**INTRODUCTION**

Rett Syndrome (RTT) is a congenital X-chromosome linked disorder that is characterized by neurodevelopmental delay, seizures, autonomic dysfunction and respiratory difficulties (Liyanage & Rastegar, 2014; Neul et al., 2010; Ramirez et al., 2013; Rett, 1969; Rett, 2016). Over 90% of patients with classic RTT have mutations to the MECP2 gene, which encodes an X-chromosome linked transcriptional regulator Methyl-Cp-binding protein 2 (Amir et al., 1999; Bienvenu
et al., 2000; Huppke et al., 2000; Kim & Cook Jr., 2000; Liyanage & Rastegar, 2014; Neul et al., 2008; Neul et al., 2010). Due to this X-linkage, males exhibit more severe disease phenotypes and most die within a year of birth (Liyanage & Rastegar, 2014); so the majority of RTT patients are female. RTT has an annual mortality rate of 1.2%, a little over a quarter of which is accounted for by sudden deaths (Kerr et al., 1997). Consistent with a potential cardiac contribution to sudden death in the syndrome, there is evidence that some RTT patients exhibit abnormalities in ventricular repolarization (Clark et al., 2020; Crosson et al., 2017; Ellaway et al., 1999; Guideri et al., 2001; McCauley et al., 2011; Sekul et al., 1994). It is now firmly established that a proportion of RTT patients show prolongation of the rate corrected QT (QTc) interval of the ECG, although the prevalence of QTc prolongation differs between studies (from ~7 to 55%) (Clark et al., 2020; Crosson et al., 2017; Ellaway et al., 1999; Guideri et al., 2001; McCauley et al., 2011; Sekul et al., 1994). There is some evidence that particular MECP2 mutations (R255*, T158M, or large deletions) are more likely to predispose to QTc prolongation in RTT (McCauley et al., 2011). These experiments were conducted on isolated ventricular myocytes (McCauley et al., 2011). In the same study, additional experiments were conducted on isolated ventricular myocytes from MeCP2<sup>Null/Y</sup> males that showed an increased “late” sodium current, I<sub>Na,Late</sub>, in RTT compared to wild-type (WT) myocytes (McCauley et al., 2011). The anti-seizure drug phenytoin both decreased I<sub>Na,Late</sub> and reduced QTc interval and arrhythmia in MeCP2<sup>Null/Y</sup> males (McCauley et al., 2011). Some subsequent studies have also reported QTc prolongation in RTT mice (Herrera et al., 2015; Mucerino et al., 2017), although one of these showed that phenytoin exacerbates breathing problems in RTT animals, which may limit its therapeutic value for shortening the QTc interval (Herrera et al., 2015). In contrast to these studies, a different investigation of MeCP2<sup>Null/Y</sup> mice did not observe prolonged QTc intervals in experiments performed at between 6 and 8 weeks of age (Hara et al., 2015).

Until recently, no study has investigated ventricular action potential (AP) repolarization per se in any RTT model. Very recently, however, the results of experiments on 2–3 month old MeCP2<sup>Null/Y</sup> males have confirmed the development of QTc interval prolongation in the ‘Bird’ model; they also demonstrated that APs from ventricular myocytes isolated from RRT mouse hearts are prolonged (increased APD<sub>90</sub>) and exhibit increased triangulation and APD instability compared to WT controls (Cheng et al., 2022). Similar to the study by McCauley and colleagues, an increased I<sub>Na,Late</sub> was found in MeCP2<sup>Null/+</sup> compared to WT myocytes. I<sub>Na,Late</sub> in MeCP2<sup>Null/Y</sup> myocytes retained sensitivity to the investigational I<sub>Na,Late</sub> inhibitor GS-6615 (also known as eliclazine), which was also found to abbreviate AP duration (Cheng et al., 2022). To date, no such study has been performed on MeCP2<sup>Null/+</sup> females. This is perhaps unsurprising given that in order for QTc prolongation to be observed MeCP2<sup>Null/+</sup> females must be kept for at least 10 months, increasing the complexity and cost of such an undertaking. The slower development of a QT phenotype in females than males is attributable to the fact that the MeCP2 gene is located on the X chromosome (as is also the case in humans). Males possess only one copy of the gene and thus in MeCP2<sup>Null/Y</sup> animals the gene product is entirely absent; MeCP2<sup>Null/+</sup> females have one normal copy of the gene and so are heterozygous for ‘null’ protein. Comparable information in females to that for males would be valuable, given that the RTT patient population is predominantly female. Accordingly, this study was undertaken to characterize repolarization in 12–13 month old MeCP2<sup>Null/+</sup> females at the level of the ECG in intact mice and through measurement of APs from isolated ventricular myocytes.

2 | METHODS

2.1 | Mouse model of RTT employed in this study

All experiments were approved by the University of Bristol Animal Welfare Ethical Review Board (AWERB)
and were carried out in accordance with UK Home Office legislation. The murine model of RTT employed for this investigation (the “Bird” strain (Guy et al., 2001)), had deletions of the third and fourth exons of Mecp2. Mice were genotyped as described previously (Cheng et al., 2022). McCauley et al reported no significant difference between QTc intervals in WT and Mecp2Null/+ mice at 4–5 months of age, with differences becoming evident at older ages (10 months) (McCauley et al., 2011). Consequently, for this study all experiments were performed on female mice of 12–13 months of age. This age-range was chosen to allow sufficient time for progressive changes to develop and thereby to optimize the likelihood of observing repolarization differences between WT and Mecp2Null/+ strains.

2.2 | Electrocardiogram (ECG) measurement

Mice were anesthetized by 1.5% isoflurane and ECG measurements were obtained 5 mins after anesthesia had been established. Surface lead II ECG measurements were obtained as described previously (Cheng et al., 2022). Transcutaneous needle electrodes were placed as follows: positive electrode in left hind leg; negative electrode in right front leg; ground electrode in right hind leg. Signals were high-pass filtered at 10 Hz, with a low-pass setting of 1 kHz. Measurements were made of RR interval (and from this heart rate); PR interval; QRS interval; QT and QTc intervals. Mean values for each ECG parameter for each mouse were obtained from 5 consecutive ECG complexes, avoiding complexes upon which breathing noise was superimposed. The duration of the QT interval duration was taken as the time between onset of the QRS complex and time-point after the T-wave peak (Cheng et al., 2022; McCauley et al., 2011).

As previously (Cheng et al., 2022), two rate correction methods were employed in this investigation to obtain QTc interval values (Equation 1: [Mitchell et al., 1998; Speerschneider & Thomsen, 2013; Cheng et al., 2022]; Equation 2: [Cheng et al., 2022; McCauley et al., 2011]):

\[
QT_c = QT / (RR/100)^{0.5} \\
QT_c = QT + 0.3173(170 − RR)
\]

2.3 | Whole body plethysmography

Respiratory patterns were monitored using unrestrained whole-body plethysmography (Emka Technologies, France) as described by Cheng et al. (2022). After a WT or Mecp2Null/+ mouse had been placed in the recording chamber an adaption period of 20 mins was allowed; data for analysis were derived from a subsequent one-hour recording period. Time-series respiratory flow data were analyzed using a published custom analysis method in Spike 2 (V8.22, Cambridge Electronic Design, UK) (Abdala Sheikh, 2022). A running average of the total expiration time for each breath was taken every minute. If an expiration time was longer than 4 times this average, it was counted as an apnoea. Both apnoea count and length were recorded (Cheng et al., 2022).

2.4 | Ventricular myocyte isolation

Animals were killed by cervical dislocation, the heart then excised and placed in ice-cold isolation solution (composition given below) supplemented with 0.1 mM CaCl2 and 10 U/mL heparin. The heart was then cannulated and was Langendorff-perfused for 3 minutes at 37°C at constant pressure of gravity (~80–100 cm H2O) with an isolation solution comprised of (in mM) 130 NaCl, 5.4 KCl, 0.4 NaH2PO4, 4.2 HEPES, 10 glucose, 1.4 MgCl2, 20 taurine, and 10 creatine (pH 7.4 with NaOH) (Cheng et al., 2022; Gadeberg et al., 2017). A 15-minute period of perfusion with enzyme solution followed. Enzyme solution comprised of isolation solution to which were added 0.1 mM CaCl2, 0.07 mg/mL protease (Sigma, Type XIV), and 0.7 mg/mL collagenase (Worthington, Type 1). At the end of this period, the ventricles were removed from the Langendorff apparatus and were shaken in enzyme solution for 5 min before filtration and centrifugation. Ventricular myocytes were then resuspended in isolation solution plus 0.1 mM CaCl2 and stored at room temperature. Cells were used for up to 10 hours following myocyte isolation.

2.5 | Action potential measurement

Ventricular myocytes were placed in a recording chamber (Cheng et al., 2022) and were superfused with a Tyrode’s solution containing (in mM): 140 NaCl, 4 KCl, 1.5 CaCl2, 1 MgCl2, 10 glucose, 5 HEPES (pH 7.4 with NaOH). The superfusate temperature was 35-37°C. A home-built superfusion device allowed local superfusate to be rapidly (<1 s) exchanged (Levi et al., 1996). Patch pipettes were made from borosilicate glass (A-M Systems Inc, Sequim, WA) pulled and fire polished to resistances of 2–3 MΩ (PP-830 and MF83, Narishige, Japan). Pipettes were filled with a solution containing (in mM): 110 KCl, 10 NaCl, 0.4 MgCl2, 10 HEPES, 5 glucose, 5 K2ATP, 0.5 GTP-Tris (pH 7.1 with KOH).
Protocols were generated and data recorded online with pClamp 10 and a Digidata 1440A interface (Molecular Devices, USA). The digitization rate was 10 kHz; the signal was low-pass filtered at 2 kHz. Action potentials (APs) were evoked at 1 second intervals by brief (3 ms) depolarizing current injection in membrane potential recording mode (Cheng et al., 2022). The threshold amplitude for these current pulses was monitored and is given in Results Table 2. Instability of repolarization was evaluated through measurement of beat-to-beat variability of AP repolarization (BVR). As previously (Cheng et al., 2022), this was quantified at 90% of AP amplitude for these current pulses was monitored and is given in Results Table 2. Instability of repolarization was evaluated through measurement of beat-to-beat variability of AP repolarization (BVR). As previously (Cheng et al., 2022), this was quantified at 90% of AP amplitude for these current pulses was monitored and is gives...

\[ \text{BVR} = \sum \left( \frac{|\text{APD}_{90}(n+1) - \text{APD}_{90}(n)|}{(n \text{ beats} \times \sqrt{2})} \right) \]

(3)

2.6 | Data analysis and statistics

The numbers of ventricular myocytes and animals from which results were derived are given in the relevant Results text and accompanying Figure or Table Legends. Data are presented as mean±SEM. Statistical comparisons utilized, as appropriate, a paired t-test, unpaired t-test with equal or unequal variances, and Mann–Whitney test. Statistical analysis was performed using Microsoft Excel (Microsoft Corporation, USA), Prism 8.4.3 (Graphpad Software Inc., USA) and Clampfit of pClamp 10.7 (Molecular Devices, USA). p < 0.05 was taken to be statistically significant. The data that support the findings of this study are available from the authors on reasonable request.

2.7 | Ranolazine and GS-6615 (eleclazine)

Ranolazine dihydrochloride was obtained from Sequoia Research Products Ltd, and 30 mM stock solution was made in distilled water. GS-6615 was obtained from SYNthesis Med Chem, and 10 mM stock solution was made in DMSO. Stock solutions were diluted with standard Tyrode’s solution to arrive at the final concentrations as given in the Results section.

3 | RESULTS

3.1 | ECG changes in Mecp2Null/+ mice

ECG measurements were compared between anesthetized female Mecp2Null/+ and WT mice at 12–13 months of age. Figure 1a shows exemplary ECG traces from WT (upper panel) and Mecp2Null/+ (lower panel) mice, with Figure 1ai showing series of successive ECG complexes and the QT interval illustrated for single ECG complexes from WT and Mecp2Null/+ animals in Figure 1aii. Table 1 summarizes mean ECG data from 12 WT and 15 Mecp2Null/+ animals. Heart rate was significantly greater in Mecp2Null/+ (462.5 ± 21.4 bpm) than in WT mice (399.2 ± 18.5 bpm; p < 0.05); whilst RR interval was significantly smaller in Mecp2Null/+ (134.9 ± 8.2 ms) than in WT mice (154.4 ± 8.3 ms, p < 0.05). PR interval was similar between the two strains. The QRS interval duration was numerically smaller in Mecp2Null/+ than WT mice, but the difference between the two values was not statistically significant. The uncorrected QT interval duration was slightly longer (by 3 ms) in Mecp2Null/+ than in WT mice, but the difference was not statistically significant. However, application of the two different rate correction methods to the QT interval (McCauley et al., 2011; Mitchell et al., 1998; Speerschneider & Thomsen, 2013; Cheng et al., 2022; see Methods) revealed a significant prolongation of QTc interval (Figure 1b and Table 1). The two different correction formulae produced different absolute QTc values, but with each method the mean QTc interval of Mecp2Null/+ mice was significantly greater than that of WT animals (by 5.7–9.2 ms; Table 1 and see Figure 1b for plotted mean and individual QTc values). J-waves were not uniformly observed; they were present in 8 out of 12 WT mice (absent in 4), and in 12 out of 15 Mecp2Null/+ mice (absent in 3). The J-wave amplitude was 0.22±0.04 mV in 8 WT mice and 0.25±0.03 mV in 12 Mecp2Null/+ mice (p > 0.05 vs. WT; unpaired t-test), meaning that there was no significant difference of J-wave amplitude between WT and Mecp2Null/+ mice in which these were present. Respiratory rates monitored during ECG measurement were 106.6±9.9 and 122.4±8.9 breaths per minute, respectively, from WT and Mecp2Null/+ mice (n = 12 and 15 respectively; p > 0.2). These results demonstrate that at 12–13 months of age Mecp2Null/+ mice have prolonged QTc intervals compared to WT controls.

Body plethysmography measurements were made separately from ECG measurements to evaluate periods of apnoea (Abdala et al., 2014; Cheng et al., 2022). Mean results (with superimposed data from measurements from individual animals) are shown in Figure 2. In all, measurements were made from 24 WT and 24 Mecp2Null/+ animals. We observed no significant difference in the number of apnoea episodes in the two strains (Figure 2a). However, the mean duration of apnoea episodes was longer in Mecp2Null/+ mice (Figure 2b). Figure 2c shows a plot of QTc interval values (obtained using equation 1) against apnoea length. No significant correlation between the two values was found (R = 0.0239 and p = 0.9115).
3.2 | AP changes in Mecp2Null/+ mice

Isolated ventricular myocytes were stimulated at 1 Hz with fixed duration (3 ms) depolarizing current injection to elicit APs (see Methods and Cheng et al., 2022). Figure 3ai,ii show exemplar APs from WT and Mecp2Null/+ myocytes, respectively. Table 2 summarizes mean AP data gathered from 24 myocytes from 10 WT mice and 18 myocytes from 12 Mecp2Null/+ animals. Several notable differences were observed between myocytes from the two mouse strains. First, the threshold amplitude of the current stimulus required to elicit APs was significantly smaller in myocytes from Mecp2Null/+ animals (see Table 2). Second, the resting membrane potential (RMP) was ~2.8 mV less negative in RTT myocytes (p < 0.05). A similar observation has been made for myocytes from male Mecp2 Null/Y myocytes for which RMP was ~2.7 mV less negative than in WT control myocytes (Cheng et al., 2022). Neither of mean AP overshoot potential nor mean AP amplitude significantly differed between RTT and WT myocytes. However, mean AP upstroke velocity was smaller in RTT (122.5 ± 6.7 V.s⁻¹) than WT myocytes (150.1 ± 6.4 V.s⁻¹; p < 0.01). AP duration (APD) parameters were quantified at multiple time-points as indicated in Table 2. No statistically significant differences in APD between the two strains were
found at time points up to and including APD<sub>50</sub> (duration at 50% of complete repolarization). However, significant differences in APD<sub>75</sub> and APD<sub>90</sub> were seen (see Table 2 and, for APD<sub>90</sub> also Figure 3b; WT and RTT values of 112.6 ± 9.4 and 151.8 ± 12.2 ms, respectively; \( p < 0.05 \)).

The difference between APD<sub>25</sub> and APD<sub>90</sub> was measured as an index of AP triangulation and was observed to be significantly greater in RTT than WT myocytes (Table 2).

In recent AP measurements from ventricular myocytes from Mecp2<sup>Null/Y</sup> males, APD<sub>90</sub> instability was found to be greater than that for WT myocytes (APD<sub>90</sub> BVR; [Cheng et al., 2022]). This is a significant because increased AP instability is a pro-arrhythmic marker (Hondeghem, 2007; Hondeghem et al., 2001). Figure 3c contains a Poincaré plot that shows examples of beat-to-beat variability of APD<sub>90</sub> for WT and Mecp2<sup>Null/+</sup> APs. Figure 3d shows mean and individual BVR values calculated using equation 3 (Methods) for WT and Mecp2<sup>Null/+</sup> APs. Although there was marked overlap in the values of BVR recorded from the two groups, the largest three values were from the Mecp2<sup>Null/+</sup> group (5.6 ± 1.4 ms from 14 myocytes from 12 Mecp2<sup>Null/+</sup> mice; 4.1 ± 0.6 ms from 18 myocytes from 9 WT mice; \( p > 0.05 \), Mann–Whitney test).

### 3.3 | Effects of GS-6615 on Mecp2<sup>Null/+</sup> APs

GS-6615 (eleclazine) is an I<sub>Na,Late</sub> inhibitor that has effectiveness against LQT3 Na channel mutations (El-Bizri et al., 2018). Recent data from Mecp2<sup>Null/Y</sup> AP recordings is suggestive that GS-6615 retains effectiveness and abbreviates APD<sub>90</sub> in the RTT setting. To determine whether this also applies to female RTT mice APs, we applied 10 \( \mu \)M GS-6615. Figure 4a shows Mecp2<sup>Null/+</sup> APs in control Tyrode’s solution and following exposure to GS-6615; AP abbreviation was observed. Figure 4b shows mean (and superimposed individual experiment) data showing the percentage reduction in APD<sub>90</sub> observed for Mecp2<sup>Null/+</sup> myocyte APs and comparable data from WT myocytes, showing that GS-6615 retained effectiveness in the RTT setting. GS-6615 also decreased AP triangulation in the RTT setting (from 134.1 ± 19.6 ms to 110.2 ± 11.0 ms; \( n = 8 \) myocytes from 6 Mecp2<sup>Null/+</sup> mice; \( p < 0.05 \), paired \( t \)-test). AP triangulation in WT myocytes was also decreased (from 109.0 ± 18.4 ms to 90.5 ± 16.6 ms; \( n = 8 \) myocytes from 5 WT mice; \( p < 0.05 \), paired \( t \)-test). Similar experiments were also performed with the lignocaine relative ranolazine; however, as reported recently in experiments on male mouse WT and RTT myocytes (Cheng et al., 2022), ranolazine prolonged rather than abbreviated AP (data not shown).

### 4 | DISCUSSION

### 4.1 | Results in context

This is the first study to report lengthening of cardiac APs from a female RTT model of any species and only the second in a RTT model from either sex (Cheng et al., 2022). The Bazett’s corrected QT (QT<sub>c</sub>) interval of female cynomolgus monkeys with MECP2 knockout is prolonged compared to controls (Chen et al., 2017), but
further cardiac electrophysiological characterization of this model has not been published. In contrast with this simian model, mouse RTT models do not exhibit embryonic lethality for males, with significant male survival and with heterozygous female mice developing symptoms more slowly than in humans; marked deficits become visible in adulthood (Novarino, 2017; Vashi & Justice, 2019). In the original study that reported QTc prolongation in RTT mice, female $\text{Mecp2}^{+/−}$ mice of 4 months of age showed no significant alterations in ECG parameters, whilst males of 2–3 months of age showed marked QT and QTc prolongation and an increase in QRS width (with QTc intervals of 53.7 ms and 67.6 ms in WT and $\text{Mecp2}^{null/+}$ animals, respectively [a 13.9 ms difference]; [McCauley et al., 2011]). Qualitatively similar changes to these ECG parameters were observed in older females, with mean QTc intervals in WT and $\text{Mecp2}^{+/−}$ animals at 50.3 and 58.1 ms, respectively (a 7.8 ms difference). A separate study attempted to group 11 month-old $\text{Mecp2}^{+/−}$ animals into groups with and without QTc prolongation (Mucerno et al., 2017), without any significant differences evident in RR interval between WT and $\text{Mecp2}^{+/−}$ animals.
In the present study a clear pattern of QTc prolongation in 12–13 month Mecp2Null/+ animals was seen (Figure 1bi,ii). In our previous investigation of Mecp2Null/Y animals, we observed significant increases in QT and QTc interval without significant changes in heart rate; QRS interval width also increased—observations that are similar to the original findings of McCauley et al (McCauley et al., 2011). In the older females investigated in this study, a significant increase in heart rate was seen (Table 1) and, perhaps due to this no significant changes in QRS width or uncorrected QT interval were observed, though marked QTc prolongation (i.e., following rate correction) was found (Table 1).

The direct measurement of ventricular APs has the dual advantages of (i) control of stimulation rate and (ii) direct measurement of membrane potential. The same recording conditions and stimulation rate (1 Hz) were used here for female Mecp2Null/+ myocytes as in our recent report that included AP data from myocytes from Mecp2Null/Y male animals from the same RTT model. It is instructive, therefore, to compare the differences in AP parameters between WT females observed in this study with those seen in males under identical experimental conditions (Cheng et al., 2022). In ventricular myocytes from 8–10 week Mecp2Null/Y animals, AP durations at 75% and 90% of complete repolarization were increased in Mecp2Null/Y compared to WT myocytes (with APD90 prolonged by ~50 ms); AP triangulation (APD90-APD25) was also increased by ~50 ms in Mecp2Null/Y myocytes (Cheng et al., 2022). The present study shows that these parameters also differ between WT and Mecp2Null/+ myocytes, with both APD90 and AP triangulation greater by ~39 ms in Mecp2Null/+ than in WT control myocytes. The modest depolarization in resting potential and decrease in current required to elicit APs (Table 2) from Mecp2Null/+ myocytes were also seen in myocytes from Mecp2Null/Y compared to those from WT controls (Cheng et al., 2022). Two differences from prior data on male myocytes were seen, however. First, AP upstroke velocity ($V_{max}$) was significantly reduced in Mecp2Null/+ compared to WT control myocytes (Table 2); in Mecp2Null/Y myocytes there was a tendency toward a reduction in $V_{max}$ but this was not statistically significant (Cheng et al., 2022). Second, AP instability (BVR) was significantly greater in male RTT than control myocytes; here there was a trend toward an increase in BVR, but this did not reach statistical significance (Figure 3c,d). The overall similarity between AP observations from male and female animals is consistent with common underlying changes in the model. The differences between females and males in the extent of APD changes and in the

T A B L E 2  Ventricular action potential (AP) parameters

| Parameter | WT | Mecp2Null/+ |
|-----------|----|-------------|
| Resting potential (mV) | −72.8 ± 0.8 | −70.0 ± 1.0 * |
| Overshoot (mV) | 43.6 ± 1.9 | 40.7 ± 3.1 |
| Amplitude (mV) | 116.4 ± 2.0 | 110.7 ± 3.6 |
| $V_{max}$ (V s⁻¹) | 150.1 ± 6.4 | 122.5 ± 6.7** |
| APD10 (ms) | 0.4 ± 0.0 | 0.6 ± 0.1 |
| APD25 (ms) | 1.6 ± 0.2 | 2.2 ± 0.5 |
| APD50 (ms) | 5.2 ± 0.7 | 6.8 ± 1.4 |
| APD75 (ms) | 65.5 ± 7.5 | 94.0 ± 10.4* |
| APD90 (ms) | 112.6 ± 9.4 | 151.8 ± 12.2* |
| APD90 - APD25 (ms) | 111.0 ± 9.3 | 149.7 ± 12.3* |
| Threshold stimulus (pA) | 707.3 ± 45.6 | 576.4 ± 41.1* |

Note: Mean ± SEM AP parameter values for APs recorded from isolated ventricular myocytes. 24 cells from 10 WT mice and 18 cells from 12 Mecp2Null/+ mice. APs were elicited by 3 ms duration depolarizing current pulses applied at a stimulation frequency of 1 Hz. Threshold values are included in the table. * denotes $p < 0.05$ and ** denotes $p < 0.01$ from unpaired $t$-test assuming equal or unequal variances, as appropriate.

FIGURE 4  Effect of GS-6615 on ventricular APs. (a) Example APs in control and 10 µM GS-6615 for a Mecp2Null/+ myocyte (AP stimulation frequency was 1 Hz). (b) Plots showing mean % change in AP duration with GS-6615 at 90% repolarization (APD90) for each of WT and Mecp2Null/+ conditions. Plots show data from 8 myocytes from 5 WT mice and 8 myocytes from 6 Mecp2Null/+ mice. There was no significant difference between the magnitude of response between WT and Mecp2Null/+; GS-6615 abbreviated APD90.
McCauley et al., 2011) and a reduced fast \( I_{\text{Na}} \) magnitude. Male Mecp2\(^{\text{Null/Y}}\) myocytes exhibit increased \( I_{\text{Na,Late}} \) (Cheng et al., 2022; McCauley et al., 2011) and a reduced fast \( I_{\text{Na}} \) magnitude (Cheng et al., 2022) compared to myocytes from WT controls. Such changes are commensurate both with a reduction in AP upstroke velocity and APD prolongation. The augmented \( I_{\text{Na,Late}} \) in male RTT myocytes was not accounted for by altered “window” current for \( I_{\text{Na}} \) as that was found to be smaller rather than augmented in Mecp2\(^{\text{Null/Y}}\) myocytes (Cheng et al., 2022). As highlighted in considering the male AP data previously (Cheng et al., 2022), the modest depolarization in RMP and reduction in current required to elicit APs are suggestive of additional changes to (a) conductance(s) at the RMP, the underlying basis for which is not known at the present time (Cheng et al., 2022).

There was a lack of observable correlation between QT\(_{\text{c}}\) prolongation and apnoea duration in Mecp2\(^{\text{Null/+}}\) animals (Figure 2), which is inconsistent with repolarization delay being a direct consequence of breathing abnormalities (this contrasts with obstructive sleep apnoea which can lead to QT\(_{\text{c}}\) prolongation [e.g., Walker et al., 2020; Sillanmäki et al., 2022]). This is also the case for male mice from this model (Cheng et al., 2022). McCauley et al showed that similar changes to repolarization and \( I_{\text{Na,Late}} \) were seen with both global Mecp2 knockout and with knockout selective to the nervous system (McCauley et al., 2011). Thus, cardiac changes in this model are secondary to those in the nervous system, but once established can persist in isolated myocytes that are no longer under direct nervous system control. Data from the present study do not address the mechanism(s) underlying these changes. However, Herrera et al have shed additional light on arrhythmogenesis in the model (Herrera et al., 2016). They observed that death in this model was associated with spontaneous arrhythmias and conduction block; atropine mitigated these effects, suggestive of parasympathetic over-activity. Mice were generated with cholinergic neurone specific Mecp2 deletion and these exhibited QT\(_{\text{c}}\) prolongation and increased susceptibility to induced arrhythmias (Herrera et al., 2016). Restoration of Mecp2 in cholinergic neurones rescued the cardiac phenotype (Herrera et al., 2016). Herrera et al discussed the possibility that changes in parasympathetic tone may be related to seizures (Herrera et al., 2016). Direct comparison with the present study (in which mean heart rate was not decreased) is difficult, however, because ECGs were monitored by telemetry (Herrera et al., 2016), whereas in the present study ECGs were measured from anesthetized animals. Additionally, the mechanism(s) that link altered nervous system activity to changes in cardiac electrophysiology that can persist in the absence of acute neural modulation remain(s) to be elucidated.

### 4.2 Consideration of the basis for altered repolarization in RTT mice

The need to keep females for at least 10 months (McCauley et al., 2011) to 12 or more months (this study) for repolarization abnormalities to be evident poses logistical and cost problems for the detailed interrogation of cellular electrophysiology in this model. With the exception of the AP recordings in the present study, the available cellular electrophysiology data in this RTT model come from experiments on myocytes from younger males (Cheng et al., 2022; McCauley et al., 2011). Male Mecp2\(^{\text{Null/Y}}\) myocytes exhibit increased \( I_{\text{Na,Late}} \) (Cheng et al., 2022; McCauley et al., 2011) and a reduced fast \( I_{\text{Na}} \) magnitude (Cheng et al., 2022) compared to myocytes from WT controls. Such changes are commensurate both with a reduction in AP upstroke velocity and APD prolongation. The augmented \( I_{\text{Na,Late}} \) in male RTT myocytes was not accounted for by altered “window” current for \( I_{\text{Na}} \) as that was found to be smaller rather than augmented in Mecp2\(^{\text{Null/Y}}\) myocytes (Cheng et al., 2022). As highlighted in considering the male AP data previously (Cheng et al., 2022), the modest depolarization in RMP and reduction in current required to elicit APs are suggestive of additional changes to (a) conductance(s) at the RMP, the underlying basis for which is not known at the present time (Cheng et al., 2022).

There was a lack of observable correlation between QT\(_{\text{c}}\) prolongation and apnoea duration in Mecp2\(^{\text{Null/+}}\) animals (Figure 2), which is inconsistent with repolarization delay being a direct consequence of breathing abnormalities (this contrasts with obstructive sleep apnoea which can lead to QT\(_{\text{c}}\) prolongation [e.g., Walker et al., 2020; Sillanmäki et al., 2022]). This is also the case for male mice from this model (Cheng et al., 2022). McCauley et al showed that similar changes to repolarization and \( I_{\text{Na,Late}} \) were seen with both global Mecp2 knockout and with knockout selective to the nervous system (McCauley et al., 2011). Thus, cardiac changes in this model are secondary to those in the nervous system, but once established can persist in isolated myocytes that are no longer under direct nervous system control. Data from the present study do not address the mechanism(s) underlying these changes. However, Herrera et al have shed additional light on arrhythmogenesis in the model (Herrera et al., 2016). They observed that death in this model was associated with spontaneous arrhythmias and conduction block; atropine mitigated these effects, suggestive of parasympathetic over-activity. Mice were generated with cholinergic neurone specific Mecp2 deletion and these exhibited QT\(_{\text{c}}\) prolongation and increased susceptibility to induced arrhythmias (Herrera et al., 2016). Restoration of Mecp2 in cholinergic neurones rescued the cardiac phenotype (Herrera et al., 2016). Herrera et al discussed the possibility that changes in parasympathetic tone may be related to seizures (Herrera et al., 2016). Direct comparison with the present study (in which mean heart rate was not decreased) is difficult, however, because ECGs were monitored by telemetry (Herrera et al., 2016), whereas in the present study ECGs were measured from anesthetized animals. Additionally, the mechanism(s) that link altered nervous system activity to changes in cardiac electrophysiology that can persist in the absence of acute neural modulation remain(s) to be elucidated.

### 4.3 Clinical relevance

The direct measurement of ventricular AP prolongation in female Mecp2\(^{\text{Null/+}}\) myocytes seen in this study together with prior data from Mecp2\(^{\text{Null/Y}}\) male myocytes (Cheng et al., 2022; McCauley et al., 2011) provide an explanation for QT\(_{\text{c}}\) prolongation observed in RTT patients. Acute administration of the \( \beta \) adrenoceptor inhibitor propranolol to both WT and male Mecp2\(^{\text{Null/Y}}\) mice failed to reduce QT\(_{\text{c}}\) interval and it failed also to protect Mecp2\(^{\text{Null/Y}}\) mice subject from arrhythmias provoked by programmed electrical stimulation (McCauley et al., 2011). In contrast, the anticonvulsant agent, phenytoin, reduced QT\(_{\text{c}}\) interval and ventricular tachycardia in RTT mice and also reduced \( I_{\text{Na,Late}} \) in Mecp2\(^{\text{Null/Y}}\) myocytes (McCauley et al., 2011). In a follow-on study, chronic administration of propranolol had no effect on QT\(_{\text{c}}\) interval or arrhythmia susceptibility both in young Mecp2\(^{\text{Null/Y}}\) males and 10-month-old Mecp2\(^{\text{Null/+}}\) females (Herrera et al., 2015). By contrast chronic phenytoin corrected the prolonged QT\(_{\text{c}}\) interval and decreased ventricular arrhythmia susceptibility in Mecp2\(^{\text{Null/Y}}\) males and 10-month-old Mecp2\(^{\text{Null/+}}\) females (Herrera et al., 2015). Unfortunately, however, chronic phenytoin also worsened breathing patterns in RTT mice (Herrera et al., 2015). Retrospective analysis in the same study of data from the RTT Natural History Study
identified 68 individuals with ECG measurements before drug treatment and who had received either propranolol or anti-epileptic drugs with Na⁺ channel-blocking properties. Numbers were insufficient to evaluate effects of β-blocking drugs on the QTc interval. However, of 64 individuals with multiple ECGs, 10 had a prolonged QTc interval (>450 ms) prior to anti-epileptic drug treatment; 7 of 10 had QTc values below the 450 ms threshold for QTc prolongation after anti-epileptic drug treatment (Herrera et al., 2015). These observations were suggestive that drugs with Na⁺ channel-blocking activity are likely to be beneficial in RTT patients with QTc prolongation, though none of the anti-epileptic drugs other than phenytoin were tested in the RTT mouse model.

We have recently shown that acute application of GS-6615 (eleclazine) and ranolazine (which is structurally related to lignocaine [Hancox & Doggrell, 2010]) reduce INa,Late from male Mecp2Null/Y myocytes; GS-6615 also reduced APD90 and AP triangulation in Mecp2Null/Y myocytes (Cheng et al., 2022). The data shown in Figure 4 of this study indicate that GS-6615, at the same concentration as applied previously to male myocytes, exhibits the ability to shorten APD90 and reduces triangulation in female Mecp2Null/+ ventricular myocytes. This raises the possibility that GS-6615 could have potential value for treatment of prolonged QTc intervals in RTT patients. GS-6615 is an investigational drug; however, it would be valuable to identify further agents that could be used to abbreviate repolarization. Ranolazine produces a prolongation of murine ventricular APs (Cheng et al., 2022; Lowe et al., 2012) and so it is difficult to establish its potential utility against QTc prolongation in RTT using a murine model. European Society of Cardiology (ESC) guidelines for the treatment of ventricular arrhythmias and prevention of sudden death note that because both mexiletine and flecainide (class IB and IC antiarrhythmic drugs, respectively) can inhibit both fast INa and INa,Late (Hézsco et al., 2021; Nagatomo et al., 2000); they may have utility in the treatment of congenital long QT variant 3 (LQT3) (Priori et al., 2015). It would be valuable to establish effects of both drugs on QTc interval and AP characteristics of this RTT model. Similarly, systematic investigation of Na⁺ channel blocking anti-epileptic drugs other than phenytoin (Herrera et al., 2015) on repolarization in this model would be useful, particularly given their existing use to treat seizures in RTT.

5 | CONCLUSIONS

The results of this study are consistent with earlier data showing QTc interval prolongation in this model of RTT (Cheng et al., 2022; Herrera et al., 2015; Herrera et al., 2016; McCauley et al., 2011). The direct measurement of APs from Mecp2Null/+ ventricular myocytes has also demonstrated delayed repolarization and showed results that are similar, albeit not identical, to those from younger Mecp2Null/Y male myocytes (Cheng et al., 2022). The beneficial effects of GS-6615 in reducing APD90 and AP triangulation are similar to those reported recently in myocytes from male RTT mice and support the further preclinical investigation of this as a potential antiarrhythmic strategy in RTT. Additional investigation of existing (class IB and IC) anti-arrhythmic and Na⁺ blocking anticonvulsant agents on repolarization in the model would also be desirable. Finally, further mechanistic work is required to determine the signaling pathway(s) that mediate changes in ventricular repolarization secondary to nervous system changes in the model. The earlier development of repolarization delay in males may make such an investigation more easily approachable, at least in the first instance, using Mecp2Null/+ males from this model.

AUTHOR CONTRIBUTIONS
Conceptualization: JCH and APA; Funding acquisition: JCH, APA, AFJ; Supervision: JCH, APA, AFJ; Experimental Design: JCH, APA, AFJ, HC; Data acquisition and analysis: HC and IC; Manuscript drafting: JCH, APA, HC, AFJ, IC.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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