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ANAVEX®2-73 (blarcamesine), a Sigma-1 receptor agonist, ameliorates neurologic impairments in a mouse model of Rett syndrome

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ABSTRACT

Rett syndrome (RTT) is a severe neurodevelopmental disorder that is associated in most cases with mutations in the transcriptional regulator *MECP2*. At present, there are no effective treatments for the disorder. Despite recent advances in RTT genetics and neurobiology, most drug development programs have focused on compounds targeting the IGF-1 pathway and no pivotal trial has been completed as yet.

Thus, testing novel drugs that can ameliorate RTT's clinical manifestations is a high priority. ANAVEX2-73 (blarcamesine) is a Sigma-1 receptor agonist and muscarinic receptor modulator with a strong safety record and preliminary evidence of efficacy in patients with Alzheimer's disease. Its role in calcium homeostasis and mitochondrial function, cellular functions that underlie pathological processes and compensatory mechanisms in RTT, makes blarcamesine an intriguing drug candidate for this disorder.

Mice deficient in MeCP2 have a range of physiological and neurological abnormalities that mimic the human syndrome. We tested blarcamesine in female heterozygous mice carrying one null allele of *Mecp2* (HET) using a two-tier approach, with age-appropriate tests. Administration of the drug to younger and older adult mice resulted in improvement in multiple motor, sensory, and autonomic phenotypes of relevance to RTT. The latter included motor coordination and balance, acoustic and visual responses, hindlimb clasping, and apnea in expiration. In line with previous animal and human studies, blarcamesine also showed a good safety profile in this mouse model of RTT. Clinical studies in RTT with blarcamesine are ongoing.

1. Introduction

Rett syndrome (RTT) is a progressive, non-inherited, X-linked neurodevelopmental disorder, with an incidence of approximately 1 in 10,000 female births (Neul et al., 2010; Laurvick et al., 2006). Most patients carry a mutation in the methyl-CpG-binding protein 2 (*MECP2*) gene that encodes a transcriptional regulator that is particularly abundant in brain (Amir et al., 1999; Neul et al., 2014). Male cases are rare because of their severity and differ in clinical presentation from females with mutations in *MECP2* (Neul et al., 2019). The disorder is characterized by a wide variety of neurologic impairments affecting, among others, cognition (i.e., developmental delay, intellectual disability, communication deficits), behavior, and motor and autonomic function (Neul et al., 2010; Kaufmann et al., 2016). RTT has a dynamic evolution in young children, with a distinctive period of loss of expressive language and fine motor skills after relatively normal development until 6 to 18 months of age. Impairment in ambulation and stereotypic hand movements are additional diagnostic features. Other common neurologic and systemic problems that can manifest over time include seizures, breathing abnormalities, aberrant behaviors, and gastrointestinal dysfunction (Kaufmann et al., 2016; Pozzo-Miller et al., 2015). Neurologic manifestations are relatively stable until late childhood or early adolescence when decline in motor function becomes evident. Adult RTT is characterized by Parkinsonian features and other motor impairments, limited communication abilities and internalizing behavioral abnormalities (Kaufmann et al., 2016; Buchanan et al., 2019). There are no specific or effective treatments for RTT, with therapeutic management being primarily symptomatic.

Knowledge of the genetic abnormality affecting most individuals with RTT has enabled the development of several animal models that have led to an active period of drug development. Initially they consisted only of male hemizygous mice with null mutations but have

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recently expanded to animals carrying common point mutations and female heterozygous mice (Samaco et al., 2013; Lombardi et al., 2015; Tan and Zoghbi, 2018). The latter carry only one non-expressing *Mecp2* allele and are therefore considered more consistent models of the almost exclusively female human condition. Mouse models have already been instrumental in the characterization of the neurobiology of RTT as well as in the search for novel treatments (Kaufmann et al., 2016, 2017). In general, they display a variety of neurobehavioral phenotypes of relevance to the clinical features of RTT, including an equivalent to the characteristic hand stereotypies (i.e., hindlimb clasping) (Samaco et al., 2013; Lombardi et al., 2015; Tan and Zoghbi, 2018). Thus, Mecp2-deficient mice have become an *in vivo* system for testing the effect of new genetic and pharmacological approaches on RTT-relevant phenotypes. Based on studies of affected individuals and mouse models, it has been concluded that RTT is a synaptic disorder that is associated with both intrinsic and circuit-level abnormalities (Kaufmann and Moser, 2000; Banerjee et al., 2019). These include maladaptive homeostatic synaptic plasticity, which plays a key role in compensatory responses to synaptic defects (Wondolowski and Dickman, 2013; Banerjee et al., 2019). Pathogenetic mechanisms in RTT not only implicate neurons but also astrocytes and microglia, with glial cells involved in increasing levels of glutamate and reactive oxygen species which are also detected in affected patients (O'Driscoll et al., 2013; Muller, 2019; Banerjee et al., 2019).

To date, no drug treatment has shown effectiveness in pivotal trials and, consequently, new pharmacological strategies are needed. Most of the clinical translational research in RTT has been based on compounds targeting the Insulin-like Growth Factor 1 (IGF-1) pathway, although treatments focused on specific circuits and neurotransmitters are also under investigation (Katz et al., 2016; Kaufmann et al., 2016; O'Leary et al., 2018; Glaze et al., 2019). Drugs that can restore cellular homeostasis and promote intrinsic compensatory synaptic mechanisms (Banerjee et al., 2019) are attractive candidates in RTT, particularly if they have already demonstrated promise in other neurologic disorders (Campbell et al., 2019; Grünewald et al., 2019; Tong et al., 2018; Jha et al., 2019). Compounds that target the Sigma-1 receptor (S1R) have the potential of correcting a number of metabolic and synaptic abnormalities, as this receptor has been shown to restore cellular homeostasis and homeostatic synaptic plasticity when activated by targeting a number of disruptive mechanisms, namely mitochondrial dysfunction and related oxidative stress, protein misfolding, proteostasis and related autophagy, and neuroinflammation (Ruscher and Wieloch, 2015; Su et al., 2016; Smith-Dijak et al., 2019). Of particular relevance to RTT, activation of S1Rs has been shown to attenuate oxidative stress linked to inflammatory glial responses (Wang and Zhao, 2019) and to increase the release of brain-derived neurotropic factor (BDNF), mechanisms known to be altered in Mecp2 deficiency (Banerjee et al., 2019; Francardo et al., 2014). Mechanistically, there is evidence that calcium signaling from the endoplasmic reticulum to the mitochondria, a key process modulated by S1R activation, not only improves mitochondrial function and reduces production of reactive oxygen species but also regulates synaptic processes such as hippocampal firing rate set points and homeostatic synaptic scaling (Vaccaro et al., 2017; Lee et al., 2018; Rossi and Pekkurnaz, 2019; Styr et al., 2019). Indeed, emphasizing the potential of improving maladaptive homeostatic plasticity in RTT, a recent study with a S1R agonist corrected homeostatic synaptic scaling in a mouse model of Huntington disease (Smith-Dijak et al., 2019). S1Rs are widely distributed in the brain with relative enrichment in the limbic system; these regions include hippocampus (pyramidal, non-pyramidal layers, granular layer of the dentate gyrus), septum, paraventricular nucleus of the hypothalamus, anterodorsal thalamic nucleus, dorsal raphe, substantia nigra, locus coeruleus, and cerebellum (Guitart et al., 2004). With such broad distribution, S1R activation has the potential of correcting multiple neural circuits and corresponding phenotypes in RTT and other neurologic disorders.

ANAVEX2-73 (blarcamesine), a lead compound from Anavex Life Sciences Corp., has demonstrated good safety, bioavailability, and tolerability in Phase 1 and Phase 2 clinical trials. In addition, data from an ongoing Phase 2a clinical trial in Alzheimer's disease have shown longitudinal dose-dependent cognitive and adaptive functional improvements as assessed by the Mini Mental State Examination (MMSE) and the Alzheimer's Disease Cooperative Study-Activities of Daily Living (ADCS-ADL), respectively (Hampel et al., 2018). Previously, blarcamesine has demonstrated anticonvulsant, anti-amnesic, neuroprotective and antidepressant properties in various animal models (Lahmy et al., 2013, 2015; Maurice, 2016; Villard et al., 2011), indicating its potential to treat CNS disorders of both neurodegenerative and neurodevelopmental nature. Modest muscarinic modulatory activity (Villard et al., 2011), in addition to its S1R-activating properties, may also play a role in blarcamesine's net effects. Based on these findings, we hypothesized that blarcamesine would have a symptomatic and potentially disease-modifying effect on RTT. The present study investigates this possibility in female *Mecp2* heterozygous mice (HET) by evaluating basic neurologic functions, via RTT-relevant paradigms (Lombardi et al., 2015), with a focus on sensory, motor and autonomic aspects.

2. Materials and methods

2.1. Animals

Female *Mecp2*$^{tm1.1Bird}$/*J* HET (C57 background) mice and C57BI/6J WT mice were purchased from Jackson Laboratories and received at 4.5 weeks of age. Mice were housed in same-genotype groups of 4 mice in opti-MICE cages (Animal Care Systems, CO). All animals were examined and weighed prior to initiation and throughout the study to ensure adequate health and suitability and to minimize non-specific stress associated with manipulation. During the course of the study, 12/12 light/dark cycles were maintained. The room temperature was between 20 and 23 °C with a relative humidity maintained at 50%. Chow and water were provided *ad libitum* for the duration of the study. The tests were performed during the animal's light cycle phase. All animal work was conducted in accordance with standard protocols and was approved by the respective Institutional Animal Care and Use Committees.

2.2. Experimental design

Neurobehavioral studies were performed using a two-tier approach, with initial tests carried out in potentially more treatment-receptive younger animals. Tier assignment was also based on previous published phenotyping studies and on our empirical evidence demonstrating significant differences between different age groups (i.e., Tier 2 tests do not yield robust or reproducible responses at younger ages) and between WT and HET mice. It has been reported that, in HET mice, most of the sensory and motor abnormalities are evident as early as 7 weeks and develop completely by 12 weeks. Other phenotypes, like expiratory apnea, are present at these early ages, but they are not consistent until 6–8 months of age (Samaco et al., 2013; Li et al., 2017). In addition, for younger animals, performing the first test at 8 weeks of age allows for a 2-weeks treatment period in order to achieve sufficient drug exposure prior to the test.

The evaluations were selected on the basis of the wide distribution of S1Rs and on previously reported key phenotypes, both reliable and functionally meaningful (Lombardi et al., 2015; Robinson et al., 2012; Samaco et al., 2013; Schaevitz et al., 2013; Allemang-Grand et al., 2017; Smith et al., 2018). Older animals were then tested in a second tier using complementary paradigms.

The Tier 1 assays utilized mice approximately 5 weeks of age that were administrated a once daily dose of blarcamesine (10 and 30 mg/kg PO) throughout the testing phase with evaluations at 8 and 12 weeks. The same animals were assessed at these two ages. No habituation has

been observed with repeat testing for the reported assays. Assessments included rotarod, gait analysis, hindlimb clasping, acoustic startle, and prepulse inhibition. 4 experimental groups of female mice (N = 20/group, 80 total) were used:

WT mice – vehicle (saline)
HET Mecp2 mice – vehicle (saline)
HET Mecp2 mice – blarcamesine (10 mg/kg/day)
HET Mecp2 mice – blarcamesine (30 mg/kg/day).

Blarcamesine was administrated at a dose volume of 10 ml/kg. The drug was weighed and mixed twice weekly, then aliquoted for daily use and refrigerated at 4 °C. On the day of testing, blarcamesine was administered as a 60-minute pretreatment.

The Tier 2 assays utilized older mice (approximately 6.5 months of age), different from those employed in Tier 1 assays, and the more effective dose as identified in Tier 1, namely 30 mg/kg/day. Blarcamesine was administrated once daily throughout the testing phase with evaluations at age 7.5 months. The latter included an optomotor/optokinetic response and plethysmography. 3 experimental groups of female mice (N = 18–20/group, 54–60 total) were used:

WT mice – vehicle (saline)
HET Mecp2 mice – vehicle (saline)
HET Mecp2 mice – blarcamesine (30 mg/kg).

Body weight was recorded daily for both younger and older mouse groups.

2.3. Motor testing

2.3.1. Rotarod – balance and motor coordination

Mice were taken to the experimental room and placed on a rotarod apparatus (Curzon et al., 2009; Robinson et al., 2012; Schaevitz et al., 2013), capable of rotating at a constant or accelerating speed. Mice were first exposed to the apparatus for 5 min of training at a constant speed. The time in seconds that it took for an animal to fall (latency) was automatically recorded by the equipment; paper towels or diaper pads cushioned the fall. After a resting period of at least 1 h, animals were placed back on the rotarod apparatus for testing. In this instance, a program of accelerating speed (0–40 RPM) was employed over 5 min and the latency to the first fall and the RPM speed at the time of the fall were recorded. The test was repeated 3 consecutive times per animal and the average performance was used in subsequent analyses.

2.3.2. NeuroCube® - gait geometry and dynamics

The NeuroCube® system is a PsychoGenics proprietary gait platform that employs computer vision to detect changes in gait geometry and gait dynamics in rodent models of neurological disorders, pain, and neuropathies (Alexandrov et al., 2015, 2016). This platform is unique for gait testing for the following reasons: (1) it is completely automated; thus, it removes any bias or subjectivity, and (2) the system captures both gait geometry (stride length, step length, base width) and gait dynamics (stride duration, step duration and swing duration). Other parameters evaluated by the system include average speed of the animal; paw image intensity, paw contact area, perimeter of contact zone, and paw diameter; paw position relative to the center of the body; body position as it pertains to movement of the subject; and rhythmicity and limb coordination. The sensitivity of the computer vision and bioinformatics components of NeuroCube® enable it to capture symptoms of the disease model and in some cases detect changes earlier compared to standard tests.

Mice were placed in the NeuroCube® for a 5-minute test. The most dominant of the features collected, which define the disease phenotype (symptom descriptors), were identified and ranked. Bioinformatic algorithms were employed to capture gait changes that can discriminate between HET Mecp2 and WT mice. The same features were used to objectively quantify disease progression and treatment, as previously reported in various rodent models of neurodegenerative and neurodevelopmental disorders (Alexandrov et al., 2015, 2016).

The following individual parameters were evaluated in this study: stride length, front step length, mean hind paw area, and front base width. Stride length is the average distance between the centers of two consecutive steps of the same paw, while step length is the average of the distance between the centers of a step and the immediate next step by the opposite paw.

2.3.3. Clasping (abnormal movements)

Hindlimb clasping is an abnormal posture that is characteristic of mouse models with Mecp2 deficiency (Lalonde and Strazielle, 2011; Lombardi et al., 2015). As such, it resembles the distinctive hand stereotypies of RTT (Neul et al., 2010). To evaluate the presence of hindlimb clasping or splay, mice were observed when held by the base of the tail and gently lifted until the front paws just lifted off the counter surface. After testing, animals were placed back into the test or home cage.

2.4. Sensory testing

2.4.1. Acoustic startle and prepulse inhibition of the startle response (PPI)

The acoustic startle measures an unconditioned reflex response to external auditory stimulation (Curzon et al., 2009). PPI consists of an inhibited startle response (reduction in amplitude) to an auditory stimulation following the presentation of a weak auditory stimulus or prepulse. PPI has been used as a tool for the assessment of deficiencies in sensory-motor gating, such as those seen in schizophrenia, neurodevelopmental disorders (e.g., fragile X syndrome), and neurodegenerative disorders (e.g., Huntington disease) (Abbruzzese and Berardelli, 2003; Kohl et al., 2013). Previous work reported strain-dependent decreases in acoustic startle and enhanced PPI in Mecp2 HET mice (Samaco et al., 2013).

Mice were placed in PPI chambers (Med Associates Inc., Fairfax, Vermont) for a 5-minute session of white noise (70 dB) habituation. The test session automatically started with a habituation block of 6 presentations of the startle stimulus alone, followed by 10 PPI blocks of 6 different types of trials. Trial types included null (no stimuli), startle (120 dB), startle plus prepulse (4, 8 and 12 dB over background noise i.e. 74, 78 or 82 dB), and prepulse alone (82 dB). Trial types were presented at random within each block. Each trial started with a 50-millisecond null period during which baseline movements were recorded. There was a subsequent 20- millisecond period during which prepulse stimuli were presented and responses to the prepulse were measured. After further 100 ms, the startle stimuli were presented for 40 ms and responses recorded for 100 ms from the startle onset. Responses were sampled every millisecond. Inter-trial intervals were variable, with an average of 15 s (range from 10 to 20 s). In startle-alone trials the basic auditory startle was measured, while in prepulse plus startle trials the amount of inhibition of the normal startle was determined and expressed as a percentage of the basic startle response (from startle alone trials) that excluded the startle response of the first habituation block.

2.4.2. Optokinetic response (OKR)

This paradigm depends on the automatic visual response of head-tracking to a moving vertical stripe pattern presented on a rotating drum to an animal placed at its center. The movement of the head is measured in synchrony with the stripe movement. Although the test measures 'relative visual acuity', it has considerable utility as a repeatable, non-stressful and reproducible measure of visual function (Pinto and Enroth-Cugell, 2000; Stahl, 2004).

The OKR device consists of a stationary holding platform that is surrounded by a rotatable cylinder with interchangeable panels covered

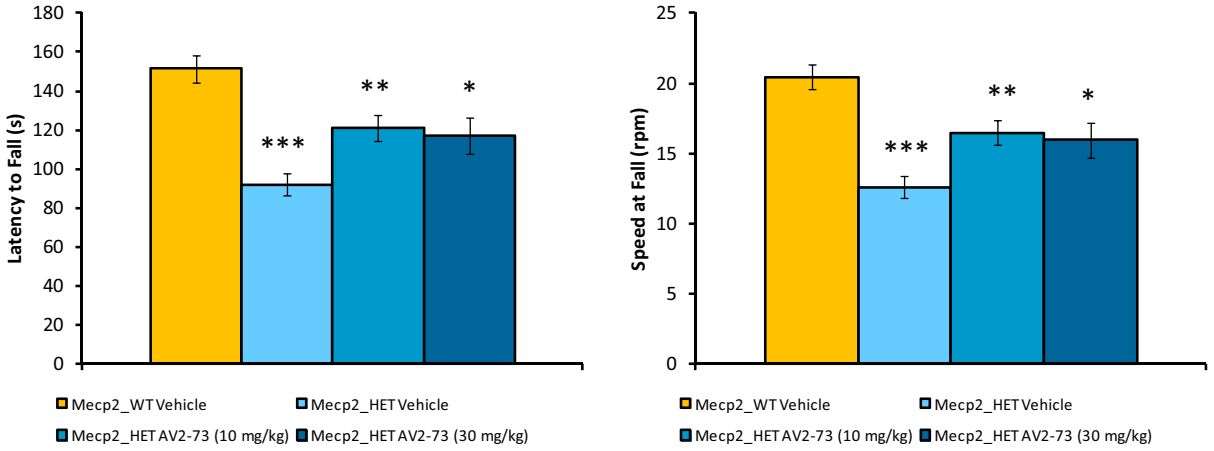

Fig. 1. Blarcamesine (AV2-73) improved the 2 rotarod parameters examined at 12 weeks, specifically latency to fall and speed at fall. Error bars are SEM. One-way ANOVA with Dunnett's post-hoc tests. *, **, indicate $p < 0.05$ and $p < 0.01$, HET on 30 mg/kg/day and HET on 10 mg/kg/day, respectively, compared with HET on vehicle. *** corresponds to $p < 0.001$, HET on vehicle vs. WT on vehicle.

with a vertical stripe pattern. The mouse tracks the rotation of the grating with reflexive head movements recorded via a camera mounted above the apparatus. Visual sensitivity can be quantified by increasing the spatial frequency of the grating.

Mice were placed on an elevated platform in the stationary chamber in the center of the cylinder and habituated to the apparatus for 5 min the day before the test. On the day of testing, the cylinder was rotated for 1 min in both clockwise and counterclockwise directions, testing left and right eye sensitivities at 3 different speeds: 0 rev/min (0 cycles per degree), 1.5 rev/min (0.07 cycles per degree), and 2.8 rev/min (0.26 cycles per degree). Maximum testing time was set at 30 min per session, and the number of responses in the direction of and opposite to the direction of the drum at each speed was measured.

2.5. Plethysmography (autonomic function)

Respiratory disturbances, including increased respiratory rate, atypical pauses, and apneas, are prominent features of RTT (Tarquinio et al., 2018) and have been described in a variety of Mecp2 deficient mouse models (Abdala et al., 2014; Castro et al., 2014; Johnson et al., 2015; Lombardi et al., 2015; Gogliotti et al., 2017; Howell et al., 2018). Most of these studies have employed plethysmography, as in the present investigation.

Specialized whole-body plethysmographs for the measurement of ventilation in conscious animals were used throughout (item 601-1425-001, Data Sciences International, New Brighton, MN). Mice were habituated to the chamber for ~15 min before recording began for 1 h. Greater stability of the ventilator parameters was observed during the second half hour of the total recording; therefore, the reported data are based on these later assessments. In line with previous publications, apneas were defined as an expiratory time (Te) > 1 s (Abdala et al., 2014). In addition, enhanced pauses were also monitored as this parameter is commonly used to assess airway responsiveness or obstruction (Hamelmann et al., 1997; Zhu et al., 2011; Oldenburg et al., 2012). In the context of RTT and Mecp2 deficiency, enhanced pause provides another metric of Te, in particular the early expiration phase that appears to be more affected in Mecp2 deficient animals (Katz et al., 2009).

2.6. Other testing

Body weights were recorded twice weekly in both the younger and older groups of mice.

2.7. Statistical analysis

Data were first subjected to descriptive statistical analyses and represented as mean and standard error to the mean (SEM). All neurobehavioral tests in Tier 1 and Tier 2 were analyzed by analysis of variance (ANOVA) followed by post-hoc comparisons where appropriate. An effect was considered significant if $p < 0.05$. Analyses were carried out with and without outliers, as reported below in Results. Outliers were defined as those animals with values on a specific behavioral test above or below 2 SD of the mean. For each test, we identified 1–2 outliers. Most analyses included comparisons between vehicle-treated HET and WT groups, and comparisons between vehicle-treated and drug-treated HET mice. Genotype and gender and their interaction were considered in all of the models, plus any required session, trial or time factor. Significant interactions were followed up with simple main effects, if there was a main repeated measure factor.

3. Results

3.1. Chronic administration of blarcamesine ameliorates motor deficits in Mecp2 HET mice as a function of age

Mice tested on the rotarod task were judged by their latency to fall and the speed at which they fell. At 8 weeks of age (after 2.5 weeks of once daily PO administration), vehicle-treated female HET mice fell more quickly and at a lower rotation speed than mice in the vehicle-treated WT group (both comparisons: post-hoc unpaired, two-tailed *t*-test, $p < 0.0001$). No changes in this endpoint were noted in the blarcamesine-treated groups at either daily dose (10 or 30 mg/kg/day). At 12 weeks of age (after 6.5 weeks of once daily PO administration), vehicle-treated Mecp2 HET mice continued to fall more quickly and at lower rotation speeds (both comparisons: post-hoc unpaired, two-tailed *t*-test, $p < 0.0001$). In this instance, however, blarcamesine treatment appeared to reverse the deficits observed in the HET animals (post-hoc Dunnett's multiple comparison test, both latency and speed: $p = 0.01$ with/without outliers), although there were no differences in the rotarod endpoints between the 10 and 30 mg/kg/day doses (Fig. 1).

Beyond these simple tasks, the effects of blarcamesine on various aspects of gait geometry and gait dynamics were determined by analysis with the NeuroCube®. In vehicle-treated HET mice vs. vehicle-treated WT mice, stride length, front step length, and front base width were abnormal at 8 weeks (the first two parameters decreased while the third increased), while at 12 weeks the differences were in stride length (decreased), mean hind paw area (reduced), and front base width (increased). Treatment with once daily administration of blarcamesine

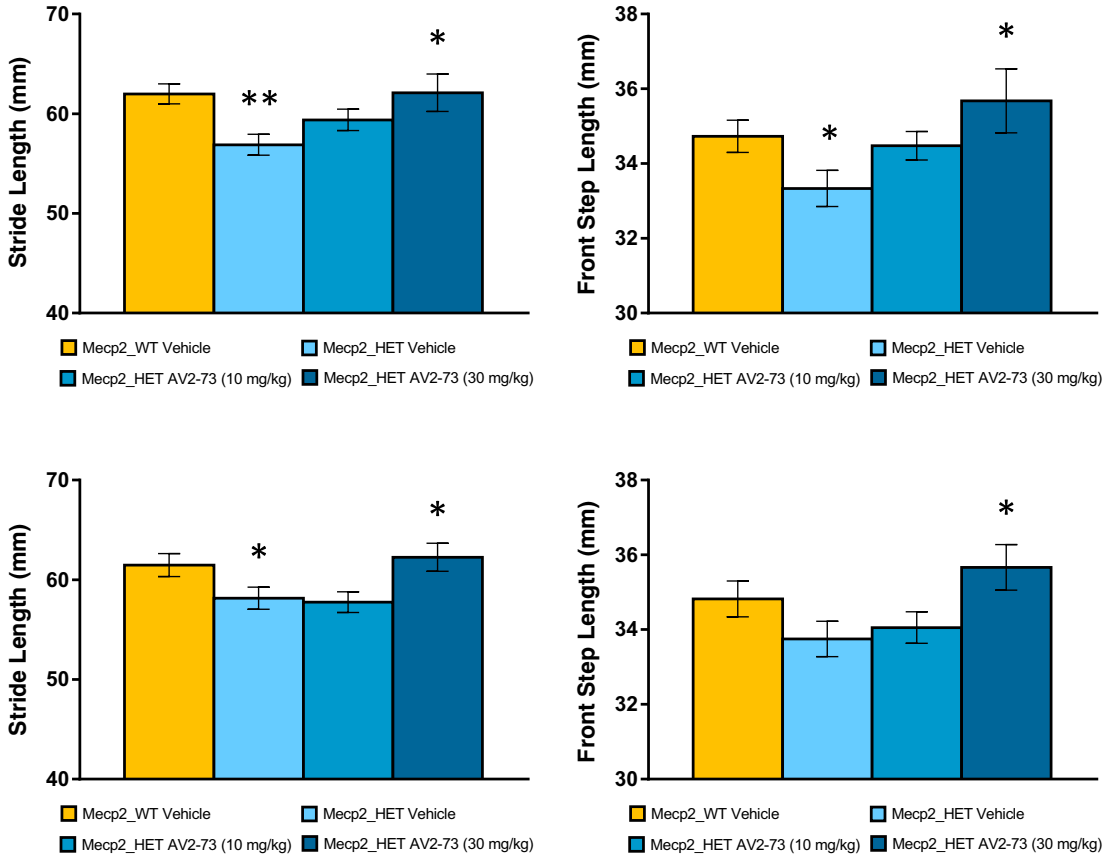

Fig. 2. Blarcamesine (AV2-73) improved selective gait parameters measured by NeuroCube® at 8 weeks (top two graphs) and 12 weeks (bottom two graphs). Error bars are SEM. One-way ANOVA with Dunnett's post-hoc tests. *, **, indicate $p < 0.05$ and 0.001, respectively. Asterisks at the Mecp2 HET vehicle bar represent comparison with Mecp2 WT vehicle group. The other asterisks correspond to comparisons between the Mep2 HET on 30 mg/kg/day and Mecp2 HET vehicle groups.

improved stride length at 8 and 12 weeks and front step length at 8 weeks only at the 30 mg/kg/day dose, with the latter effect completely restored relative to vehicle-treated WT mice (Fig. 2 top). The effect of treatment on front base width at 12 weeks was also significant (Fig. 2 bottom), in this instance by increasing the differences with the WT group (i.e., larger front base width).

When assessing clasping response among the Mecp2 HET mice among the various treatment groups, a somewhat different pattern emerged. Normal mice (or, in this context, WT mice) lifted by their tails reflexively splay their hindlimbs (Lalonde and Strazielle, 2011); Mecp2 HET mice, however, more characteristically display a clasping phenotype (Lombardi et al., 2015), and this difference was robustly evident in the current study (two proportion z-test, $p < 0.0001$, Fig. 3). Blarcamesine reduced clasping at the 30 mg/kg/day at both testing times, 8 and 12 weeks ($p = 0.023$ and $p = 0.046$, respectively); no effect was observed at the lower blarcamesine dose, although the effect trended in the same direction as in the higher dose group.

3.2. Chronic administration of blarcamesine ameliorates acoustic startle deficit in Mecp2 HET mice

Acoustic startle measures an unconditioned reflex response to external auditory stimulation (Curzon et al., 2009). Vehicle-treated HET mice startled significantly less than vehicle-treated WT mice ($p < 0.0001$) at both 8 weeks (Fig. 4) and 12 weeks. Once daily treatment with blarcamesine at 30 mg/kg/day partially reversed this decrease at 8 weeks compared to the vehicle-treated HET mice ($p < 0.05$, with/without outliers). Although at 12 weeks there was an increase in startle response after blarcamesine administration, this effect was at a trend level ($p = 0.08$) and became significant only after removing outliers ($p = 0.02$).

Prepulse Inhibition (PPI), a refinement of the acoustic startle response, assesses sensory-motor gating resulting from a low threshold stimulus (Abbruzzese and Berardelli, 2003; Kohl et al., 2013). In contrast to previous reports (Samaco et al., 2013), no differences in the PPI response were observed between the WT and HET mice at either age; correspondingly, there were no differences in animals treated with blarcamesine at either dose (Fig. 4).

3.3. Chronic administration of blarcamesine reverses deficits in visual acuity in older Mecp2 HET mice using the optokinetic response

Head tracking responses to a moving stripe pattern comprise the so-called Optokinetic Response (OKR), as utilized in both laboratory animals and human subjects (Pinto and Enroth-Cugell, 2000; Stahl, 2004), from which an assessment of visual acuity can be made. These were dampened in 7-month old female Mecp2 HET mice compared to their WT counterparts (post-hoc Dunnett's multiple comparison test, $p < 0.05$, Fig. 5). This observation held whether the pattern was presented in a clockwise or counter-clockwise direction and regardless of the rotating speed (1.5 or 2.8 RPM), suggesting a general decrease of visual acuity. Daily dosing with blarcamesine at 30 mg/kg/day for approximately 4 weeks ($p < 0.05$, Fig. 5) eliminated WT vs. HET differences with the effect at the lower rotating speed (1.5 RPM) driving this result. Findings with the 10 mg/kg/day dosing regimen were not generated as the higher dose was deemed to be generally more effective vs. the motor endpoints in the younger animal cohorts (Figs. 2, 4).

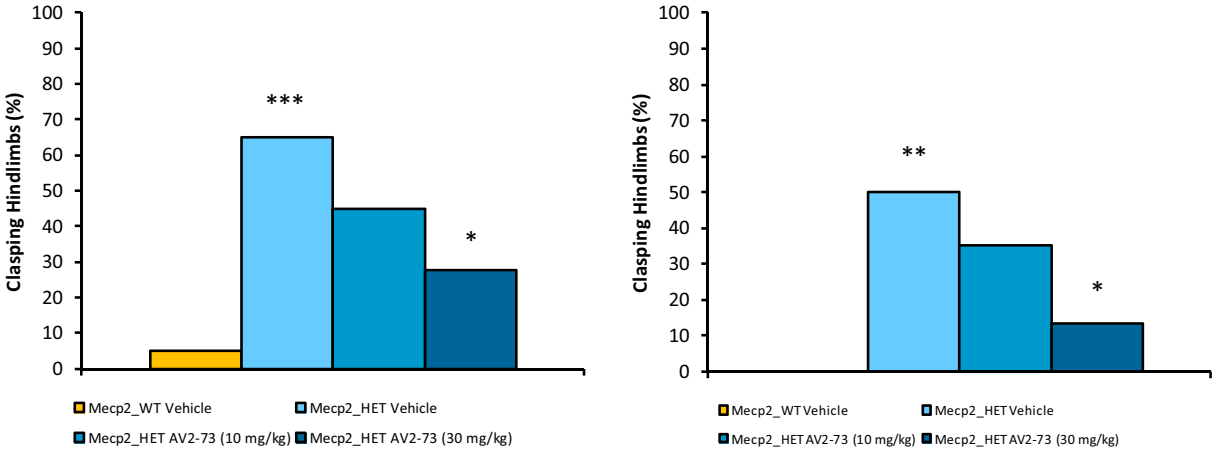

Fig. 3. Blarcamesine (AV2-73) markedly reduced the atypical hindlimb clasping response at both doses at 8 weeks (left) and 12 weeks (right). Error bars are SEM. One-way ANOVA with Dunnett's post-hoc tests. *, **, *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Asterisks at the Mecp2 HET vehicle bar represent comparison with Mecp2 WT vehicle group. The other asterisks correspond to comparisons between the Mep2 HET on 30 mg/kg/day and Mecp2 HET vehicle groups.

3.4. Chronic administration of blarcamesine reverses expiratory apnea measured by whole body plethysmography in older adult Mecp2 HET mice

Respiratory rate was significantly increased in vehicle-treated Mecp2 HET mice, when compared with vehicle-treated WT mice, at 7 months of age ($p < 0.05$). This difference decreased, although not significantly, after administration of blarcamesine 30 mg/kg/day for 1 month (Fig. 6). Apnea count (per hour) was increased in Mecp2-deficient mice when compared with their WT counterparts. Treatment with blarcamesine restored apnea count to WT-level in Mecp2 HET mice but only after removing outliers ($p = 0.18$ and $p = 0.003$ with and without outliers, respectively) (Fig. 6). Enhanced pause count (per minute) but not average duration of enhanced pauses was different in vehicle-treated Mecp2 HET mice when compared with vehicle-treated WT mice. The increase in enhanced pause count was reduced, but not significantly, after blarcamesine administration to Mecp2 HET animals (Fig. 6).

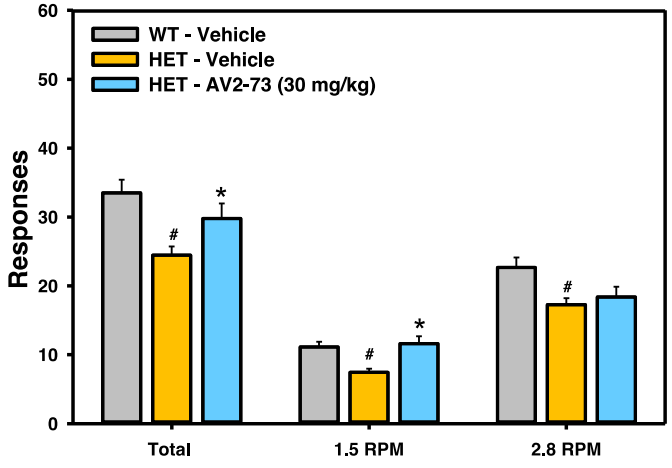

Fig. 5. Blarcamesine (AV2-73) ameliorated the decreased OKR in Mecp2 HET mice observed at 7 months. All bar graphs represent total clockwise and counter-clockwise responses. Error bars are SEM. One-way ANOVA with Dunnett's post-hoc tests. #, * indicate $p < 0.05$, for comparisons between vehicle-treated Mecp2 HET and vehicle-treated WT mice and between vehicle-treated Mecp2 HET and drug-treated Mecp2 HET mice, respectively.

3.5. Chronic administration of blarcamesine produces minimal effects on body weight gain in Mecp2 HET mice

As described above, a younger cohort of female HET mice, dosed daily beginning at 5.5 weeks of age and continuing to the conclusion of

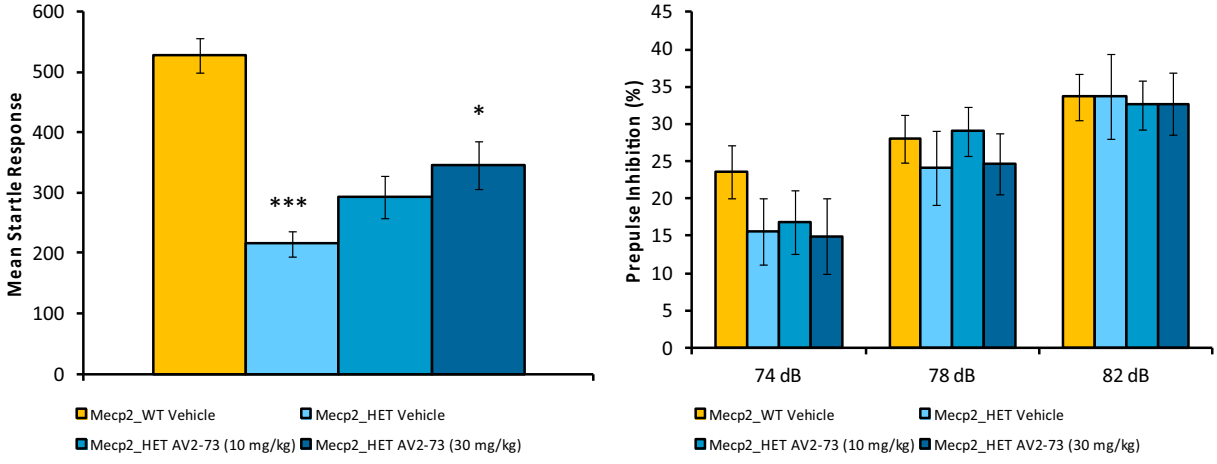

Fig. 4. Blarcamesine (AV2-73) increased the decreased acoustic startle response in Mecp2 HET mice observed at 8 weeks. Error bars are SEM. One-way ANOVA with Dunnett's post-hoc tests. *, *** indicate $p < 0.05$, and $p < 0.0001$, respectively. Asterisk at the Mecp2 HET vehicle bar represents comparison with Mecp2 WT vehicle group. The other asterisk corresponds to a comparison between the Mep2 HET on 30 mg/kg/day and Mecp2 HET vehicle groups.

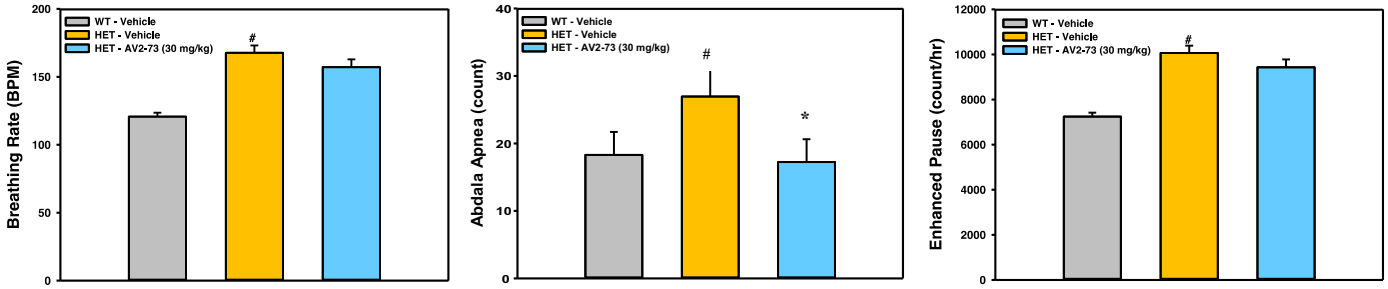

Fig. 6. Blarcamesine (AV2-73) markedly reduced apnea count in Mecp2 HET mice at 7 months. Other abnormal respiratory parameters, specifically breathing rate and enhanced pause count also decreased but not significantly. Error bars are SEM. One-way ANOVA with Dunnett's post-hoc tests. #, * indicate $p < 0.05$, for comparisons between vehicle-treated Mecp2 HET and vehicle-treated WT mice and $p < 0.01$ between vehicle-treated Mecp2 HET and drug-treated Mecp2 HET mice, respectively.

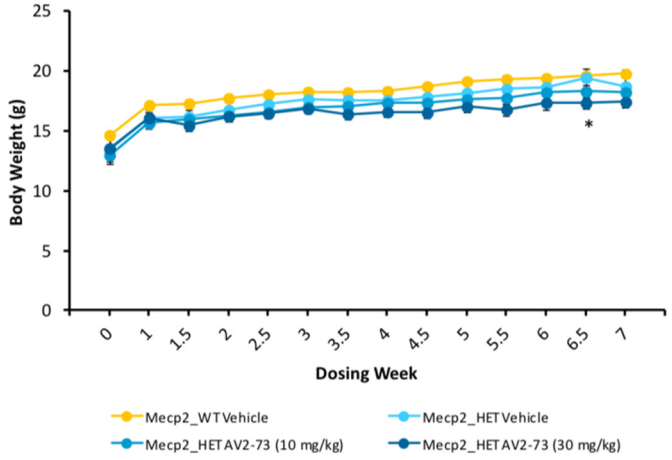

Fig. 7. Daily dosing with blarcamesine (AV2-73) had a negligible effect on body weight gain in the younger cohort of mice employed for various assessments of motor and sensory function. Error bars are SEM. One-way ANOVA with Dunnett's post-hoc tests. * indicates $p < 0.05$, for comparisons between vehicle-treated Mecp2 HET and vehicle-treated WT mice.

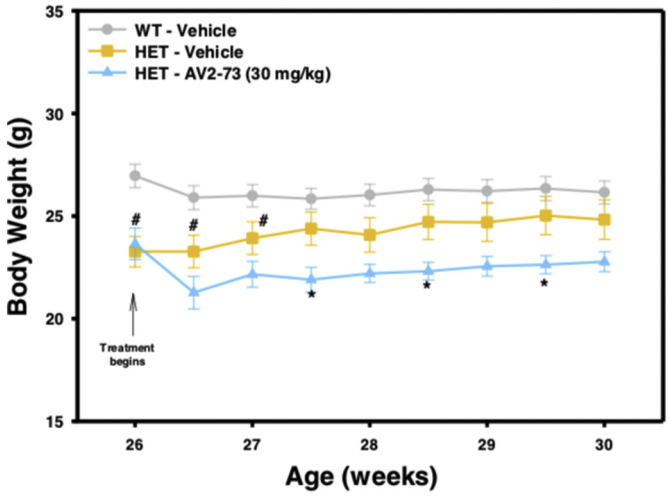

Fig. 8. Daily dosing with blarcamesine (AV2-73) had a more systematic effect on body weight gain in the older cohort of mice employed for assessments of visual and respiratory function. More specifically, a decrease was observed at the time of the first assessment (0.5 weeks) but subsequent measures revealed a similar rate of weight gain as in the vehicle-treated HET animals. Error bars are SEM. One-way ANOVA with Dunnett's post-hoc tests. #, * indicate $p < 0.05$, for comparisons between vehicle-treated Mecp2 HET and vehicle-treated WT mice and $p < 0.01$ between vehicle-treated Mecp2 HET and drug-treated Mecp2 HET mice, respectively.

the study at 12 weeks, was used to assess changes in performance of various motor and sensory tasks at 8 and 12 weeks of age (or following 2.5 and 6.5 weeks of dosing). No changes in normal weight gain were observed throughout this drug treatment period at the 10 mg/kg/day dose and only a minor change at the 30 mg/kg/day dose was observed at 6.5 weeks into the regimen (repeated measures ANOVA; Dunnett's multiple comparisons test, $p < 0.05$, Fig. 7).

The older cohort of female HET mice was also dosed daily, in this case beginning at 26 weeks of age and continuing to the conclusion of the study at 30 weeks. Rather than the motor endpoints employed in the younger animals, these mice were used to assess changes in other phenotypic signs (visual acuity and respiratory function) following the period of chronic dosing. Changes in normal weight gain were more apparent in this older cohort with a decrease in body weight in vehicle-treated WT vs. HET mice throughout the study (repeated measures ANOVA; Dunnett's multiple comparisons test). The impact on weight gain was most apparent at the time of the first assessment (0.5 weeks) in that following this time point the drug-treated HET mice appeared to resume the same rate of weight gain as the vehicle-treated animals (Fig. 8).

4. Discussion

RTT is a severe neurodevelopmental disorder without effective or specific treatments. Advances in understanding of the genetics and neurobiology of RTT have led to an active period of drug development but no pivotal trial has been completed as yet. Therefore, testing novel compounds that can ameliorate RTT's clinical manifestations is a priority in the field. Blarcamesine is a S1R agonist and muscarinic receptor modulator with a strong safety record and preliminary evidence of efficacy in Alzheimer's disease. Its role in calcium homeostasis and mitochondrial function makes it an attractive candidate for RTT. Although there are multiple mouse models of RTT, current preclinical studies have mainly employed female heterozygous animals carrying one null allele (HET) of *Mecp2*. Administration of blarcamesine to HET mice resulted in an improvement in multiple motor, sensory, and autonomic phenotypes of relevance to RTT. In line with previous animal and human studies, blarcamesine showed a good safety profile in this mouse model of RTT.

Our characterization of the effects of blarcamesine in Mecp2-deficient mice focused on basic neurologic endpoints considered to be relevant to RTT (Samaco et al., 2013; Lombardi et al., 2015) and to be of widespread use in numerous drug testing paradigms. Blarcamesine testing in Mecp2 HET mice was conducted using a two-tier approach (Samaco et al., 2013; Li et al., 2017), based on previously published tests and our own experience. Tests in presumably more treatment-receptive younger animals were followed by complementary assessments in older adult mice, if the initial findings proved to be positive. Younger animals were tested for motor and sensory function while older mice

were assessed for visual acuity and breathing. Paradigms included a combination of widely used tests (Curzon et al., 2009) and paradigms of particular relevance to Mecp2 deficiency (Robinson et al., 2012; Samaco et al., 2013; Schaevitz et al., 2013; Lombardi et al., 2015; Allemang-Grand et al., 2017).

Motor coordination and balance were evaluated by the well-established rotarod test (Robinson et al., 2012; Schaevitz et al., 2013) and gait was assessed by NeuroCube®. Both tested doses of blarcamesine showed effectiveness in preventing falls from the rotarod in young adult HET mice after approximately 6 weeks of treatment. A shorter drug administration, between 2 and 3 weeks, did not change motor stability in the HET mice. Gait impairment is one of the four diagnostic criteria of the disorder (Neul et al., 2010). Contrary to the rotarod effects, only the higher dose was consistent in improving some but not all gait parameters. However, positive effects were detected at 8 weeks and continued into the 12-week assessments. Two gait abnormalities, stride length and mean hind paw area, were ameliorated while a third one, front step length, showed an apparently exaggerated compensatory response. A fourth parameter, front base width, displayed an exacerbation of the abnormal pattern in Mecp2 HET mice, which could be an adaptive response given, as in cases of complex and dynamic functions, it is difficult to separate these from dysfunction. Overall, blarcamesine had a positive effect on different aspects of motor function in Mecp2 deficient mice. This beneficial action appears to be dependent on dose and length of treatment.

Hand stereotypies characterized by abnormal posture and repetition are the hallmark of RTT (Neul et al., 2010). Hindlimb clasping, an atypical postural response characteristic of several mouse models of genetic abnormalities (Lalonde and Strazielle, 2011), is a distinctive feature of early Mecp2 deficiency that resembles the human abnormality (Lombardi et al., 2015). In the present study, vehicle-treated Mecp2 HET mice showed a 50–70% clasping response that was virtually absent in vehicle-treated WT mice. Blarcamesine dramatically reduced the frequency of clasping, in a dose response-dependent fashion, at both time points of testing and to as little as 10% at 12 weeks. This finding stresses the potential of blarcamesine in ameliorating one of the most prevalent and impairing features of RTT (Stallworth et al., 2019).

Two tests of sensory function were performed. The acoustic startle response is one of the most widely applied sensory tests in animal studies (Curzon et al., 2009). Assessment is frequently carried out with the related PPI response that measures sensory-motor gating, a process of filtering out redundant or unnecessary sensory stimuli (Abbruzzese and Berardelli, 2003; Kohl et al., 2013). While acoustic startle was reduced in Mecp2 HET mice, PPI response was comparable to WT mice. It is unclear whether the PPI responses were not affected in Mecp2 HET mice because of their reduced acoustic startle (i.e., easier to inhibit milder response) since a previous study had reported a strain-dependent decrease in startle but enhanced PPI in Mecp2 HET mice (Samaco et al., 2013). Blarcamesine ameliorated the sensory deficit at both time points in young adult mice, again only at the higher dose of 30 mg/kg/day. The optokinetic response (OKR), a test of visual function (Pinto and Enroth-Cugell, 2000; Stahl, 2004), was performed only in the older group of animals and at the more effective dose of 30 mg/kg/day. OKR decreases to different orientation and speed of the visual stimulus were mildly improved by drug treatment for one month. In sum, blarcamesine at a dose of 30 mg/kg is able to correct sensory deficits at different ages in Mecp2-deficient mice.

Breathing abnormalities are a major feature of RTT (Neul et al., 2010; Tarquinio et al., 2018) and have been replicated in multiple mouse models of Mecp2 deficiency (Lombardi et al., 2015). The most concerning breathing abnormality in RTT is respiratory apnea, representing predominantly breath-holding in expiration. For this reason, apnea counts are commonly evaluated in preclinical drug studies in RTT (Tropea et al., 2009; Abdala et al., 2014; Castro et al., 2014; Kron et al., 2014; Patrizi et al., 2016; Gogliotti et al., 2017). Although there are multiple ways to assess apnea in Mecp2 deficient mice, most studies employ operational definitions based on prolonged expiratory time (Abdala et al., 2014; Gogliotti et al., 2017; Howell et al., 2018). We used Abdala's definition and found that the marked increase in apnea count in vehicle-treated Mecp2 HET mice was dramatically reduced by administration of blarcamesine. This finding was evident, however, only after removing outliers from the analyses. The latter likely reflects the technical difficulties in obtaining consistent measurements with plethysmography, as it has been reported by others (Abdala et al., 2014; Gogliotti et al., 2017; Howell et al., 2018). These authors suggest a number of strategies for reducing variability including a period of acclimation and motion-free measurements, or even quantifications of apnea variability. Blarcamesine was less effective in ameliorating other parameters such as breathing frequency and enhanced pause counts. The latter is a parameter not previously reported in the literature but of interest in Mecp2 deficiency since it reflects the relative contribution of the early and late phases of the expiration period (i.e., of greater magnitude when the early relaxation phase of expiration is brief or the total time of expiration lengthens; Hamelmann et al., 1997). In conclusion, blarcamesine appears to be effective in ameliorating apnea count, the most severe breathing abnormality in Mecp2 deficient mice.

Changes in normal body weight gain were monitored throughout the drug treatment periods in order to provide an assessment of general health. In the younger cohort of animals used to evaluate changes in motor and sensory function, there were no changes in body weight gain at either dose of blarcamesine with the exception of a minor drop at the higher dose at 6.5 weeks into the regimen. The latter is attributed to a statistical anomaly rather than an indication of weakened health. Within the older cohort of HET mice used to probe for changes in visual acuity and respiratory function, there appeared to be a single-step drop in body weight at the earliest time point that immediately returned to the same trajectory of weight gain as the vehicle-treated HET animals. It is reasonable to attribute this finding to the dose and yet there is no ready explanation as to why it should impact body weight at only a single time point and why recovery to normal absolute body weight was not achieved subsequently. In any case, the overall effect on body weight appeared to be minor in that the weight loss at the end of the dosing regimen was approximately 10% and therefore not viewed as deleterious. The observed recovery of visual acuity and respiratory function in the HET animals with blarcamesine dosing supports this interpretation.

In conclusion, the data demonstrate that blarcamesine is effective in ameliorating multiple neurobehavioral phenotypes in Mecp2-deficient mice. Corrected functions include basic motor and sensory features, such as balance and acoustic startle, as well as more complex gait parameters. Of particular significance is the fact that blarcamesine had a robust effect upon two distinctive clinical manifestations in RTT replicated by Mecp2 deficient mice: hindlimb clasping, a model of hand stereotypies, and expiratory apneas. The strong effect of the drug on the aforementioned RTT-like features is encouraging. Indeed, clinical trials of blarcamesine in RTT are ongoing given that the efficacy data are in line with the pharmacological action of blarcamesine. Enhancement of calcium homeostasis leading to improved mitochondrial function and potential improvements in synaptic function in multiple brain regions make blarcamesine a strong candidate for improving core neurologic deficits in RTT and other neurodevelopmental disorders (Banerjee et al., 2019). A first cohort of RTT patients in the ongoing ANAVEX2-73-RS-001 study (NCT03758924) demonstrated encouraging data of significant improvements of the two global efficacy endpoints, the Rett Syndrome Behaviour Questionnaire (RSBQ) Total score and the Clinical Global Impression – Improvement (CGI-I) as well as in the RSBQ Hand Behaviours and the RSBQ Breathing Problems subscale scores. Efficacy signals on *both* caregiver- & clinician-based measures of severity correlated with plasma changes of key biomarkers related to disease pathogenesis (Kaufmann et al., 2019).

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