Received: 4 August 2023 | Revised: 9 February 2024 | Accepted: 15 February 2024

DOI: 10.1111/bph.16361

SHORT REPORT

Radiprodil, a selective GluN2B negative allosteric modulator, rescues audiogenic seizures in mice carrying the GluN2A (N615S) mutation

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Funding information
This work was commissioned to S&P BRAIN srl by the company GRIN THERAPEUTICS, Inc.

Background and Purpose: *GRIN*-related disorders are neurodevelopmental disorders caused by mutations in N-methyl-D-aspartate receptor (NMDAR) subunit genes. A large fraction of these mutations lead to a 'gain of function' (GoF) of the NMDAR. Patients present with a range of symptoms including epilepsy, intellectual disability, behavioural and motor. Controlling seizures is a significant unmet medical need in most patients with *GRIN*-related disorders. Although several hundred *GRIN* mutations have been identified in humans, until recently none of the mouse models carrying *Grin* mutations/deletions showed an epileptic phenotype. The two recent exceptions both carry mutations of GluN2A. The aim of this study was to assess the efficacy of radiprodil, a selective negative allosteric modulator of GluN2B-containing NMDARs, in counteracting audiogenic seizures (AGS) in a murine model carrying the GluN2A (N615S) homozygous mutation (*Grin2a*$^{S/S}$ mice).

Experimental Approach: *Grin2a*$^{S/S}$ mice were acutely treated with radiprodil at different doses before the presentation of a high-frequency acoustic stimulus commonly used for AGS induction.

Key Results: Radiprodil significantly and dose-dependently reduced the onset and severity of AGS in *Grin2a*$^{S/S}$ mice. Surprisingly, the results revealed a sex-dependent difference in AGS susceptibility and in the dose-dependent protection of radiprodil in the two genders. Specifically, radiprodil was more effective in female versus male mice.

Conclusion and Implications: Overall, our data clearly show that radiprodil, a GluN2B selective negative allosteric modulator, may have the potential to control seizures in patients with *GRIN2A* GoF mutations. Further studies are warranted to better understand the sex-dependent effects observed in this study.

Abbreviations: AGS, audiogenic seizures; AGS-RA, audiogenic seizures followed by respiratory arrest; G, guanine; GoF, gain of function; M2, transmembrane domain 2; N, asparagine; P, postnatal day; P-loops, phosphate-binding loops; RA, respiratory arrest; S, serine; V, vehicle; WR, wild running.

KEYWORDS
audiogenic seizures, dose–response curve, gain of function mutation, knock-in mice

1 | INTRODUCTION

The N-methyl-D-aspartate receptor (**NMDAR**) is a tetrameric ionotropic channel containing two copies of the essential subunit **GluN1** and two accessory subunits from either the GluN2(A-D) or the GluN3(**A-B**) family of *GRIN* genes (Sprengel et al., 2001). The **GluN1** subunit is expressed ubiquitously in the brain throughout development, and its germ line knockout is lethal (Forrest et al., 1994). The subunits belonging to the GluN2 and GluN3 families are expressed differentially through development and across brain regions. The various heterotetrameric **NMDAR** channels have different biophysical and pharmacological properties (Paoletti et al., 2013). The tightly controlled excitatory activity of **NMDAR**s is critical to nervous system development and function, and consequently, any dysregulation has been implicated in neuropathology (Hanson et al., 2023).

Mutations, mostly de novo, in the **NMDAR** subunits genes *GRIN1*, *GRIN2A*, *GRIN2B* and *GRIN2D*, have been identified as responsible for recently described *GRIN*-related neurodevelopmental disorders (Benke et al., 2021; Chen et al., 2017; Platzer et al., 2017; Platzer & Lemke, 2019; Strehlow et al., 2019). A large fraction of these mutations leads to enhanced NMDAR activity and are defined as Gain of Function (GoF) mutations according to thorough characterization in vitro (Han et al., 2022; Myers et al., 2023).

GRIN-related disorder represents an area of clear unmet medical need, as currently available therapies cannot control the highly disabling symptoms. These include seizures that are often resistant to broad spectrum antiseizure medications. Seizures and electroclinical activity have to be controlled as their persistence during crucial periods of brain development can have long-term consequences (Löscher et al., 2020).

Unfortunately, existing preclinical models of *GRIN* mutations do not adequately recapitulate the human pathology and symptoms (Bertocchi et al., 2023). Recently, two knock-in mice with different point mutations inserted in the **GluN2A** subunit were described that show an epileptic phenotype and other symptoms akin to human *GRIN*-related disorders (Amador et al., 2020; Bertocchi et al., 2021). A careful analysis of human *GRIN*-related mutations and rare variants reported that *GRIN2A* mutations are most often associated with epileptic disorders (XiangWei et al., 2018).

One of the *GRIN2A* knock-in mice described above, the *Grin2a*$^{S/S}$ mouse, is homozygous for the **GluN2A**(N615S) mutation and exhibits high sensitivity to audiogenic seizures (AGS) (Bertocchi et al., 2021).

The asparagine (N) amino acid residue N615 of the **GluN2A** subunit is localized in the P-loop structure of the M2 membrane domain forming the ion pore and is implicated in augmenting the voltage-dependent Mg^{2+} block of the channel pore (Bertocchi et al., 2021).

In *Grin2a*$^{S/S}$ mice, the mutation induces a significant decrease in Mg^{2+} block but does not induce alterations in channel conductance or in the synaptic expression of **GluN2A**(N615S)-containing **NMDAR**s (Bertocchi et al., 2021). This reduction of the Mg^{2+} block was more prominent at postnatal day (P)42 than P14, consistent with the expression profile of *Grin2a* during development, which starts only after birth reaching its peak in adulthood (Monyer et al., 1994).

The **GluN2A**(N615S) mutation is analogous to those described in children with developmental and epileptic encephalopathies (Allen et al., 2016; Endele et al., 2010). Although in patients the mutation is only present on one allele, the *Grin2a*$^{S/S}$ homozygous mouse represents a suitable nonlethal model for *GRIN*-related developmental and epileptic encephalopathies, because the phenotypic effect of the mutation is more stable and measurable than the phenotype described in heterozygous *Grin2a*$^{S/+}$ mice (Bertocchi et al., 2021).

Radiprodil is a potent and selective negative allosteric modulator of the **GluN2B** containing **NMDAR**. The efficacy of radiprodil to inhibit **NMDAR** currents is fully retained when tested using receptors containing **GluN2B** GoF disease causing mutations (Mullier et al., 2017). Further, radiprodil is anticonvulsant in vivo in both acute and subchronic seizure models (Auvin et al., 2020). Moreover, initial paediatric clinical experience with radiprodil showed that in three infants with drug-resistant infantile spasms, radiprodil administered for up to 34 days was safe and well tolerated and reduced spasms in all three infants, with one becoming spasm-free (Auvin et al., 2020).

More recently, radiprodil's inhibitory activity in vitro has also been confirmed in heterotrimeric **NMDAR**s containing GluN2 GoF mutations (Han et al., 2022), suggesting that it might have anticonvulsant effects in rodents or humans with *GRIN2A* GoF mutations. Indeed, increasing evidence suggests that, in the cortex and hippocampus, heterotrimeric **GluN1**/**GluN2A**/**GluN2B** receptors constitute the largest portion of the **NMDAR** pool (Han et al., 2022).

Previously, radiprodil was reported to extend the survival of mice carrying the homozygous **GluN2A**(S644G) mutation, that usually have lethal spontaneous seizures by P17 (Amador et al., 2020). Interestingly, *Grin2a*$^{S/S}$ mice express normal levels of **GluN2A** but increased levels of **GluN2B** protein in the forebrain membrane fraction, and hippocampal long-term potentiation (LTP) in both *Grin2a*$^{S/S}$ and *Grin2a*$^{S/+}$ mutated mice is supported almost exclusively by **GluN2B**-containing **NMDAR**s (Bertocchi et al., 2021).

Based on its mechanism of action and available data, radiprodil may have the potential to control seizures in patients with *GRIN2A* GoF mutations, targeting the underlying pathophysiology leading to hyperactive **NMDAR**s. To test this hypothesis, the present study aimed to test whether radiprodil can control AGS in the *Grin2a*$^{S/S}$ mouse model.

2 | METHODS

2.1 | Animals

All animal experiments at the Neuroscience Institute Cavalieri Ottolenghi (NICO) in Orbassano (Turin) were conducted in accordance with the European Community Council Directive of 24 November 1986 (86/EEC) and approved by the University of Turin Ethical Committee for animal research and by the Italian Ministry of Health (licence no. 209/2022-PR).

Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

Mice with the **GluN2A**(N615S) mutation (Bertocchi et al., 2021) (Grin2Atm2Rsp strain, RRID:IMSR_EM:09319), available for purchase at the EMMA European Mutant Mice Archive (EM:09319:B6.129-Grin2atm2Rsp/Kctt, https://www.informatics.jax.org/allele/MGI:6406404), were bred and housed in a temperature (22 ± 1°C) and humidity (50 ± 10%) controlled room, in groups of two to four per cage, with ad libitum access to food and water. Nesting paper materials were used for environmental enrichment, and diurnal rhythm was maintained with a 12:12 h light–dark cycle (08:00 AM to 08:00 PM).

Grin2a$^{S/S}$ mice were generated by breeding *Grin2a*$^{+/S}$ mice, and homozygous mice were identified by genotyping and the presence of clasping reflex, as described by Bertocchi et al. (2021).

The number of animals used was determined by the use of a statistical program for power analysis (G*Power Version 3.1.2; Franz Faul, University of Kiel, Germany). Precautions have been taken to reduce the number of animals used.

2.2 | Experimental design and radiprodil administration

Young adult male and female *Grin2a*$^{S/S}$ mice (average age is between P70 and 80) were treated either with vehicle (V) or radiprodil at different doses (1.5, 3 or 10 mg·kg^{-1}) by intraperitoneal injection (i.p.) 30 min before being exposed to an audio stimulus. Each mouse was placed individually inside a sound-attenuating box (cubicle by Ugo Basile, Gemonio (VA), Italy) 5 min before the presentation of a high-frequency acoustic stimulus (11 kHz, ∼100 dB).

As the ED_{50} of radiprodil in adult DBA/2 mice (also sensitive to AGS) is 2.1 mg·kg^{-1} ip (Auvin et al., 2020), we first tested a dose of 3 mg·kg^{-1} (n = 8 vehicle- and n = 8 radiprodil-treated *Grin2a*$^{S/S}$ males; n = 7 vehicle- and n = 9 radiprodil-treated *Grin2a*$^{S/S}$ females), and then the highest (10 mg·kg^{-1}, n = 8 vehicle- and n = 9 radiprodil-treated *Grin2a*$^{S/S}$ males; n = 8 vehicle- and n = 8 radiprodil-treated *Grin2a*$^{S/S}$ females). Lastly, we tested the lowest dose of radiprodil (1.5 mg·kg^{-1}, n = 13 vehicle- and n = 11 radiprodil-treated *Grin2a*$^{S/S}$ males; n = 9 vehicle- and n = 12 radiprodil-treated *Grin2a*$^{S/S}$ females). The mice were tested under the same conditions during the day and randomly assigned to the control or treatment groups one at a time in three different sessions (i.e., for each dose being tested). Males and females were tested separately during the day (first the males and then the females). Each mouse was tested only once. More details and information related to the experimental mice used for the pharmacological treatments can be found in Tables S1–S6.

2.3 | Audiogenic seizure induction protocol

The AGS testing was conducted in a standardized sound-attenuating chamber (isolation cubicle by Ugo Basile), provided with a speaker for high-frequency acoustic stimulus (11 kHz, ∼100 dB) and with an infrared camera for recording. The cubicle was connected to a laptop running AnyMaze Software (Stoelting Co, Europe, Churchtown Dublin, Ireland) to control the protocol induction. Immediately after drug treatment, mice were left undisturbed in their home cage in the induction room for 25 min. Subsequently, they were gently introduced into the isolation cubicle for tone presentation. After 5 min of acclimatization to the cubicle, the stimulus was presented until the onset of AGS or for a maximum of 80 s (four repetitions of a 20-s tone, interrupted by 2-s breaks). At the end of tone presentation, if there was no AGS or AGS recovery (no death from respiratory arrest), mice were left undisturbed inside the cubicle for another 5 min. Subsequently, mice were deeply anaesthetised with 10% isoflurane and killed by cervical dislocation.

to control the protocol induction. Immediately after drug treatment, mice were left undisturbed in their home cage in the induction room for 25 min. Subsequently, they were gently introduced into the isolation cubicle for tone presentation. After 5 min of acclimatization to the cubicle, the stimulus was presented until the onset of AGS or for a maximum of 80 s (four repetitions of a 20-s tone, interrupted by 2-s breaks). At the end of tone presentation, if there was no AGS or AGS recovery (no death from respiratory arrest), mice were left undisturbed inside the cubicle for another 5 min. Subsequently, mice were deeply anaesthetised with 10% isoflurane and killed by cervical dislocation.

2.4 | Behavioural analysis

Video recordings of mice inside the cubicle were analysed by an investigator blinded to the mouse treatment. A typical complete response to the tone stimulus by a *Grin2a*$^{S/S}$ mutant mouse includes the following phases: (1) wild running (WR), (2) loss of posture with clonic seizures, tonic extension of limb extremities and (3) respiratory arrest (RA) (Bertocchi et al., 2021). Based on this, behaviours were categorized as follows:

- **only WR**: mice who experienced only wild running (WR);
- **AGS**: mice who experienced WR, loss of posture with clonic seizures, tonic extension of limb extremities, but recover and were alive at the end of the protocol;

- **AGS-RA**: mice who experienced all categories and died from respiratory arrest (AGS-RA);
- **normal behaviour**: mice that did not show any of the phases described above.

In the survival plots shown in Section 3, mice exhibiting only WR, the first stage of an audiogenic fit, were considered not susceptible to AGS.

2.5 | Data and statistical analysis

Data were recorded and analysed in Excel (2020, Microsoft, Redmond, Wasghington, USA), which was used also for the construction of the pie charts. For survival plots, bar graphs and dose response curves, Graphpad Prism software 9.0 (GraphPad Prism, RRID: SCR_002798, Boston MA 02110, USA) was used. For statistical analysis of survival plots, we used the most widely used method of comparing two or more survival curves, namely, the Log-rank (Mantel–Cox) test. Differences were considered to be statistically significant when $P < 0.05$.

The dose response curves report the percentages obtained in the survival plots and the *P* value refers to those obtained with the Log-rank (Mantel–Cox) test.

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2022). All animals tested were treated as independent values, no technical replicates are present.

2.6 | Materials

Radiprodil (2-[4-(4-fluoro-benzyl)-piperidin-1-yl]-2-oxo-N-(2-oxo-2,-3-dihydro-benzooxazol-6-yl)-acetamide) was provided by GRIN Therapeutics Inc, Brussels, Belgium. DMSO, NaCl and methylcellulose were obtained from Sigma-Aldrich (Merck, Darmstadt, Germany). Drug and vehicle solutions were freshly prepared on the day of the experiment with 5% (w/v) DMSO and 1.0% (w/v) methylcellulose in 0.9% saline. For dosing, 1.5-, 3- or 10-mg radiprodil was added to 5 ml of vehicle, giving concentrations of 0.6, 0.3 and 2 $mg{\cdot}ml^{-1}$, respectively; 100 µl of the drug or vehicle solution was used for i.p. injection in a mouse weighing 20 g.

2.7 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY (http://www.guidetopharmacology.org) and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2023).

3 | RESULTS

3.1 | Radiprodil protects *Grin2a*$^{S/S}$ mice against AGS

Adult (8–13 weeks old) male and female *Grin2a*$^{S/S}$ mice, homozygous for the mutation, were used for these experiments, because both sexes proved to be equally sensitive to audiogenic seizures (AGS) (Bertocchi et al., 2021).

When exposed to an audiogenic stimulus (11 kHz, ~100 dB), most vehicle-treated *Grin2a*$^{S/S}$ mutant mice responded after tone onset with a stereotypical response, composed of WR followed by clonic seizures, tonic extension of limb extremities and, in most severe cases, death by respiratory arrest (RA).

In contrast, a single dose of radiprodil, administered intraperitoneally 30 min before exposure to tone, was shown to significantly decrease the incidence of AGS in *Grin2a*$^{S/S}$ mutant mice (Figure 1). The percentage of AGS-susceptible *Grin2a*$^{S/S}$ mice was reduced in a dose-dependent manner, going from a rate of 75.5% in vehicle-treated *Grin2a*$^{S/S}$ mice to only 6% in *Grin2a*$^{S/S}$ mice treated with the highest dose of radiprodil used in this study (10 $mg{\cdot}kg^{-1}$). Even the lowest dose of radiprodil (1.5 $mg{\cdot}kg^{-1}$) reduced AGS susceptibility by 50%. Furthermore, radiprodil-treated *Grin2a*$^{S/S}$ mice that continued to be sensitive to AGS showed an increased latency to seizure onset with increasing doses of radiprodil (Figure 1, Tables S1–S6).

3.2 | The protective effect of radiprodil is dose-dependent and differs between genders

The protective effect of radiprodil differed in magnitude between male and female *Grin2a*$^{S/S}$ mice (Figure 2). The incidence of AGS decreased in a dose-dependent manner in both genders but as shown by the dose–response curves in Figure 2a, radiprodil's potency and efficacy was higher in *Grin2a*$^{S/S}$ females. In female mice, radiprodil significantly protected against AGS at the lowest dose of 1.5 $mg{\cdot}kg^{-1}$ and resulted in a full protection (100%) at higher doses (3 and 10 $mg{\cdot}kg^{-1}$). By contrast, in male *Grin2a*$^{S/S}$ mice, which in this study showed a lower susceptibility to AGS (65%) than their female littermates (88%), only the higher doses of radiprodil (3–10 $mg{\cdot}kg^{-1}$) were able to significantly protect against AGS, whereas the protection achieved with the lowest dose (1.5 $mg{\cdot}kg^{-1}$) did not reach statistical significance (Figure 2b).

3.3 | Sex-dependent protection at different stages of AGS by radiprodil

Pie charts in Figure 3 represent the different phases of AGS and demonstrated strong efficacy of radiprodil at increasing doses in

counteracting AGS in both male and female *Grin2a*$^{S/S}$ mice (Figure 3a,b, respectively). However, it is important to understand this finding in the context of differences between male and female mice administered vehicle. When injected with vehicle, both sexes were equally susceptible to the most severe form of AGS, which culminated in RA and death (41% vs. 42%, Figure 3a,b, left pie chart). However, control vehicle-treated *Grin2a*$^{S/S}$ male mice showed lower susceptibility to AGS, in fact showing a lower percentage than females of the form of AGS not followed by death (26% against 46%, Figure 3a,b, respectively). This difference affected overall susceptibility to AGS (AGS with recovery and AGS-RA) between males and females (Figure 2). Overall, 20% more *Grin2a*$^{S/S}$ males exhibited 'normal' behaviour during tone presentation than females (Figure 3a,b, left pie chart).

Moreover, in *Grin2a*$^{S/S}$ males, a low dose of radiprodil (1.5 mg·kg^{-1}) was not able to significantly reduce AGS (Figure 2b) as mentioned above, but it was able to halve the incidence of AGS-RA (Figure 3a). A significant protective effect was seen in male mice at 3 mg·kg^{-1} radiprodil, with a complete rescue of AGS-RA, but with a quarter of males (25%) still presenting AGS (with recovery). The highest dose of radiprodil (10 mg·kg^{-1}) was even more effective (Figure 3a), with only a small percentage of *Grin2a*$^{S/S}$ male mice still susceptible to the WR stage, the earliest sign of an audiogenic seizure.

In contrast to male mice, in *Grin2a*$^{S/S}$ females, a low dose of radiprodil was highly effective in reducing both AGS with and without RA (from 42% to 8% and from 46% to 17%, respectively; Figure 3b). At higher doses, radiprodil completely abolished the incidence of AGS in all the *Grin2a*$^{S/S}$ females tested, leaving just a fraction manifesting WR (from 33% at 3 mg·kg^{-1} to 25% at 10 mg·kg^{-1}; Figure 3b).

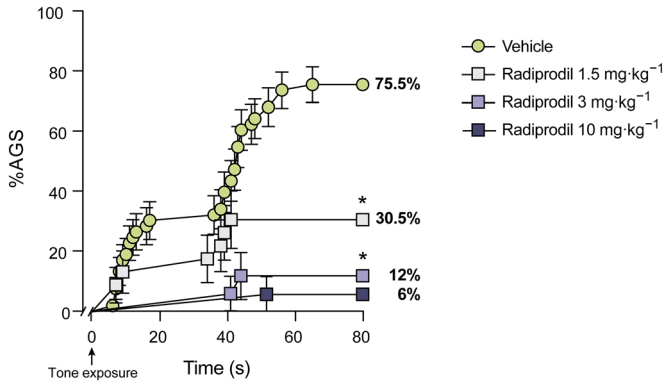

FIGURE 1 Survival plot showing the incidence of audiogenic seizures (AGS) in all *Grin2a*$^{S/S}$ mice tested, with males and females pooled. The incidence of AGS dropped from 75.5% of the vehicle-treated group (n = 53) to 30.5% of the group treated with radiprodil at the lower dose (1.5 mg·kg^{-1}, n = 23), to 12% of the medium dose (3 mg·kg^{-1}, n = 17) and to 6% of the group treated with the higher dose (10 mg·kg^{-1}, n = 17) [*P < 0.05 by log-rank (Mantel–Cox) test].

4 | DISCUSSION

GRIN-related disorders are severe neurodevelopmental syndromes for which there is no effective treatment. The research and development of new treatments is made difficult by the complex and multifaceted nature of these syndromes, which are associated with symptoms in multiple domains, regardless of the type of *GRIN* mutation (Xie et al., 2023). Taking into account the causal association of *GRIN* variants with epilepsy, compounds targeting **NMDAR**s are of considerable medical interest. However, given that 'classical' nonselective **NMDAR** channel blockers have side effects limiting their therapeutic index, more selective **NMDAR** modulators have been

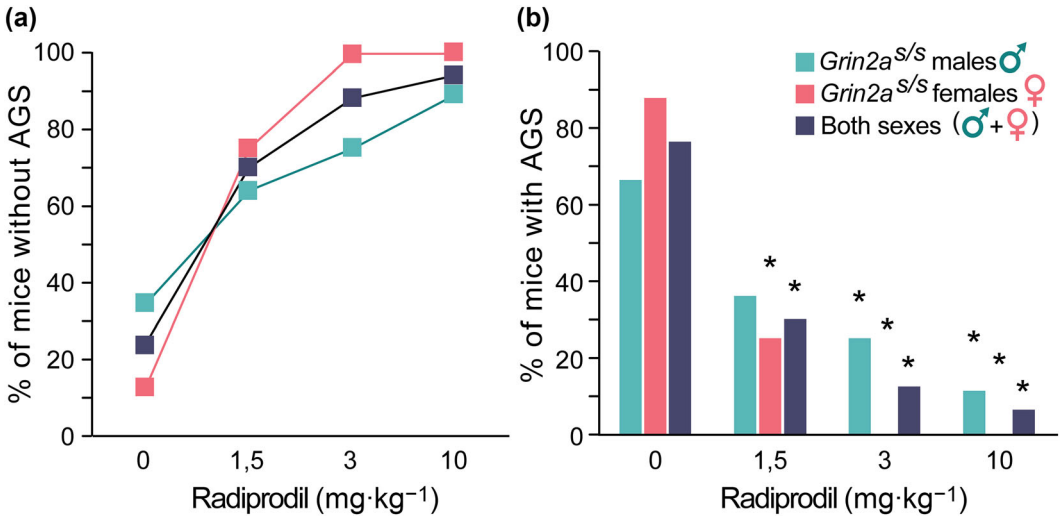

FIGURE 2 (a) Dose-dependent protection against AGS in the *Grin2a*$^{S/S}$ mouse model (black curve). Green and pink curves represent the same result when only male and female *Grin2a*$^{S/S}$ mice are considered, respectively. (b) A sex-dependent difference in audiogenic seizure (AGS) incidence (not significant) can be observed in vehicle-treated mice (at 0) and also in the efficacy of radiprodil in preventing AGS in males and females at different doses [*P < 0.05 versus dose 0, by log-rank (Mantel–Cox) test].

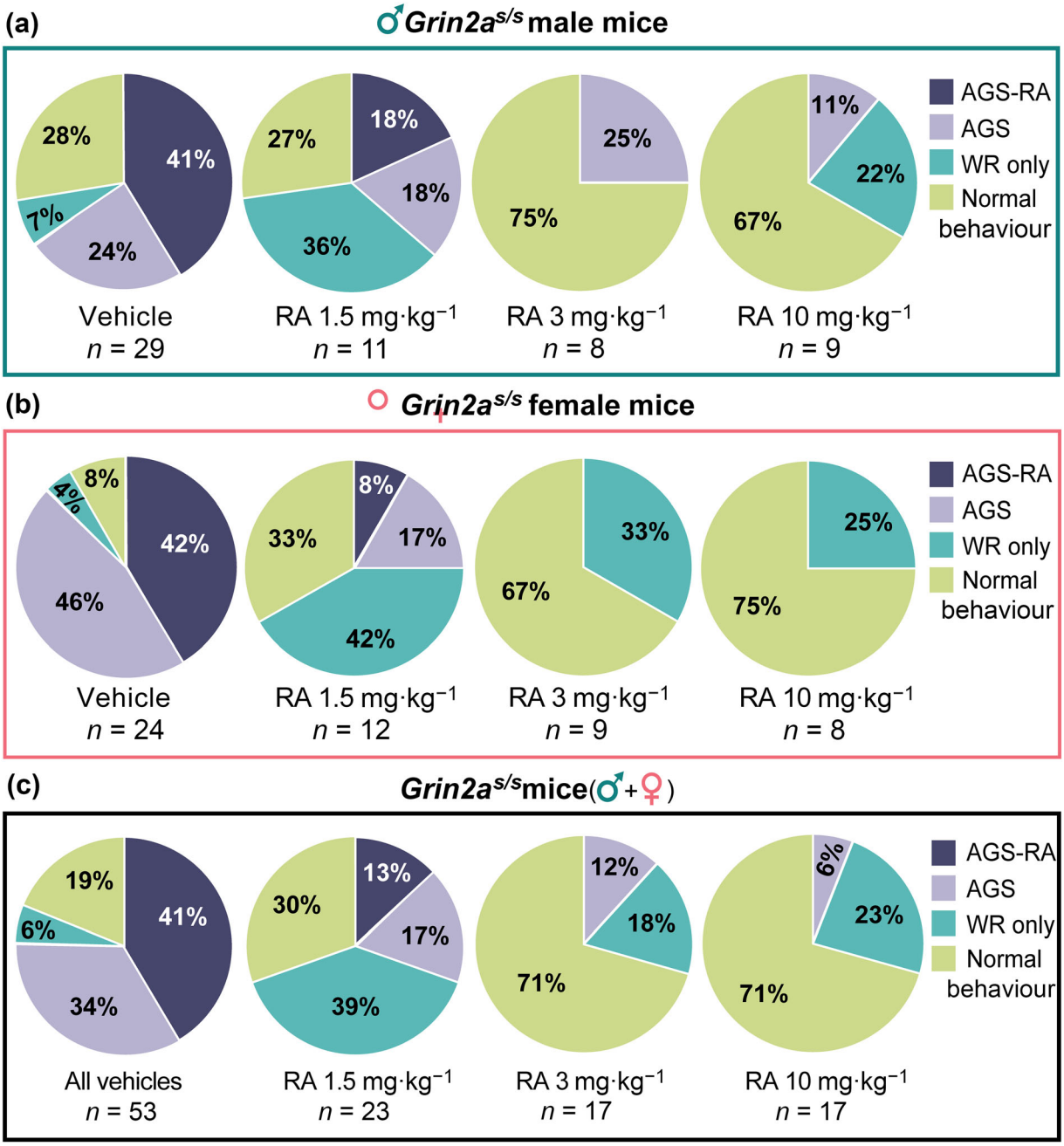

FIGURE 3 Pie charts showing the efficacy of increasing doses of radiprodil in counteracting the different phases of a typical audiogenic fit in order of severity: from normal behaviour in blue, to wild running (WR) only in yellow, to AGS (audiogenic seizure) with recovery in red and AGS-RA (AGS followed by respiratory arrest) in brown-red, in (a) males *Grin2a*$^{S/S}$ mice; (b) females *Grin2a*$^{S/S}$ mice and (c) male and female *Grin2a*$^{S/S}$ mice pooled.

designed to have fewer side effects, thus having greater therapeutic potential in various disorders, including syndromes associated with GoF *GRIN* mutations.

Here, we tested the efficacy of different doses of radiprodil, a novel negative modulator selective for GluN2B-containing NMDARs, in counteracting the epileptic phenotype caused by a GoF *Grin2a* mutation in *Grin2a*$^{S/S}$ mice. The *Grin2a*$^{S/S}$ mouse, carrying the GluN2A (N615S) mutation in homozygosity, is a model of *GRIN2A*-related developmental and epileptic encephalopathies and is highly vulnerable to AGS (Bertocchi et al., 2023).

GluN2A subunit expression starts only after birth and increases during postnatal development to progressively 'replace' GluN2B in an activity-dependent manner in different brain areas related to cognition (Bellone & Nicoll, 2007). GluN2B, on the other hand, is highly expressed during embryonic development and in the 'excitable' immature brain (Monyer et al., 1994).

Despite this so-called 'developmental switch', the expression levels of **GluN2B** remain high even after brain circuits have matured, being far more abundant than **GluN2A** in the forebrain and continuing to have a significant impact on synaptic activity into adulthood (Wong et al., 2021). As in other epilepsy models (Ghasemi & Schachter, 2011), the **GluN2B** subunit appears to be up-regulated in adult *Grin2a*$^{S/S}$ mice, and the **GluN2B**-containing **NMDAR**s mediate hippocampal LTP in these mutants, whereas in adulthood, this role is usually subserved predominantly by **GluN2A**-containing **NMDAR**s (Bertocchi et al., 2021).

It has previously been shown, in vitro, that radiprodil maintains its inhibitory effect at NMDARs containing GluN2B GoF mutations (Mullier et al., 2017). However, it is also clear that GluN2B selective inhibitors can also control seizures in rodent models not directly caused by dysfunctions of the NMDAR (Mareš et al., 2021). In this study, we have demonstrated a significant dose-dependent anticonvulsant effect of radiprodil in adult *Grin2a*$^{S/S}$ mice. Radiprodil's ability to protect against AGS caused by a mutation present on the **GluN2A** subunit indicates that this compound is able to reduce the excitation provoked by the **GluN2A**(N615S) GoF mutation and suggests radiprodil has therapeutic potential for treating seizures in patients caused by *GRIN2A* and other *GRIN* subunit GoF mutations. Given that it is increasingly recognized that many **NMDAR**s in cortical and hippocampal areas are triheteromeric, such as those containing both **GluN2A** and **GluN2B** subunits alongside two essential **GluN1** subunits (Han et al., 2022), the selective activity of radiprodil at the **GluN2B**subunit, likely extends its therapeutic potential to disorders caused by mutations in other *GRIN* subunits.

Given that there are various nonseizure clinical symptoms in *GRIN*-related disorders, the mechanism of action of radiprodil, which targets the root cause of the disorder, suggests that **GluN2B** selective inhibition might also be effective in alleviating other neurological symptoms associated with *GRIN*-related disorders (Benke et al., 2021). Whilst we have not explicitly tested for these other symptoms in the current study, our profiling of *Grin2a*$^{S/S}$ mice show that nonseizure symptoms (e.g., muscle tone, hippocampal associative learning, attention and hyperactivity) are prominent (Bertocchi et al., 2021).

Surprisingly, the efficacy of radiprodil in protecting *Grin2a*$^{S/S}$ mice from AGS showed a gender effect. In addition to the different susceptibility of males versus females to AGS, with the males being more resistant (albeit not significantly), all doses of radiprodil showed greater efficacy in counteracting AGS in females compared with males. This may be due to inherent sex differences in physiology and response to drug treatment (Soldin & Mattison, 2009). However, in our previous study (Bertocchi et al., 2021), we described 100% occurence of AGS-RA in all mice tested, without priming. This inconsistency is most likely due to the different set-up and conditions, in particular regarding the intensity of the tone. AGS are the predominant reflex epilepsies in rodents, and susceptibility is strongly associated with the interaction of genetics and environment (Faingold, 2012). Housing conditions and even the most subtle differences in the 'trigger' can influence the variability of AGS susceptibility, engendering variability between studies. Clearly, laboratory conditions and the setup used to induce AGS should be standardized as much as possible.

Nonetheless, the results of this study indicate that radiprodil effectively counteracts AGS in *Grin2a*$^{S/S}$ mice and may therefore have anticonvulsant activity in patients with a range of GoF *GRIN* mutations, and not just in patients with *GRIN2B* GoF mutations. The results of a clinical study that is currently recruiting in several European centres *GRIN*-related disorder patients caused by GoF mutations in the **NMDAR** to test the efficacy of radiprodil (EudraCT Number: 2022–000317-14) will indicate whether the observed anticonvulsant effect of radiprodil in mice translates into an effect in patients.

AUTHOR CONTRIBUTIONS

Conceptualization: Ilaria Bertocchi, Carola Eugenia Eva, Naheed Rohman Mirza and Pierandrea Muglia. *Methodology*: Ilaria Bertocchi, Carola Eugenia Eva, Naheed Rohman Mirza and Pierandrea Muglia. *Validation*: Ilaria Bertocchi and Carola Eugenia Eva. *Investigation*: Ilaria Bertocchi, Alessandra Oberto and Lorenzo Cifarelli. *Data curation*: Ilaria Bertocchi, Alessandra Oberto and Carola Eugenia Eva. *Writing—original draft*: Ilaria Bertocchi and Pierandrea Muglia. *Writing—review & editing*: Ilaria Bertocchi, Pierandrea Muglia, Naheed Rohman Mirza, Lorenzo Cifarelli, Alessandra Oberto, Rolf Sprengel and Carola Eugenia Eva. *Funding acquisition*: Pierandrea Muglia. *Resources*: Rolf Sprengel: *Supervision*: Ilaria Bertocchi.

ACKNOWLEDGEMENTS

We thank Federico Montechiaro and the team at Ugo Basile srl for their technical assistance in delivering the cubicle and its adaptation to our experimental interests. Our most sincere thanks go to our colleague Dr. Carola Eva, who was substantially involved in this work. We are sad to say that Dr. Carola Eva is no longer with us to share in the successful publication of this manuscript.

CONFLICT OF INTEREST STATEMENT

PM is founder of GRIN Therapeutics that is developing radiprodil for *GRIN*-related disorders. The other authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from GRIN THERAPEUTICS, Inc. Data are available with the permission of GRIN THERAPEUTICS, Inc.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis and Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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How to cite this article: Bertocchi, I., Cifarelli, L., Oberto, A., Eva, C. E., Sprengel, R., Mirza, N. R., & Muglia, P. (2024). Radiprodil, a selective GluN2B negative allosteric modulator, rescues audiogenic seizures in mice carrying the GluN2A (N615S) mutation. *British Journal of Pharmacology*, 1–9. https://doi.org/10.1111/bph.16361