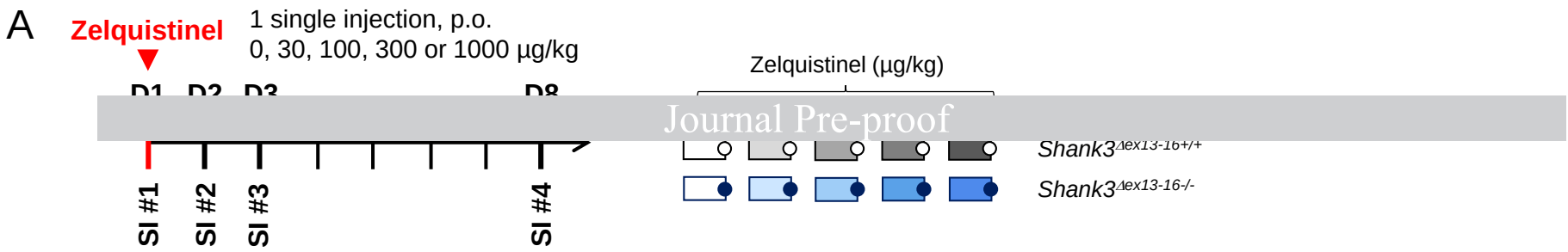
1. TABLE

ASD mouse model	Treatment	Route	Duration	Dose (µg/kg)	Sociability	Stereotypies	Anxiety
***Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$**	zelquistinel	p.o.	acute	0-1000	+	++	NA
		p.o.	subchronic	100	++	+	NA
		i.p.	chronic	100	+++	++	+++
	D-cycloserine	p.o.	subchronic	40000	+	+	NA
***Fmr1*$^{-/-}$**	zelquistinel	p.o.	subchronic	100	+++	++	NA
VPA	zelquistinel	p.o.	subchronic	100	++	NA	NA

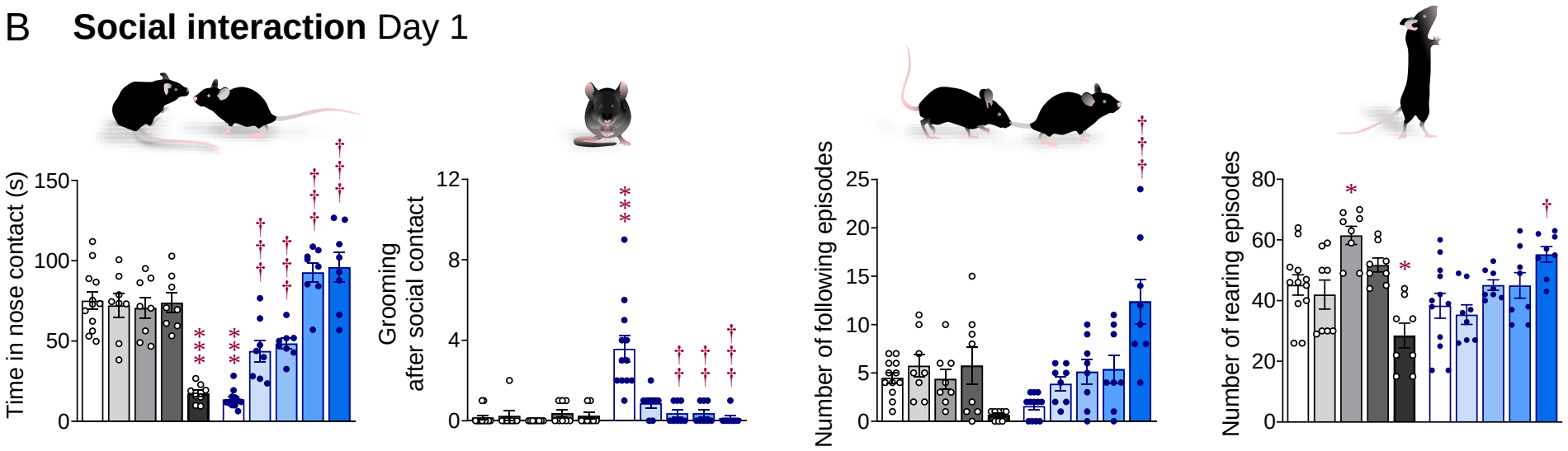
Table 1. Summary of treatment effects over the different ASD mouse models

ASD: autism spectrum disorder; i.p.: intraperitoneal; NA: not assessed; p.o.: *per os*; VPA: valproate.

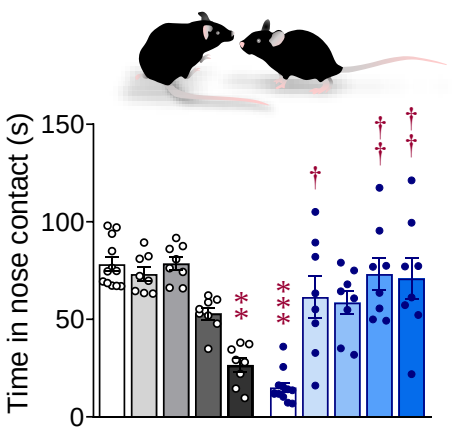
A



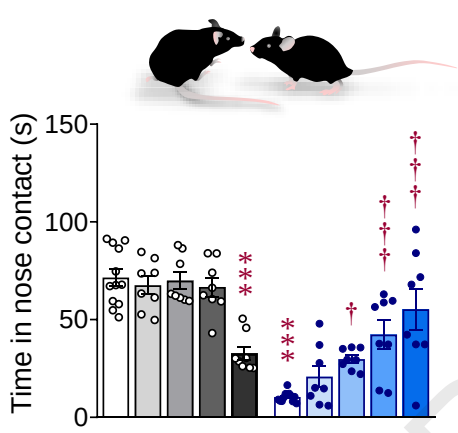
B **Social interaction** Day 1



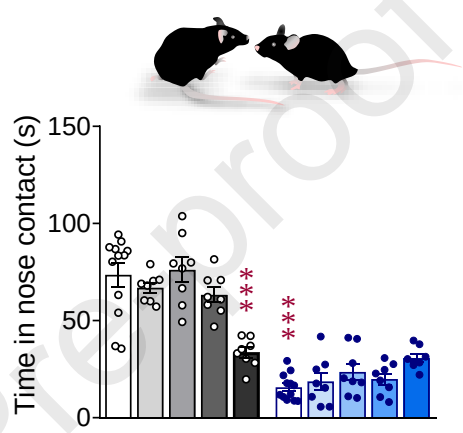
C Day 2 (24 hours)



D Day 3 (48 hours)



E Day 8 (168 hours)



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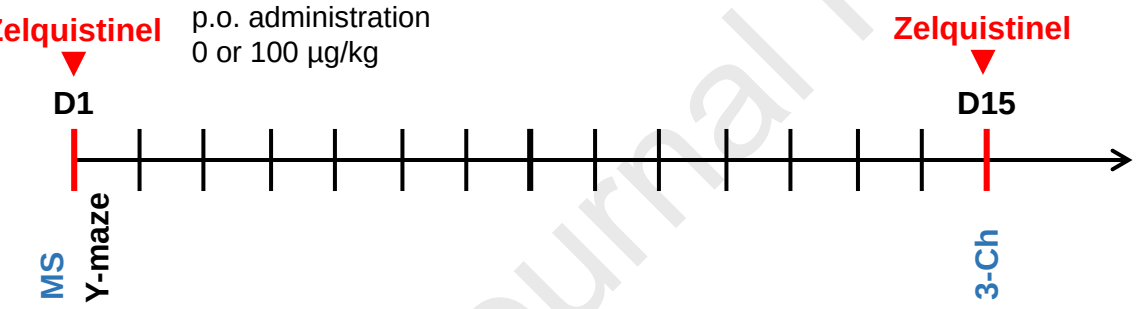
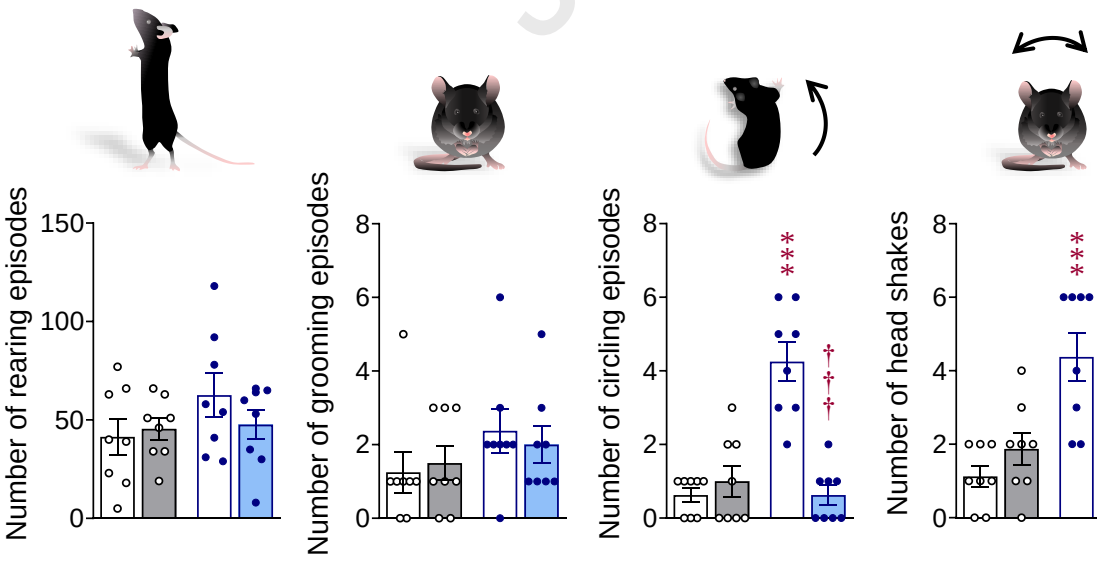
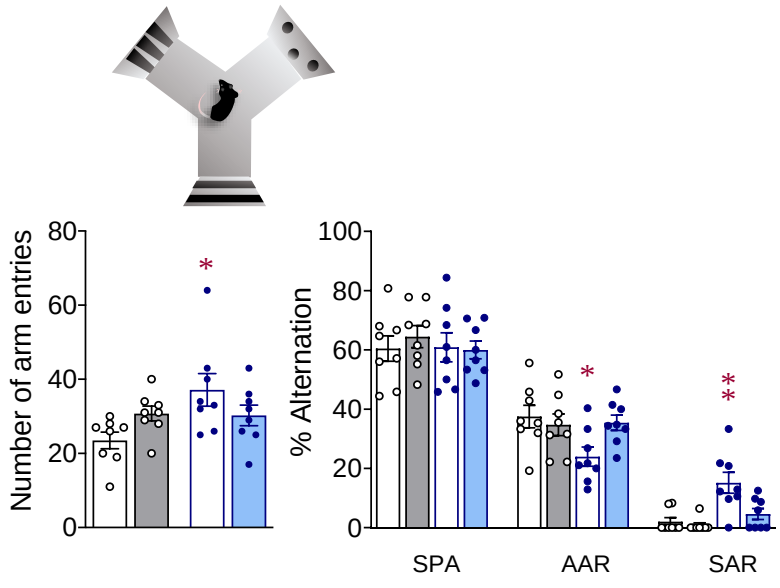


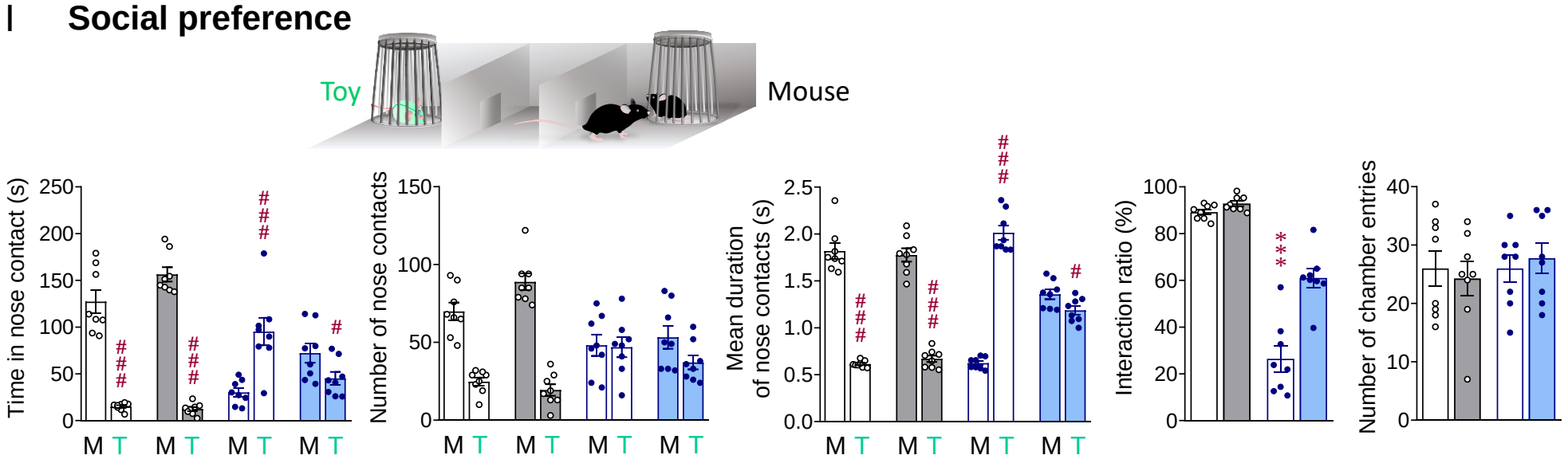
G **Motor stereotypies**



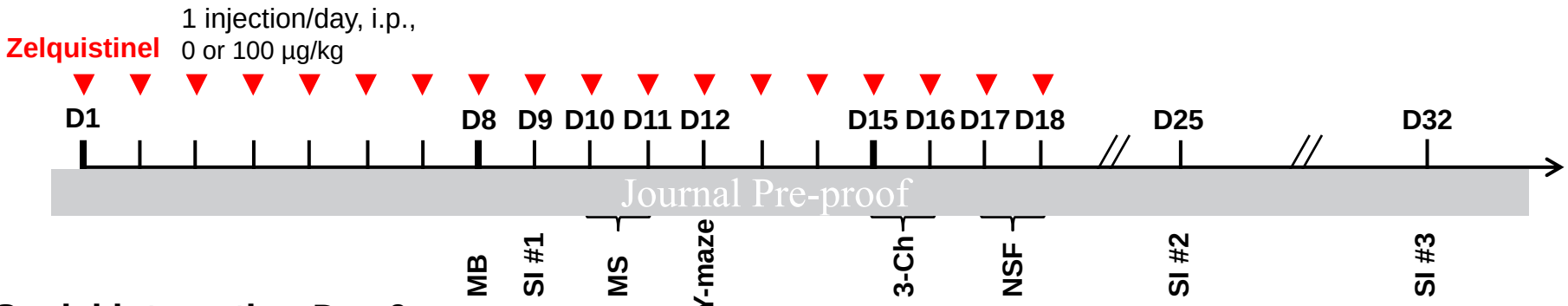
H **Y-maze exploration**



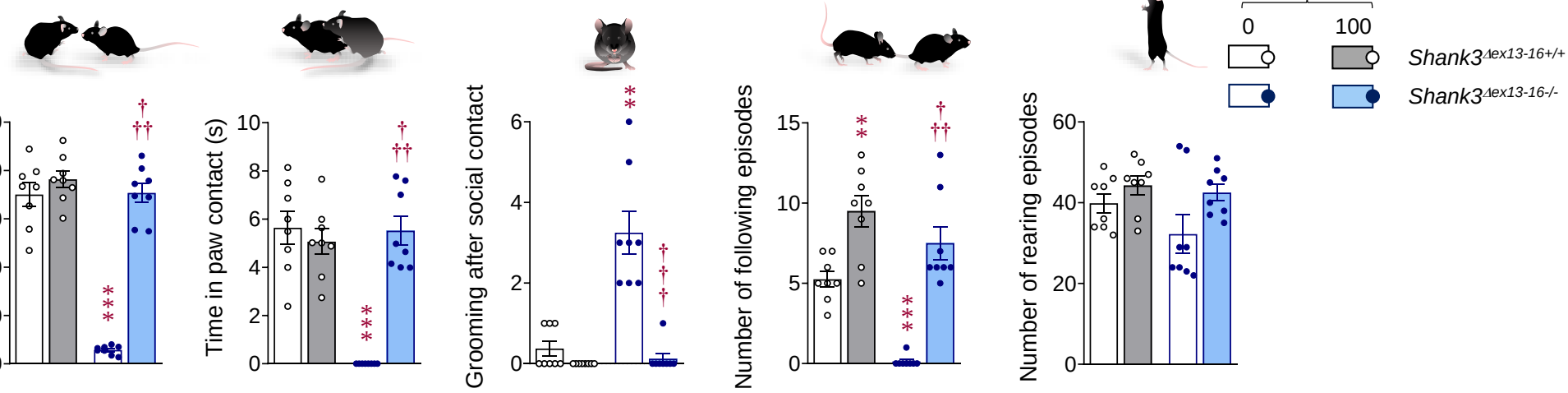
I **Social preference**



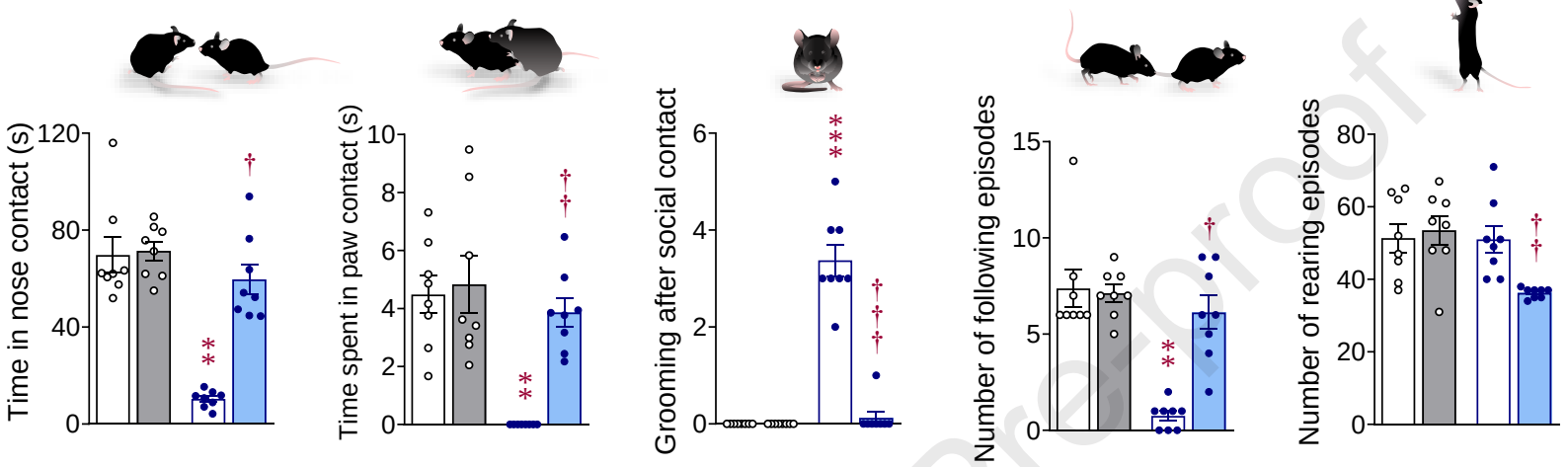
A



B **Social interaction** Day 9

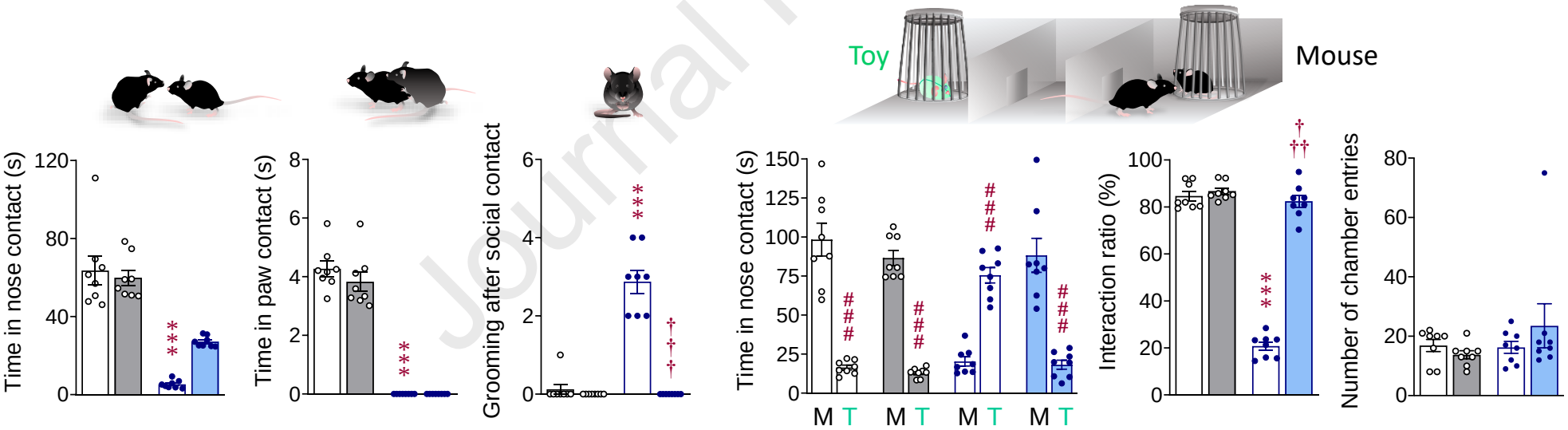


C Day 25



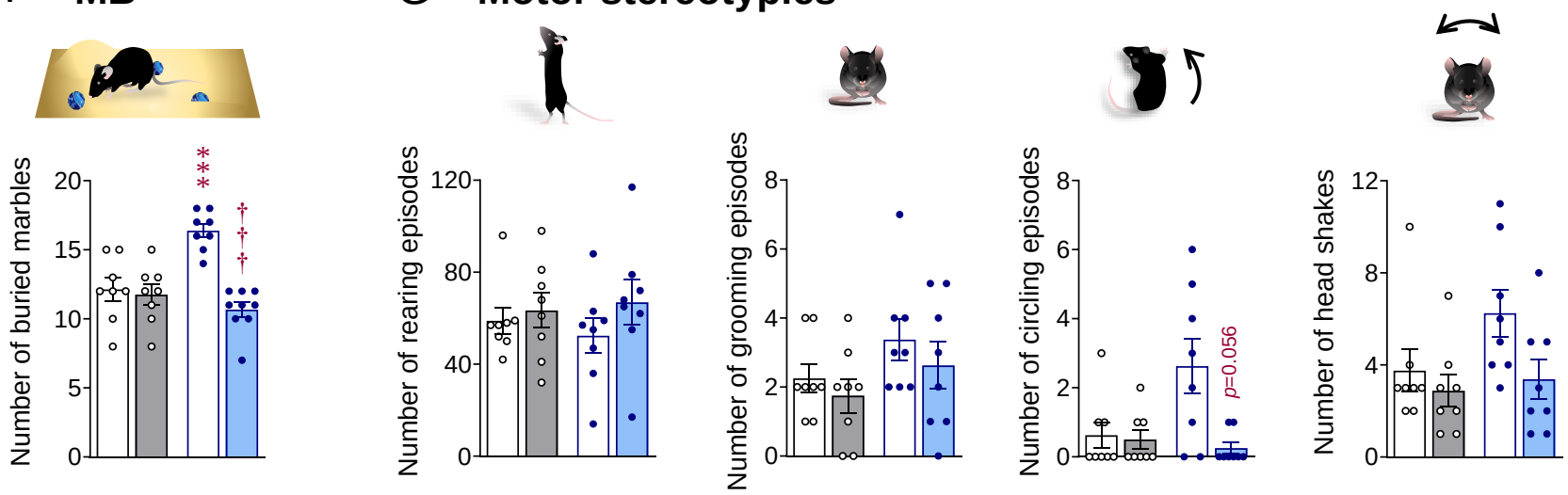
D Day 32

E **Social preference**



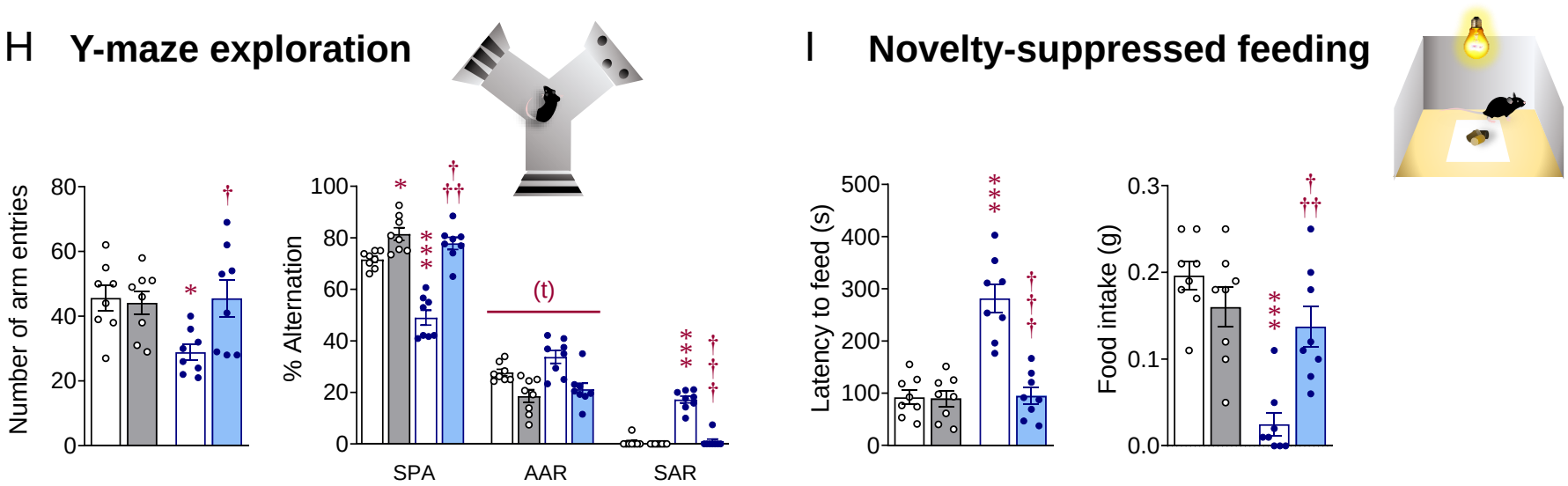
F **MB**

G **Motor stereotypies**



H **Y-maze exploration**

I **Novelty-suppressed feeding**



A

Zelquistinel

Repeated administration, p.o.
0 or 100 µg/kg

MS SI #1 SI #2 SI #3

D-cycloserine

Repeated administration, p.o.
0 or 40 mg/kg

Zelquistinel (µg/kg)
0 100

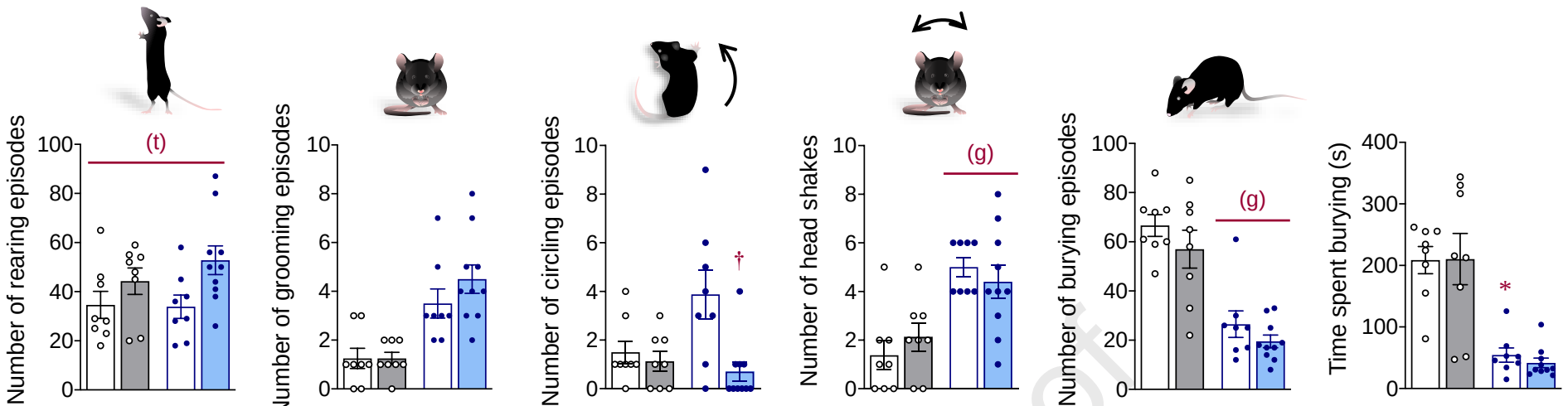
Shank3Δex13-16+/+
Shank3Δex13-16-/-
Fmr1+/+
Fmr1-/-

D-cycloserine (mg/kg)
0 40

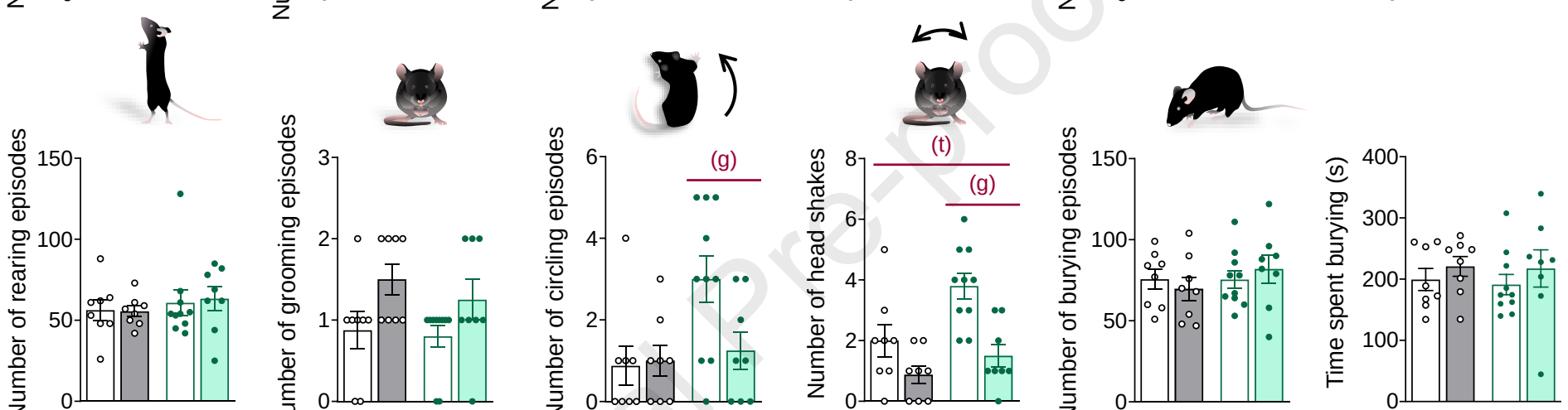
Shank3Δex13-16+/+
Shank3Δex13-16-/-

Motor stereotypies (Day 4)

B



C



D

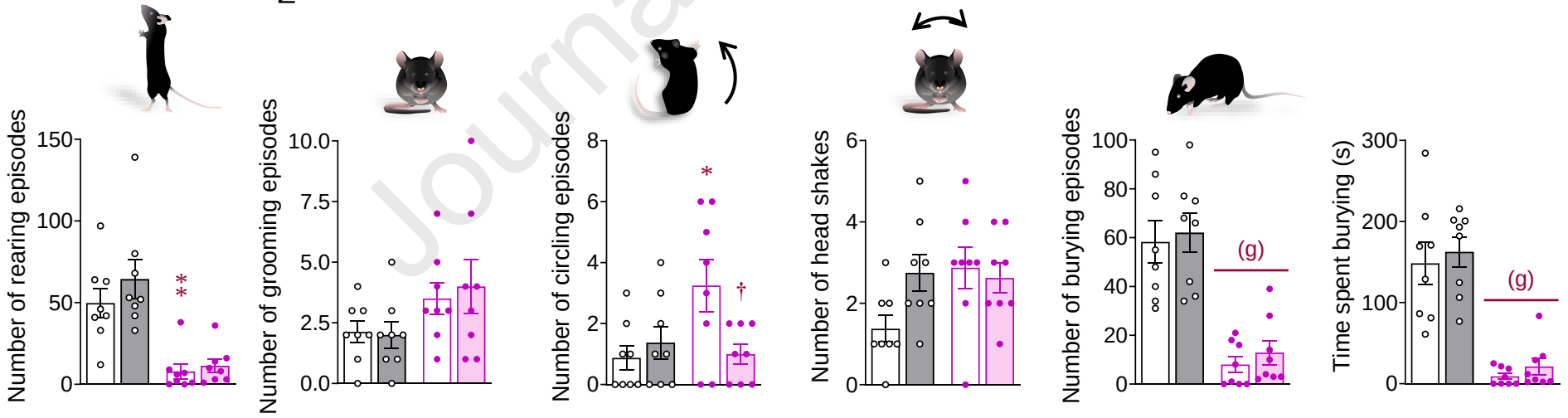
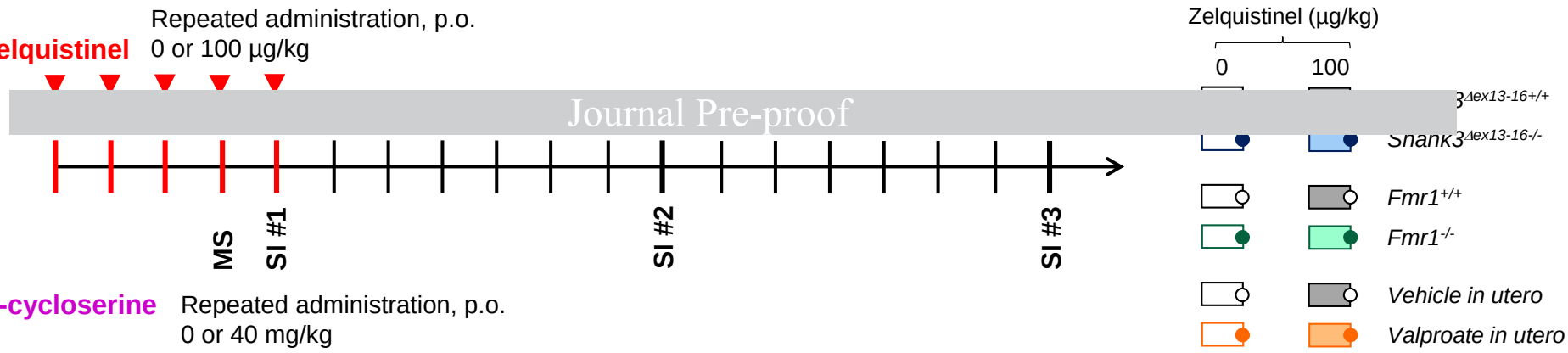
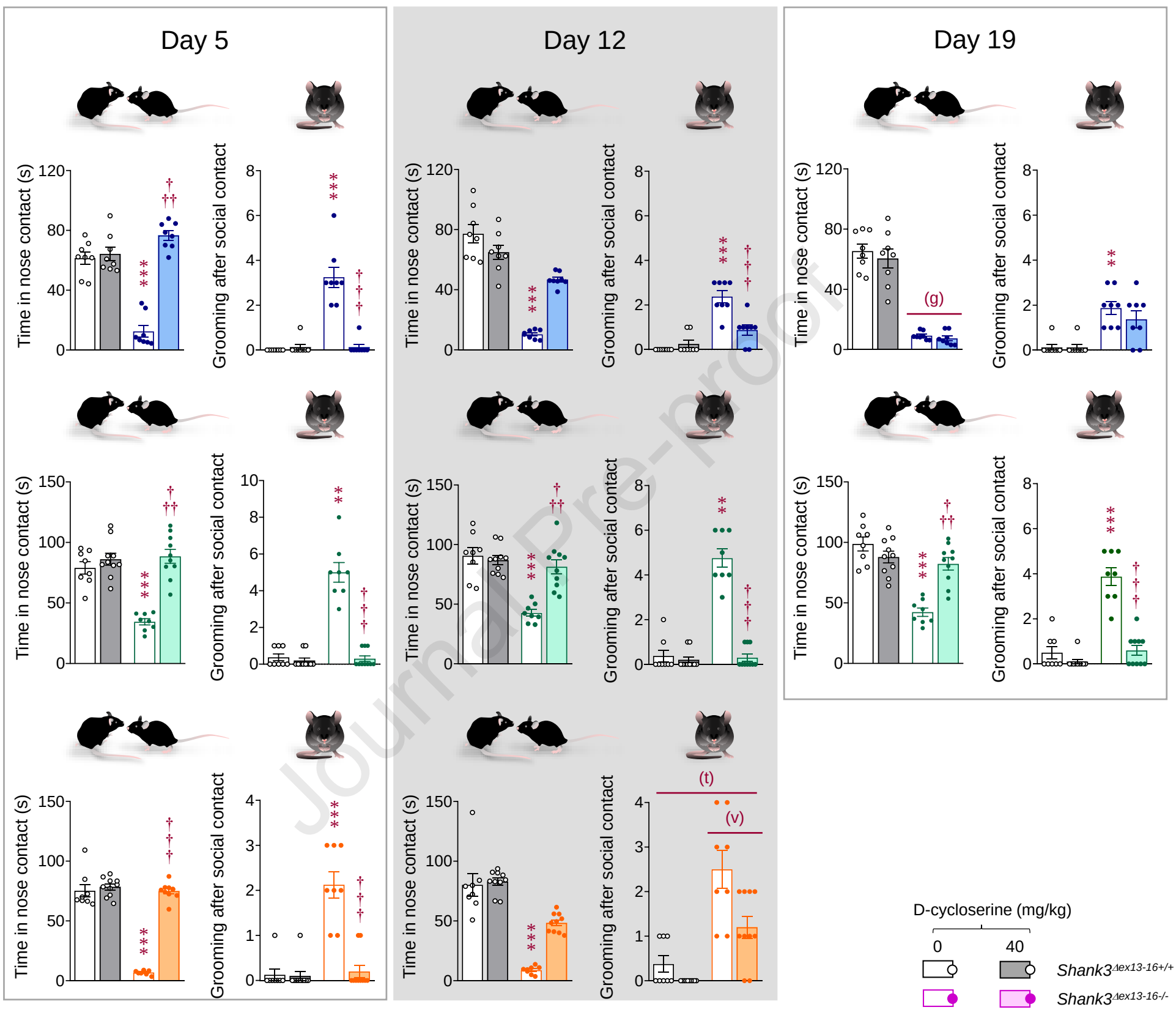


Figure 3. Subchronic PO stereotypies

A



Social interaction



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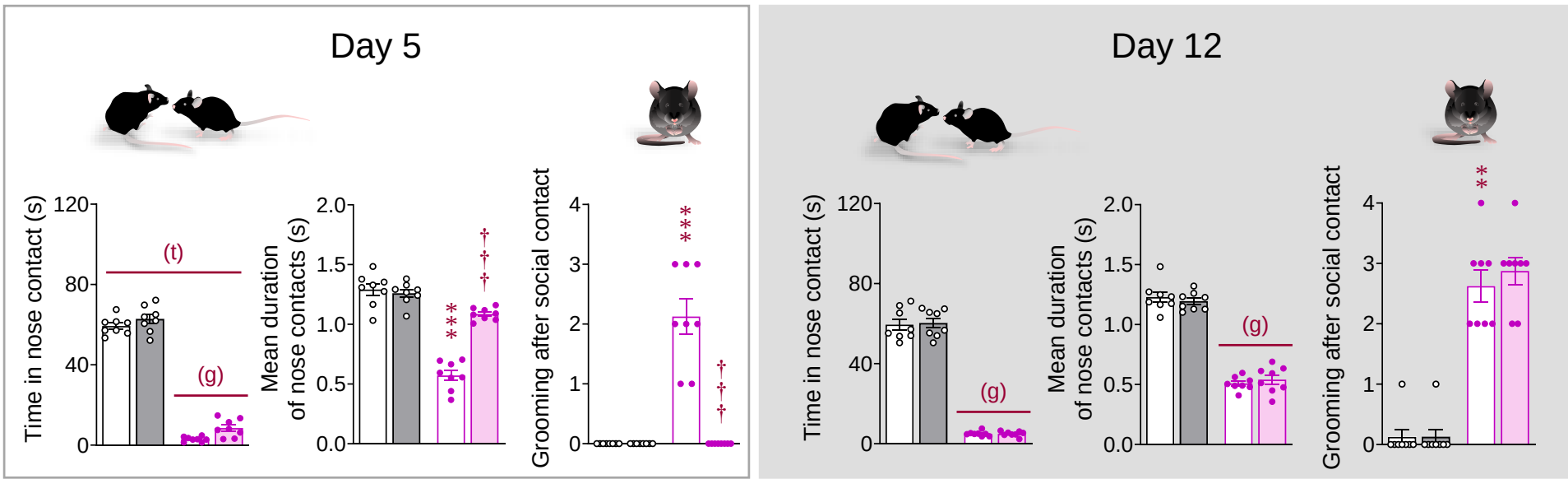
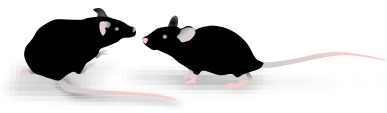
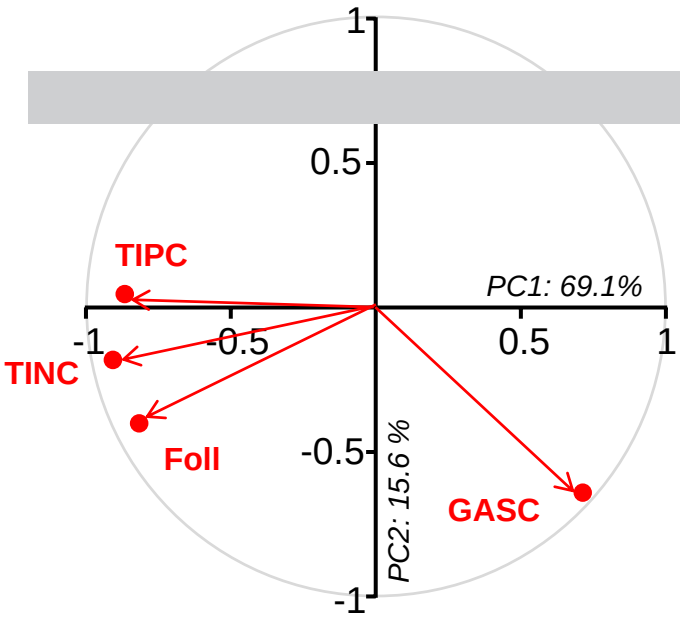


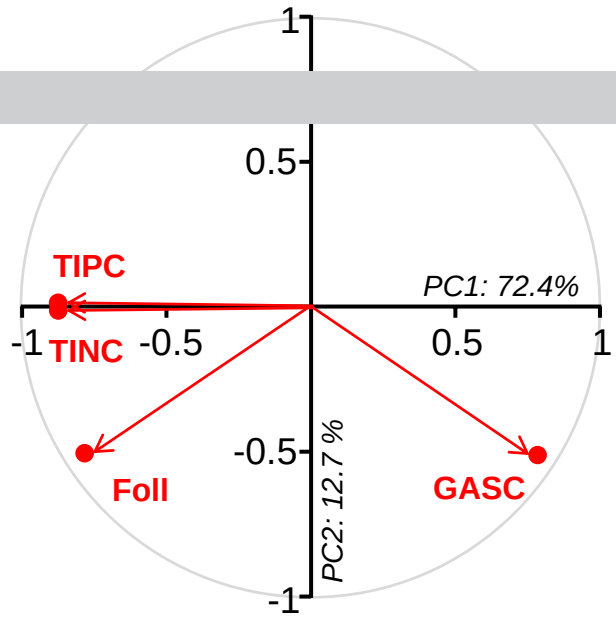
Social interaction



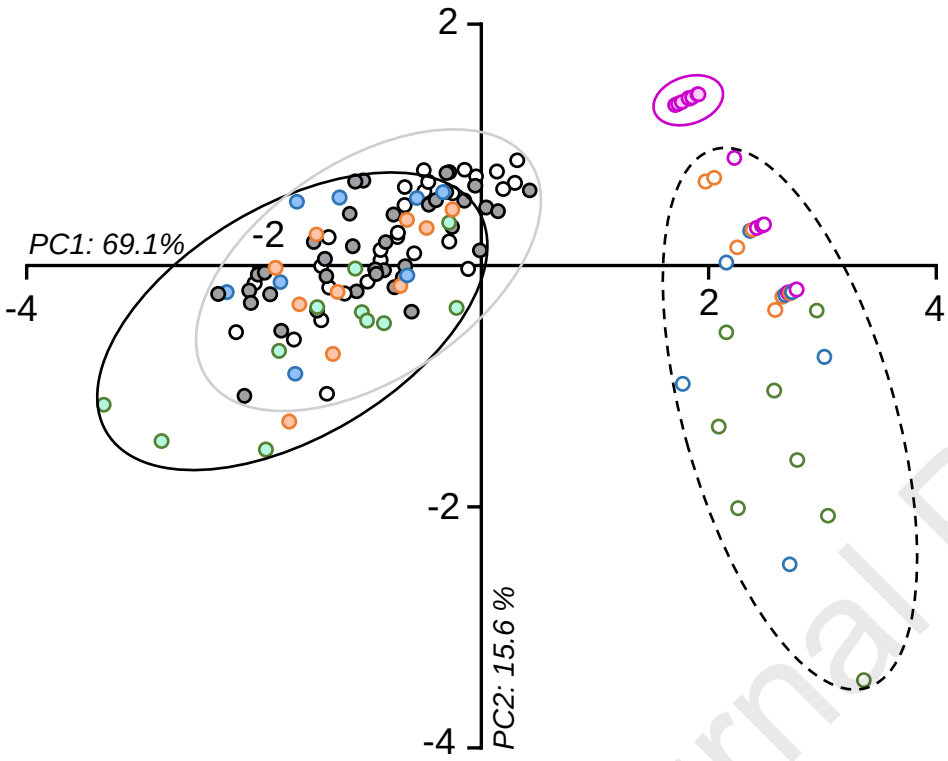
A Under *per os* treatment (Day 5)

B One week after end of *per os* treatment (Day 12)





- *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ + vehicle
- *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ + zelquistinel
- *Fmr1*$^{-/-}$ + vehicle
- *Fmr1*$^{-/-}$ + zelquistinel
- VPA-exposed + vehicle
- VPA-exposed + zelquistinel
- *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ + vehicle
- *Shank3*$^{\Delta ex13\text{-}16\text{-}/\text{-}}$ + D-cycloserine
- WT or saline-exposed + vehicle
- WT or saline-exposed + zelquistinel/D-cycloserine



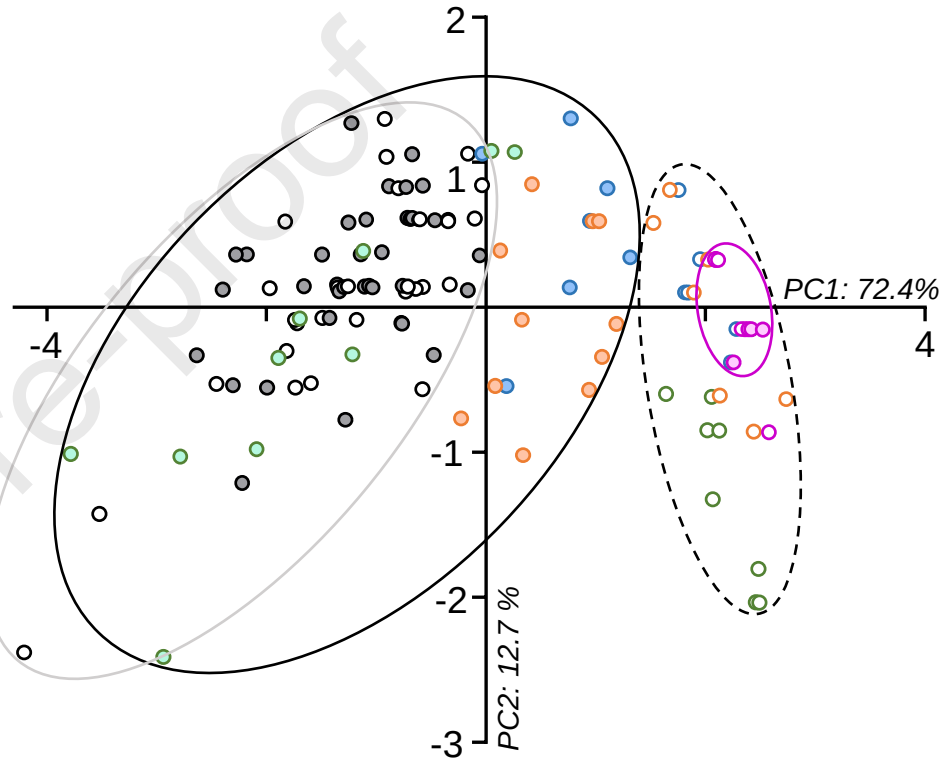


Figure 5. PCA

HIGHLIGHTS

- Acute oral zelquistinel dose-dependently reduces ASD symptoms in *Shank3B* KO mice.
- Effects of chronic i.p. zelquistinel are greater and longer lasting in this model.
- Oral subchronic treatment normalizes ASD symptoms in three different mouse models.
- Beneficial effects of zelquistinel are longer lasting in *Fmr1* null mice.
- D-cycloserine, another NMDAR modulator, is less efficient in Shank3B KO mice.

1. DECLARATION OF INTEREST

This work was supported by Allergan Laboratories, Gate Neurosciences, the Centre National de la Recherche Scientifique (CNRS), Institut National de la Santé et de la Recherche Médicale (Inserm), Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), and Université de Tours. Allergan Laboratories and Gate Neurosciences provided fundings and had a role in the study design. Other institutions had a role in funding salaries for permanent staff and providing optimal technical and scientific environment for research. Pradeep Banerjee is an employee of Allergan (now part of AbbVie). John E. Donello was an employee of Allergan and is now an advisor of Gate Neurosciences. All the other authors declare that they have no conflict of interest.