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KV7 channel activation inhibits human and murine myometrium contractility and delays delivery in a mouse model of preterm birth

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Abstract

Despite spontaneous preterm birth (PTB, delivery < 37 weeks' gestation) being a major contributor to perinatal morbidity and mortality worldwide, there is a paucity of treatments for treating preterm labor. We hypothesized that uterine smooth muscle (myometrium) Kv7 channels could be a therapeutic target for preventing preterm labor. Building on our previous work, we confirmed that K_V7 channels, proteins encoded by KCNQ2-5 genes and associated accessory KCNE1-5, are expressed and functional in pregnant human myometrium prior to and after the onset of labor. $K_V7.2$-5 activators (retigabine and ML213) effectively inhibit pregnant human and mouse myometrium contractions *in vitro*, and *in vivo* significantly delayed PTB in a non-infection preterm labor mouse model. This supports our hypothesis that augmenting K_V7 activity represents a viable mechanism to suppress uterine contractility and delay of PTB. Addressing the current drive to repurpose existing drugs for treating PTB, we propose this as a new avenue of clinical exploration.

One Sentence Summary: K_V7 channel activators inhibit human uterine contractions and delay delivery in preterm mice, identifying a new target for preterm labor prevention.

INTRODUCTION

Preterm birth (< 37 weeks' gestation) affects approximately 15 million pregnancies worldwide every year and results in unacceptable levels of neonatal death and morbidity [1]. The resultant social, economic, and healthcare burden for surviving babies is considerable [2].

A major cause of preterm birth is the spontaneous onset of preterm labor, typified by inappropriate early onset of uterine contractions and premature cervical shortening. Due to an incomplete understanding of the mechanisms driving preterm labor and the lack of effective prophylactic interventions, many women still present in threatened preterm labor and require intervention [3–5]. Most will receive, when not contraindicated due to the presence of overt infection, tocolytic therapy to delay labor and corticosteroids to improve neonatal survival and morbidity [5–7]. However, there are few tocolytic therapies available e.g., nifedipine and atosiban (a mixed oxytocin/vasopressin receptor antagonist), but the effectiveness of these agents in terms of delaying birth and impact on neonatal outcomes is limited [5,8]. As a result, there is a clinical need to identify new uterine smooth muscle (myometrium) targets to develop more effective tocolytic agents.

Plasma membrane potassium (K^+) channels are particularly apposite for targeting, as K^+ channel expression and function underpin uterine quiescence in pregnancy and contribute to the generation of rhythmic contractions in labor [9]. Gene expression of several K_V channel sub-types, and related KCNE ancillary units, have been identified in human and rodent uterine myocytes including K_V7 and K_V11 [9,10]. We have demonstrated that myometrial K_V7 channels encoded by KCNQ1-5 genes are expressed robustly throughout mouse gestation. In addition, activators of $K_V7.2$ to $K_V7.5$, such as retigabine,

inhibited myometrial contractions in mouse and human myometrium [11,12]. Considering this functional impact, we hypothesize that K_V7 channels have potential as a novel therapeutic target to delay preterm birth.

Consequently, the aim of this study was to establish which K_V7 channels are present in human myometrium from pregnant women after the onset of labor, and to use a mouse model of preterm birth to provide 'proof of principle' data that targeting K_V7 in vivo can delay preterm delivery.

RESULTS

K_V7 channel expression in late pregnant human myometrium

For K_V7 channel activators to be considered as a potential treatment of preterm labor, KCNQ and KCNE mRNA and associated proteins must be expressed and active in pregnant human myometrium and remain in tissues after labor onset. Using myometrium from women at the time of elective cesarean section (term not in labor: TNL) (Fig. 1A, n = 51), mRNA expression of all KCNQ isoforms except KCNQ2 were detected. Similar to our previous mouse studies, KCNQ4 was the most highly expressed mRNA compared to KCNQ1,3,5 ($p < 0.05$). The ranked order was KCNQ4 > > KCNQ3 > KCNQ1 > KCNQ5; Kruskal-Wallis tests confirmed significant differences between the individual KCNQ isoforms (Fig. 1A). Whilst there was a relatively wide spread of KCNQ4 expression values, individual profiles of KCNQ expression were remarkably similar, with KCNQ4 being the predominant isoform in 42 out of 51 tissues (and second highest in the remaining 9 samples).

We also assessed KCNQ expression in myometrium from women at term in active labor (TAL) (Fig. 1B; n = 46). KCNQ profiles in TAL were similar to those found in TNL (KCNQ4 > KCNQ3 > KCNQ1 > KCNQ5), with KCNQ4 remaining the most predominant transcript, providing evidence that at the mRNA level, K_V7 channel components are still detectable, and hence available for pharmacological targeting, in labor.

Accessory subunits KCNE1-5 were analyzed in the same TNL and TAL samples (Fig. 1C, D). All KCNE genes were detected in both TNL and TAL samples, with KCNE4 being the most highly expressed isoform in both groups. The expression profile of KCNE genes was the same for both groups (TNL and TAL, KCNE4 > KCNE3 > KCNE5 = KCNE1 > KCNE2). KCNE2 expression was particularly low compared with other KCNE isoforms. KCNE3 showed a slightly lower expression in TNL [median, quartile range KCNE3: TNL: 737.3 (286.0, 1166) *vs* TAL: 915.0 (502.5, 2708).

In a separate experiment, using mRNA from a small set of myometrial tissues (provided by author D Slater), we compared expression from women who had Cesarean sections from preterm deliveries not in labor (PNL, n = 4) and after spontaneous onset of preterm labor (PAL, n = 5, < 37 weeks of gestation pregnancy), with TNL (n = 6) and TAL (n = 6) controls. We focused on the most highly expressed KCNQ and KCNE genes identified in Fig. 1. Both KCNQ4 and KCNE4 mRNA (Fig. 2A and 2B respectively) were

detected in preterm tissues. Expression of KCNQ4 was high in PAL as it was in TNL and TAL samples. KCNQ4 expression in the PNL group was lower than that in TNL women ($P < 0.05$). A similar profile of mRNA expression was seen for KCNE4 and there were no significant differences between the groups.

Overall, these data shown in human myometrium that KCNQ4 tissue expression remains consistent across term and preterm (in active labour) gestations.

Kv7.1-5 and KCNE1-5 proteins in human myometrium

Following the successful detection of KCNQ1, 3–5 and KCNE1-5 transcripts in human myometrium, we determined whether transcripts were associated with protein expression in human myometrium using Western blot and commercially available antibodies Kv7.1-5/KCNE1-5 proteins [12–19]. We were able to detect Kv7.1, Kv7.3, Kv7.4, KCNE2, KCNE3 and KCNE4 proteins. Kv7.2, Kv7.5, KCNE1 and KCNE5 were not detected (Fig. 3A). Of the two KCNE2 antibodies, only one antibody (APC-054, Alomone; epitope – 88–107 aa, rat origin) detected a faint protein band (Fig. 3A). APC-054 appears to detect the fully glycosylated protein, whereas the second antibody (sc-25703, Santa Cruz, epitope – 1–70 aa, human origin) detects the semi-mature monoglycosylated protein. Focusing on $K_V7.4$, we quantified expression in whole tissue lysates from human myometrium in TNL and TAL samples. Figure 3B demonstrates that protein expression was similar in both groups.

Immunohistochemistry provided further confirmation of the presence and localization of $K_V7.1$, $K_V7.3$, $K_V7.4$, KCNE3 and KCNE4 proteins in pregnant human myometrium smooth muscle (Fig. 4). All were expressed in smooth muscle cells and widely distributed across cells. Semi-quantification of the staining indicated that $K_V7.3$ was slightly increased in TAL v TNL samples and $K_V7.4$ reduced (Fig. 4B and C). Both proteins were present in laboring samples suggesting functional availability.

Pharmacological evidence for functional K7 channels in human myometrium

Evidence for functional $K_V7.2$-5 channels was gained through a series of *in vitro* protocols which assessed the impact of pharmacological K_V7 activators and inhibitors on TNL human myometrium contractility (Fig. 5).

Application of the $K_V7.2$-5 activator retigabine (10 μM and 20 μM) to spontaneously contracting pregnant human myometrium (TNL) resulted in a reduction in mean integral tension (MIT) compared to vehicle time controls (dimethylsulfoxide, DMSO; Fig. 5A) providing evidence of functional $K_V7.2$-5 channels in these tissues. ML213, a $K_V7.2/K_V7.4$ and $K_V7.5$ activator caused a reduction in MIT at doses 5, 10 and 20 μM, indicating it is an effective uterine relaxant (Fig. 5B).

There was no significant effect of chromanol 293B (0.5–20 μM), an inhibitor of K_V7.1/KCNE1, on spontaneously contracting myometrium (Fig. 5C) indicating a limited contribution from this channel in human myometrium consistent with our previous mouse myometrium data [11].

In contrast, the pan Kv7 blocker XE991, at 10 and 20 μM had no significant effect on mean integral tension but significantly enhanced contractile frequency (Fig. 5D-E) and decreased contraction amplitude (Fig. 5F) compared to vehicle control in TNL pregnant human myometrium. This is consistent with XE991 causing a small depolarization of the resting membrane potential due to inhibition of K_V7 channels.

These data indicate that channels formed by K_V7.2-7.5 are the major regulators of contractility in term pregnant human myometrium, and that modulators reported to have greater specificity for K_V7.4 have greatest effect. K_V7.1 has a limited functional contribution to contraction and an explanation for this may be that K_V7.1 is forming channels with KCNE4 (which is the most abundant isoform) in myometrium; this combination is reported to suppress K_V7.1 currents in model systems [20,21].

K7 channels in myometrium from a mouse model of preterm birth

We have previously demonstrated the presence of functional of K_V7 channels in non-pregnant and pregnant mouse myometrium (day 6–7 and term) and in myometrium from mice injected with lipopolysaccharide (day 15, preterm gestation, but not in labor) [11]. However, there is limited knowledge of expression or function of myometrial K_V7 channels related to preterm labor. To rectify this, *Kcnq* and *Kcne* profiles were assessed in myometrium harvested from a preterm mouse model (RU486 treated on day 15 of gestation to induce progesterone withdrawal and preterm labor and birth on ~ day 16) and vehicle (DMSO) treated control mice (Suppl. Figure 1). Myometrium was obtained from mice when they showed signs of labor (PTAL) and from control non-laboring pregnant mice on day 16 (14 hours after DMSO treatment, PTNL) for experiments shown in Figs. 6 and 7

Using myometrium harvested on day 16 of pregnancy, transcripts for all *Kcnq* genes were detected in both PTNL and PTAL myometrium (Fig. 6A and B). *Kcnq1*, *Kcnq4* and *Kcnq5* were highly expressed.

A separate comparison individual *Kcnq* genes expression data from PTNL versus PTAL myometrium revealed decreases in *Kcnq4* (median [IQR]; PTNL, 139.5 [114.6, 259.7]; PTAL, 83.6 [56.7, 106.2], p = 0.02) and *Kcnq5* (PTNL, 630.8 [443.3, 1025.0]; PTAL, 113.2 [104.6, 351.1], p = 0.01) mRNA expression after the onset of preterm labor. Although *Kcnq1* showed a t reduction in PTAL, this did not reach significance (p = 0.065). No differences were observed for *Kcnq2* or *Kcnq3*.

All *Kcne* genes were detected in PTNL (Fig. 6C) and PTAL (Fig. 6D) samples with *Kcne4* being the most highly abundant in both groups (p = 0.0001 for both).

Comparison of *Kcne* gene expression data from PTNL versus PTAL myometrium showed significant reductions in *Kcne2* (median [IQR]; PTNL, 18040 [16183, 226228]; PTAL, 319.3 [231.4, 471.4]; p = 0.002) and *Kcne1* expression (PTNL, 27.7 [13.1, 35.8]; PTAL, 5.4 [2.8, 8.6]; p = 0.02).

Contribution of K_V7 channels to regulating ex vivo myometrium contractile activity in a mouse model of preterm birth

After characterizing the expression of *Kcnq* and *Kcne* genes, the functional expression of these channels was assessed using two K_V7 activators, retigabine and ML213. Figure 7 shows baseline spontaneous contractility *in vitro* in PTNL and PTL mice (n = 6 for each concentration). Application of retigabine to spontaneously contracting myometrium from PTAL mice significantly decreased MIT by more than 60% at all concentrations tested (mean ± SEM; DMSO, 79.2 ± 4.7%; retigabine 5 μM, 32.7 ± 4.5%; 10 μM, 28 ± 7.4%; 20 μM, 32.6 ± 8%; $p < 0.05$ for all as indicated by Dunn's multiple comparison post hoc test performed after Kruskal-Wallis test; Fig. 7B). In PTNL samples, significant decreases in MIT were only observed with 10 and 20 μM (DMSO, 91.2 ± 2.7%; retigabine 10 μM, 41.4 ± 10.6%; 20 μM, 16.9 ± 6.1%; Fig. 7A). Retigabine caused complete abolition of contractions in 45% and 50% of PTNL and PTAL samples, respectively.

Similar to retigabine, ML213 inhibited contractile activity of PTAL myometrium at all concentrations (DMSO: 75.8 ± 4.5%; ML213 5 μM, 11.9 ± 3.3%; 10 μM: 10.4 ± 2.8%; 20 μM: 11.4 ± 5%; $p < 0.05$ for all; Fig. 7D). In PTNL myometrium, a significant relaxatory effect was only observed with 10 and 20 μM (DMSO: 94.5 ± 2.1%; ML213 10 μM: 11.9 ± 2.1%; 20 μM: 11.9 ± 3.6%; $p < 0.05$ for both; Fig. 7C). The effect of retigabine and ML213 at 20 μM was not different between PTNL and PTAL ($p < 0.05$).

In vivo measurement of intrauterine pressure (IUP)

With confirmation that mRNA expression for all K_V7 channel components could be detected in myometrium from PTAL and the pharmacological evidence that the channels were functional in myometrium assessed *in vitro*, the next step was to determine the effect of K_V7 channel activators on myometrial contractility in vivo, assessed with intrauterine pressure telemetry measurements and timing of birth (delivery of first pup as recorded on CCTV) in RU486 treated mice *in vivo*.

Acute gavage of two doses of retigabine, 90 minutes apart starting at 14 hours post RU486 demonstrated little impact on timing of delivery and hourly averaged IUP measurements in RU486 treated mice compared to administration of retigabine vehicle (0.5% hydroxymethylcellulose) (Suppl Fig. 2.). However, since time of delivery post RU486 showed some variation that potentially could have masked effects of retigabine, data were also analyzed to account for time of delivery (IUP displayed relative to time of delivery of first pup labor; t = 0) and revealed a temporal decrease in IUP following the gavage treatment period. This suggested that short term exposure to retigabine altered uterine contractility and IUP in vivo, but not sufficiently to delay birth.

Repeat dosing of with Kv7 activators delays birth in mouse model of preterm birth

To address the above observations, a repeat dosing regimen comprising of up to six gavage treatments (retigabine, ML213, or vehicle) was given every 60 minutes starting ~ 13 hours post RU486 injection was employed.

Retigabine significantly delayed preterm birth, by increasing the median [IQR] time to delivery of first pup to 21.5 [20.5, 24.0] hours v 15.5 [15.1, 16.5] ($p < 0.001$) hours for RU486 vehicle treated animals; this equated to an average of 39% (36 to 59%) delay in time to delivery (Fig. 8). This is physiologically relevant if compared to the 1 hour it takes a mouse to deliver all pups and the ~ 16 hours it takes from RU486 administration to induce preterm delivery.

ML213 also delayed delivery (18.0 [16.5,20.1], $p < 0.01$) by ~ 3 hours (Fig. 8); this was slightly less effective at delaying delivery *in vivo* compared to the tocolytic effect displayed *in vitro*. However, this was anticipated as it is reported the drug had poor metabolic stability following incubation with rat or human liver microsomes and was predicted to be rapidly cleared after systemic exposure [22].

Fetal viability and fetal ductus arteriosus function

We were unable to assess the impact on neonatal outcomes as in this PTB model, premature neonates are either born without signs of life/cannibalized by the mother due to their prematurity. This occurred even when gestation was prolonged by K_V7 activator treatment.

However, in a separate experimental protocol, we assessed the impact of treatment with K_V7 channel activators on *ex vivo* murine fetal ductus arteriosus (DA) contractility. This is an important consideration, as agents that constrict the ductus *in utero* would impact on fetal perfusion and oxygen delivery [23]. Suppl. Figure 3 shows that under deoxygenated organ bath conditions that mimic *in utero* oxygen status, fetal ductus arteriosus segments exposed to ML213 had no significant change in lumen diameter (10^{-8} to 10^{-4} M). DAs exposed to retigabine were similarly unaffected over the pharmacologic range (10^{-8} to 10^{-6} M) although at the highest concentration applied (a concentration unlikely to be used in *in vivo*), a significant ($p = 0.0002$) constriction was observed at the highest concentration (10^{-4}M) compared to baseline diameter. Because of this, there was a significant ($p = 0.004$) difference in E_{max}-values between these two drugs.

Discussion

This study provides a clear rationale for pursuing K_V7 channels as a potential target for tocolytic therapy and the prevention of preterm labor. Two structurally different activators of $K_V7.2$-7.5 delayed early birth in a recognized mouse model of preterm birth. This important finding is supported by comprehensive molecular data and functional data from human pregnant myometrium and a preterm birth mouse

model. These novel findings build upon our previous work which described the physiological function of K_V7 channels in the non-pregnant and pregnant mouse [11,12].

K7 channels as a plausible target for tocolytic targeting

Ion channels have long been viewed as important targets for therapeutic intervention due to their fundamental role in cellular physiology and human disease. Indeed, an estimated 10–20% of small molecular targets are ion channels [24,25]. K_V channels represent a large gene superfamily that has been studied intensively in smooth muscles, including the myometrium, in terms of function [9,26–33] and in relation to K_V7 channels this has led to a number of compounds being considered for treatment of epilepsy, hypertension, stroke, lung disease, overactive bladder, and neuropathic pain [27,34–40].

However, as far as we are aware, apart from our previous work [11,12,41] and information in RNAseq datasets (e.g., [42]) here is a paucity of knowledge concerning the functional role of K_V7 channels in the uterus. In general, K channel activity contributes to suppression of uterine contractility in pregnancy through promoting a low resting potential, modifying action potentials (and hence contraction amplitude) through repolarization, and hence limiting calcium entry and contractility [41]. However, as gestation nears term and labor (in both rodents and humans), there is a transition to greater myometrial cell excitability due to a gradual increase of the resting in resting membrane potential, most likely due to change in K^+ channel populations and activity [43,44]. For a K^+ channel to be useful for inhibiting preterm labor, its expression must be maintained in labor. $K_V7.11$ channels, for example, are expressed in late pregnancy, but their ability to influence repolarization is suppressed at labor onset through a variety of pathways [31,41,45,46]. Our previous pilot work in human myometrium suggested that $K_V7.2$-5 were expressed in human myometrium in late pregnancy, but here we confirm through molecular profiling that KCNQ and KCNE mRNA are expressed both at the end of pregnancy and after the onset of labor. Of the different isoforms found, KCNQ4 and KCNE4 mRNAs were the most highly expressed transcripts in term labor and were also expressed in myometrium from women in preterm labor. This was reflected in $K_V7.4$ and KCNE4 protein expression in myometrium from women at term and in labor. This identifies $K_V7.4$ as a potential K_V7 channel target in human myometrium [23]. Whilst transcripts for KCNQ5 were found and maintained in labour, we did not detect $K_V.5$ protein. This does not fully rule out this as a target but more work on understanding protein expression is needed. KCNQ3 whilst expressed, is a key component of the neuronal M channel, so a less favorable target [47] along with Kv7.1 which is a cardiac associated K_V channel.

Pharmacological approaches using retigabine and ML213 provide clear evidence that activation of $K_V7.2$-5 channels can suppress contractions in human myometrium *in vitro*, an observation similar to that in term pregnant mice [11]. ML213 is reported as a $K_V7.4$, $K_V7.2$ and $K_V7.5$ channel activator, but given

that K_V7.2 was expressed at low levels in myometrium, these findings support the notion that K_V7.4 channels is a plausible functional human myometrium target.

The K_V7 channel inhibitor, XE991 also altered human uterine contractility *in vitro* providing additional evidence for functional K_V7. In contrast, Kv7.1, whilst detectable in human myometrium as KCNQ1 transcripts, did not contribute functionally to myometrial contractility (i.e., not affected by chromanol application).

K_V7.2-5 channel activation prevented preterm labour

Two options for inducing PTB were considered, a lipopolysaccharide induced PTB (inflammation driven) and RU486 (progesterone/glucocorticoid block). The latter was chosen because it may be more reflective of idiopathic PTB, a clinical scenario that would benefit from an effective tocolytic. RU486 induction of PTB is rapid in the mouse due to dependence on progesterone for maintenance of pregnancy, so any delay in labor in this scenario provides a strong proof of principle effect.

The effectiveness of Kv7.2-5 activation to prevent preterm labor was demonstrated in a mouse model where K_V7 channels components, particularly *kcnq4 and kcnq5*, were highly expressed in myometrium from preterm mice (prior to and after the onset of labour). Both retigabine and ML213 suppressed contractility in tissue *ex vivo* from preterm mice (not in labor and in labor).

This effect was also confirmed *in vivo* where intrauterine pressure monitoring indicated that retigabine reduced pressure (indication of reduced uterine contractility) in RU486 treated mice in the hours preceding delivery. Whilst a single dose of retigabine was insufficient to delay birth, repeated gavage of retigabine significantly delayed labor and birth of the first pup by six hours compared to controls.

ML213 was also assessed, although we were aware prior to starting experimentation that it would quickly be metabolized by the liver when given orally. With this caveat, as a K_V7.4 (and Kv7.4/5, Kv7.5) targeting agent it showed significant promise as it delayed preterm birth by three hours. Focus on the development of more favorable administration routes of an ML213 drug may be preferable.

In the context of human pregnancy, this delay of preterm birth using KV7.2-5 activators has real potential to translate to prolongation of delivery for > 7 days or more, a delay that could significantly enhance human fetal maturity.

Limitations and future work

In terms of limitations, there are several avenues available for further exploration. There is scope for further interrogation of the composition of the K_V7.4 channels (homo or heterotetramers with other KCNQ isoform such as KCNQ5) [48,49], or KCNE accessory subunits using proximity assays, imaging, patch clamp electrophysiology and transgenic mouse models.

In the current study, we restricted functional studies to myometrium from women in late pregnancy as samples from women in spontaneous preterm labor are rare, but with time these experiments could be collated. The work in mouse models of PTB were designed to circumnavigate this issue and to provide an *in vivo* solution. Uterine contraction mechanisms are similar between species, and tissues exhibited relevant KCNQ and KCNE expression profiles. However, before work can be translated into pregnant women this may necessitate additional studies in another animal model, particularly if more specific $K_V7.4$ activators are to be developed. The side effects on other tissues and smooth muscles [10,50], which also express $K_V7.2$-5 would also have to be monitored, although in the mouse model, retigabine was well tolerated.

There is still a need to assess the impact of K_V7 activators on neonatal outcome. In our study, neonatal wellbeing could not be reviewed due to the gestation-based immaturity of the mice in this model even when gestation was prolonged. However, we considered the impact of retigabine and ML213 *ex vivo* on the murine fetal ductus arteriosus, and whilst retigabine at the highest concentration caused some constriction, this was at concentrations not likely to be achieved *in vivo*. In future studies, we plan to assess compounds in term pregnancy or a modified PTB model (induced later in gestation or milder stimulus) where neonates survive to determine neonatal developmental and behavior outcomes. To support translation of these findings into $K_V7.4$ (or KV7.4/5) based tocolytics, protocols investigating different routes and drug delivery mechanisms may be useful.

In summary, this study identifies $K_V7.2$-5 activators as emerging candidates for tocolytic drug development. These activators can clearly suppress uterine contractility in human tissues and delay preterm birth in mice. There is compelling evidence that $K_V7.4$ and associated accessory subunit KCNE4 are the targets for uterine specific drug development. Many pharmaceutical companies avoid investing in maternity, but with continued increase in PTB and the greatest burden being within the low-risk pregnant population there is a need that should be addressed [5].

Whilst opportunities to explore drug repurposing have been affected by concerns about the long-term effects of retigabine and flupirtine [50,51], release of compounds from existing drug libraries and/or further development [23,34,52] could potentially facilitate research and drug development for more short-term use in this major area of health care. This could be underpinned with more precise uterine targeting using emerging and novel drug delivery systems [53,54].

Materials and Methods

Study design

This was an experimental laboratory study using human myometrial tissue, a mouse (C57BL/6J) model of preterm delivery, and an isolated fetal ductus arteriosus preparation (from CD-1 pregnant mice).

Human myometrium studies

Human myometrium was obtained from 143 women with informed written consent, from women at term (> 37 weeks' gestation) at the time of elective not in labor (TNL) or emergency in labor (TAL) cesarean section, as approved by St Thomas' Hospital Ethics Committee (and subsequently regulated NRES Committee London-Westminster (EC00/137). For molecular and isometric tension measurement all were singleton pregnancies with exclusion criteria of adverse medical and obstetric conditions (including asthma requiring steroids, chronic hypertension requiring medication, lupus/systemic lupus erythematosus, diabetes, and pre-eclampsia) and the use of any medication that had potential to alter myometrial contractility (e.g., anti-hypertensives and inhaled bronchodilators). Biopsies were obtained from the upper edge of the lower segment incision and placed into ice-cold phosphate buffered saline. Tissue was rinsed and dissected on ice into small segments which were either snap frozen and stored at -80°C for RNA and protein analysis; formalin fixed, and wax embedded for immunohistochemical analysis; or kept at 4°C until used for isometric tension recording and cell isolation for patch clamp electrophysiology. Participant and pregnancy outcome data was retrieved from electronic and hand-held patient notes.

cDNA from a smaller number of human myometrium samples were obtained from D Slater we compared expression from women who had cesarean sections from preterm deliveries not in labor (PNL, n = 4) and after spontaneous onset of preterm labor (PAL, n = 5, < 37 weeks of gestation pregnancy), with TNL (n = 6) and TAL (n = 6) controls (Conjoint Health Research Ethics Board REB15-1110).

Mouse model of preterm birth

Acclimatized female C57BL/6J mice (Charles River) were mated and a copulation plug visualized one day post copulation (1 dpc) and subsequently weighed regularly. Mice were housed in constant temperature rooms (21 ± 1°C) with fixed 12-hour light: dark intervals and ad libitum access to standard chow and water. The progesterone receptor/glucocorticoid receptor antagonist RU486 (mifepristone; Merck Life Science, UK) was used to artificially induce preterm birth as previously published [55] (Suppl. Figure 1). On 15 dpc, mice were restrained and a subcutaneous injection containing 150 µg RU486 [dissolved in DMSO (Merck) at a concentration of 1 µg/µl] administered in the nape of the neck. Control mice were injected with 150 µl of DMSO. All injections were administered at the same time of day and mice were continuously monitored by video so that the timing of gestation (defined as time to deliver first pup) could be accurately determined.

For *in vitro* contraction studies, myometrium was harvested in RU486 mice when they showed signs of labor (preterm in labor, PTL) and in parallel with tissue from control non-laboring pregnant mice at the same gestation (preterm not in labor, PTNL).

Measurement of intrauterine pressure using radio telemetry in mouse model of preterm birth

This followed the method developed by Pierce and colleagues [56,57]. C57BL/6J mice were anesthetized with 3% isoflurane/oxygen in an induction chamber at day 8–10 dpc. Once anesthesia had been

achieved, mice were transferred to a warming pad, placed in a supine position and a nose cone was used to deliver maintenance anesthesia (2% isoflurane/oxygen) for the duration of the surgical procedure. The abdomen was shaved and disinfected with a 0.5% (w/v) chlorhexidine gluconate solution (Medlock Medical Ltd., UK) and the work surface was replaced with a sterile cover. The fore- and hind-limbs were secured to the work surface, and 3 μg of buprenorphine (Vetergesic) (Sogeval UK Limited, York, UK) was injected intra-muscularly (I.M.) before a midline incision approximately 3 cm in length was made. The right and left uterine horns were identified, carefully exteriorized and the pups in each horn counted. One uterine horn was chosen for implantation of the pressure radio-telemetry probe, whilst the other horn was covered in saline-soaked gauze to prevent drying. Dumont tweezers were used to hold a small fold of the uterine wall, and a 1 mm incision was made using 3 mm Vanna scissors. Vessel cannulation forceps were used to feed the catheter of the probe into the incision of the uterus. Blunt-end forceps were used to manipulate the path of the catheter until the opening of the catheter was located between two fetal sacs. The catheter was kept in place by applying a drop of Vetbond (3M, USA) to the site of catheter insertion and the uterus was carefully placed back into the abdominal cavity, followed by the probe body, which was placed in the lower portion of the abdominal cavity. The probe was carefully placed so as to not introduce any acute bending or twisting of the catheter that could interfere with pressure measurements. The abdominal muscle layer and skin were each closed with a simple continuous suture pattern using a braided absorbable suture. Anesthesia was withdrawn and mice placed into a recovery chamber held at 28°C overnight with access to softened standard chow. Additional analgesia (3 μg buprenorphine, S.C.) was given to mice the following morning. Mice were then placed in a cage over a 'receiver mat,' which was connected via a matrix to a computer for remote data acquisition. Mice were free to move during the recording period. From day 13–14 dpc, intrauterine pressure (IUP) was recorded continuously until one day post-partum (approximately 19.5 dpc) or until delivery occurred.

Continuous IUP data was averaged over one-minute intervals for all treatment groups using the manufacturers supplied software (Dataquest A.R.T, UK). A baseline period prior to any intervention was used to standardise intrauterine pressure. All data are expressed as a fold change in IUP from baseline. For mice delivering at term, a baseline period of 12 hours prior to delivery was used so that data from all groups was comparable. Every 60 minutes, pressure recordings were averaged to obtain a mean hourly pressure over a 17-hour period. For all groups, labor was defined as time 0 as indicated by the complete delivery of first pup. Some mice displayed negative IUP values in certain hours which were due to movement and occasional blockage of the catheter (DSI, personal communication), therefore for any hourly averages which included less than 75% positive values, negative values were excluded from the analysis. In addition, mice that had a negative average IUP baseline were excluded from data analysis.

In vivo administration of K_V7 activators in preterm birth mouse model

Acute treatment: On 16 dpc, 14 hours after RU486 administration, C57BL/6J mice were administered a single 20 mg/kg retigabine (Merck) or vehicle (10% (v/v) DMSO in 0.5% hydroxymethylcellulose solution) via oral gavage. For retigabine or ML213, 5 mg was first dissolved into 100 μl DMSO to which 900 μl 0.5%

hydroxymethylcellulose was added to achieve a final concentration of 5 mg/ml. A second administration was given 90 minutes later. This was based on pharmacokinetic profiles of retigabine administration to female non-pregnant mice (carried out by Quotient Bioresearch, UK, data not shown) which reported that peak plasma concentrations of retigabine (6930 ng/ml) were achieved approximately 60 minutes after oral gavage. Repeated gavage: retigabine or ML213 (20 mg/kg, P.O.) or vehicle were prepared similarly as described for acute treatment above and were administered at 60-minute intervals starting 13 hours after RU486 injection. Doses were repeated for a maximum of 6 treatments, or until delivery of the first pup had occurred.

Preterm birth mouse model myometrial tissue collection

For murine myometrium, pregnant mice were sacrificed by cervical dislocation and the gravid uterus was carefully removed from the peritoneum. Uterine horns were cut along the axis of placental attachment, and the fetus and placenta were excised. Endometrial tissue was gently removed by brushing with a cotton bud. Tissues were either snap frozen in liquid nitrogen for mRNA extraction or used immediately for isometric tension measurement studies.

Preterm birth mouse model isometric tension recording

This is a modified protocol based on a method published previously [12,58]. In brief, small longitudinal myometrial strips (human: ~7x2x2 mm; mouse: ~ 5x2x2 mm) were mounted and maintained at 37°C in organ baths (oxygenated, 95% O2, 5% CO2) in physiological salt solution (PSS, pH 7.4 in mM: NaCl 119, KCl 4.7, MgSO4 1.17, NaHCO3 25, KH2PO4 1.18, EDTA 0.025, glucose 6 and CaCl2 2.5). Mouse and human myometrial tissues were subjected to stretch to approximately 1.5-fold their slack length (by applying between 29.34 to 38.25 mN respectively). After development of regular spontaneous contractile activity with a stable baseline, a control period of contractile activity was recorded prior to the addition of either KV7 activators [retigabine, ICA-069673 (Santa Cruz) and ML213 (Tocris)] or inhibitors [XE991 and chromanol 293B (Sigma)] (0.5–20 μM for all compounds). Vehicle control (DMSO) was carried out in parallel.

Contractility data were recorded and analyzed using LabChart 6 software (ADInstruments, UK) and mean integral tension (MIT) for an experimental period, addition of Kv7 activator or vehicle, MIT (the sum of the integrals for each contraction divided by the duration of the period assessed) was expressed as a percentage of the preceding control (baseline) period MIT. Contraction frequency and amplitude of contractions were also assessed.

Human and mouse myometrium mRNA extraction and real-time qRT-PCR

Frozen myometrial tissue (~ 100 mg of human or mouse) was homogenized using a Qiagen Tissue Lyser. Total RNA was extracted using the RNeasy mini kit (Qiagen, UK) according to the manufacturer's instructions. Complementary deoxyribonucleic acid (cDNA) was synthesized using an Omniscript RT Kit (Qiagen, UK). Real-time polymerase chain reaction (PCR) was carried out with the use of SYBR Green

chemistry (Bioline) on a RotorGene 6000 (Qiagen, UK) using human primers as reported in [59] and for mouse as listed in Supplementary Table 1. A pre-PCR cycle was run for 10 min at 95°C followed by 35 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 50 s followed by a final extension at 72°C for 15 s. Melt curve analysis was performed to confirm the presence of one single product. Cycle threshold (CT) values were used for analysis, and abundance data were obtained using quantified cDNA to generate a standard curve. All unknowns fell within the dynamic range of the standard curve. Standards were quantified using densitometry, and tenfold serial dilution from 1010 to 101 copies was run in parallel with the samples. Abundance data for the genes of interest were expressed as normalized copy number following normalization using GeNORM (http://medgen.ugent.be/~jvdesomp/genorm), with stably expressed reference genes (glyceraldehyde 3-phosphate dehydrogenase (GADPH). Data for the genes of interest were normalized (GeNorm) to mRNA copy number of a panel of genes (GAPDH, β-actin and β-2 microglobulin). All PCR products were sequenced to confirm identity. For analysis of human myometrium KCNQ4 and KCNE4 mRNA expression comparing preterm and term samples, because of limited material, only the housekeeper GAPDH was used.

Immunohistochemistry studies of pregnant human myometrium

Serial sections of paraffin embedded human myometrial tissues were cut (5 μm) and dewaxed from paraffin-embedded tissue blocks (using a Leica RM 2065 microtome, Leica Biosystems, UK) as previously described [59]. Immunohistochemical staining was performed using the Vector Stain Elite ABC kit (Vector Laboratories, UK). The optimal dilution for each antibody was established (Supplementary Tables 2 & 3). A positive control (human midbrain) was used to verify specificity. A negative control was performed for each test section by incubation with goat IgG. All slides were assessed by the same observer, blinded to labor group. Digital images of 5 randomly selected, high-power (×400 magnification) fields were captured on NIS-Elements F2.20 microscope (Nikon United Kingdom Ltd, UK). Quantifications of KCNQx and KCNEx expression was performed as described previously [60] using the Positive Pixel Algorithm of Aperio ImageScope software. A visual check was also performed to ensure accurate discrimination of immunolabeled regions.

SDS-PAGE and Western Immunoblotting

Protein samples for Western Blot were prepared by homogenisation of human myometrial tissues (TNL samples, n = 4; TAL samples, n = 4) using a Tissue Lyser II (Qiagen, UK) at 25hZ for 3 min in 20 μl lysis buffer/mg of tissue (10 mM of Hepes-KOH [pH 7], 1 mM of dithiothreitol, 1% nonident-P40, and protease-inhibitor cocktail (COMPLETE tablets; Boehringer-Mannheim Biochemicals, UK) and subsequent centrifugation for 1 min at 12470 x g to remove any tissue debris. Mouse brain, heart or aorta tissues were used as a positive control for a detection of variety KV7x/KCNEx proteins and were prepared in the same way as the human myometrium samples. Total protein concentration was calculated using BCA assay (Pierce, UK) and 30 μg of proteins were loaded to each lane of 12% Tris-Glycine precast gels (Generon, UK) and separated using the XCell SureLock™ Mini-Cell system (Invitrogen, UK). Following

electrophoresis, proteins were transferred to Immobilion™-P transfer membrane (Millipore) using the XCell SureLock™ Mini-Cell blotting module wet transfer blotting system. 50mM Tris, 150mM NaCl, 0.2% (v/v) Tween-20, pH 7.4) containing 5% fat free milk powder for 1 hour at room temperature. After the transfer, membranes were blocked in TBS-T buffer (50mM Tris, 150mM NaCl, 0.2% (v/v) Tween-20, pH 7.4) supplemented with 5% milk (Sigma-Aldrich) for 1 hour at room temperature, followed by 3 hours incubation with primary antibody at RT. All KV7x and KCNEx antibodies used in this study are listed in Supplementary Table 4. Monoclonal mouse anti-smooth muscle alpha actin (Sigma, A2547) was used as a smooth muscle protein marker and a loading control. After the first antibody incubation, membranes were washed three times in TBS-T and HRP-conjugated anti-rabbit, anti-goat or anti-mouse IgGs secondary antibody was added (1:10000 dilution, Santa Cruz). The secondary antibody was incubated for 45 minutes at room temperature, followed by three washes in TBS-T buffer. To detect the antibody, chemiluminescent reagent (Invitrogen) was added, and the blots were exposed to X-ray film.

Mouse ductus arteriosus (DA) vessel studies

Animal experiments were conducted in accordance with the National Institutes of Health Animal Care Standards and were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center. Adult female CD-1 mice were bred by timed mating between 0700–1000 hrs with the presence of a vaginal plug indicating the first day of pregnancy (d1). Pregnant females were anesthetized on the morning of d19 (term) via intraperitoneal injection of 0.4mL of 1.25% avertin (2,2,2-tribromoethanol in tert-amyl alcohol, Sigma-Aldrich, St. Louis, MO), followed by isoflurane inhalation (Baxter) for fetal anesthesia, and cervical dislocation of the dam. The anesthetized fetuses were removed by cesarean section. Their thoracic cavities were opened via partial dissection and puncture of the diaphragm, and the pups were then submerged in chilled, deoxygenated (95% N2, 5% CO2) modified Krebs (in mM; 109 NaCl, 4.7 KCl, 2.5 CaCl22H2O, 0.9 MgSO4, 1.0 KH2PO4, 11.1 glucose, 34 NaHCO3 (pH 7.3)) (2). Any pups with signs of respiration were excluded from vessel myography.

DA segments representing at least five different litters were freshly isolated from the d19 fetuses for use in myography studies. Each DA was excised, mounted in custom myography chambers (University of Vermont), and allowed to equilibrate for 40 min at 5 mmHg and 37°C in deoxygenated modified Krebs buffer. The vasoreactivity of each vessel was ascertained using cannulated, pressurized vessel myography as previously described [61–64]. Inverted light microscopes with video capture (IonOptix) were used to continuously record lumen diameter. After the equilibration period, distending pressure was raised by 5 mmHg in 10 min increments to 20 mmHg, followed by two 10-minute exposures to deoxygenated 50 mM KCl with a 20 min wash step of deoxygenated Krebs in between to validate viability and reactivity. After a second 20 min wash step, the chambers were changed from a flow-through system to a 20 mL recirculating volume, and vessels were re-equilibrated for 20 min. At the end of this period, the intraluminal diameter was recorded and used as the baseline value to compare drug-related changes in DA tone. Vessels were exposed to increasing concentrations (10 − 8 to 10 − 4 M) of either retigabine (Axon Medchem; n = 10) or ML213 (Tocris Bioscience; n = 11) in 20 min increments, and changes in lumen diameter after each concentration increase were documented and compared.

Statistics

Formal power calculations determined the samples for KCNQ and KCNE mRNA expression profiles and N numbers for main experiments. For comparison of KCNQ/KCNE genes within pregnant human myometrium tissue, an n of 21 samples per experimental group was calculated to provide 90% power to detect a ratio of five between genes at the group-wise 5% significance level (alpha = 0.001; S.D. = 0.699) correcting for multiple testing using the Sidak-Bonferroni correction. This was based on data generated from previous experiments [12]. To assess changes in KCNQ/KCNE genes in tissues taken from women at term prior to labor (TNL) versus women in labor (TAL), an n of 42 (21 per sample group) was calculated to provide 90% power to detect a ratio of five (corresponding to a standardised ratio of 3.5 from previous work) between groups (TNL Vs. TAL) at the group-wise 5% significance level (alpha = 0.0051; SD = 0.544) correcting for multiple testing as above. To determine the effect of K_V7 modulators on contractile activity of pregnant human myometrium, pilot data has found a consistent standard deviation within treatment groups of 16.6%. With at least n = 4 observations in the control and treatment group, there is 90% power to detect a 33% difference in contractility between the controls and the active treatment. For animal studies, considering the 3R's, experimental protocols used between n = 6–9 animals. For the DA vessel experiments, changes in lumen diameter were analyzed as percent change from the baseline and plotted as mean ± SEM. A comparison of fit determined whether three parameter non-linear log fit lines were significantly different between ML213 and retigabine. Two-way analysis of variance followed by a post hoc Dunnett's test was used to determine significant differences in the % change from baseline for each concentration of drug. Two-way analysis of variance followed by a post hoc Sidak's test was used to determine significant differences between the Emax values of ML213 and retigabine.

All tests were performed using SPSS for Windows version 27 and GraphPad PRISM version 9. The Kolmogorov-Smirnov test was used to evaluate normality of data distribution and summary data are presented as means ± SD or median [interquartile range (IQR)] as appropriate for data distribution. Between-group comparisons were made using Kruskal-Wallis and post hoc Dunn's test or Mann-Whitney. The null hypothesis was rejected where $P < 0.05$.

Declarations

Competing interests:

Authors declare that they have no competing interest

Data and Materials availability

All experimental data associated with this study are available in the main text or the supplementary materials. Limited residual human tissue and associated data available (subject to ethical approval and

a specifically negotiated MTA with KCL and Guys and St Thomas' Foundation Trust). Requests to be directed to the corresponding author.

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Author contributions

(CRediT): Conceptualization: RMT, IAG, HDM, MRJ, PDT, SKE, DMS, JLH, JR; Methodology: RMT, PDT, YTM, SKE, DMS, ECCS, HDM, JR; Investigation: YTM, RMT, PDT, MKZ, ECCS, RP, HDM, JR, JLH, DCS; Visualization: YTM, RMT, ECCS, HDM, MKZ, JR, JLH; Funding Acquisition: RMT, IAG, PIA, MRJ, PDT, HDM; Project Administration: RMT; Supervision: RMT, HDM, PDT, JR; Writing original draft: RMT, YTM, HDM; Writing – reviewing and editing: YTM, HDM, ECCS, MKZ, JR, JLH, DCS, RP, PIA, DMS, SKE, IAG, MRJ, PDT, RMT.

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Figures

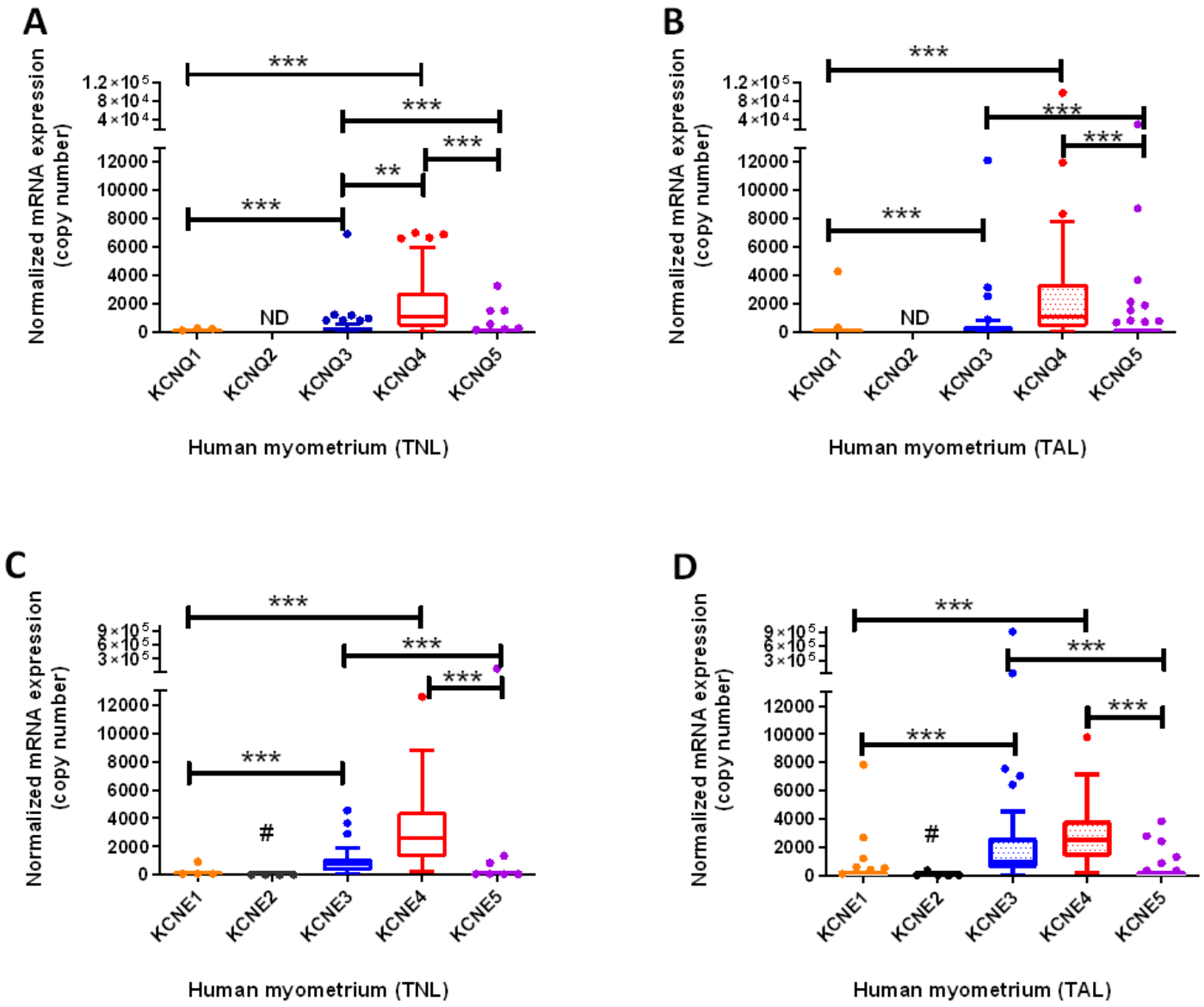


Figure 1

Normalized mRNA expression profile of KCNQ and KCNE genes in pregnant human myometrium taken at term prior to (TNL n=51, A and C) and during active labor (TAL n=46, B and D). Normalized (GeNorm) mRNA copy number expressed as median and quartile ranges. Statistical significance was determined using Kruskal Wallis and Dunn's multi-comparison post-test. ** P<0.01; *** P< 0.001, # P< 0.001 KCNE2 compared to all other KCNE genes.

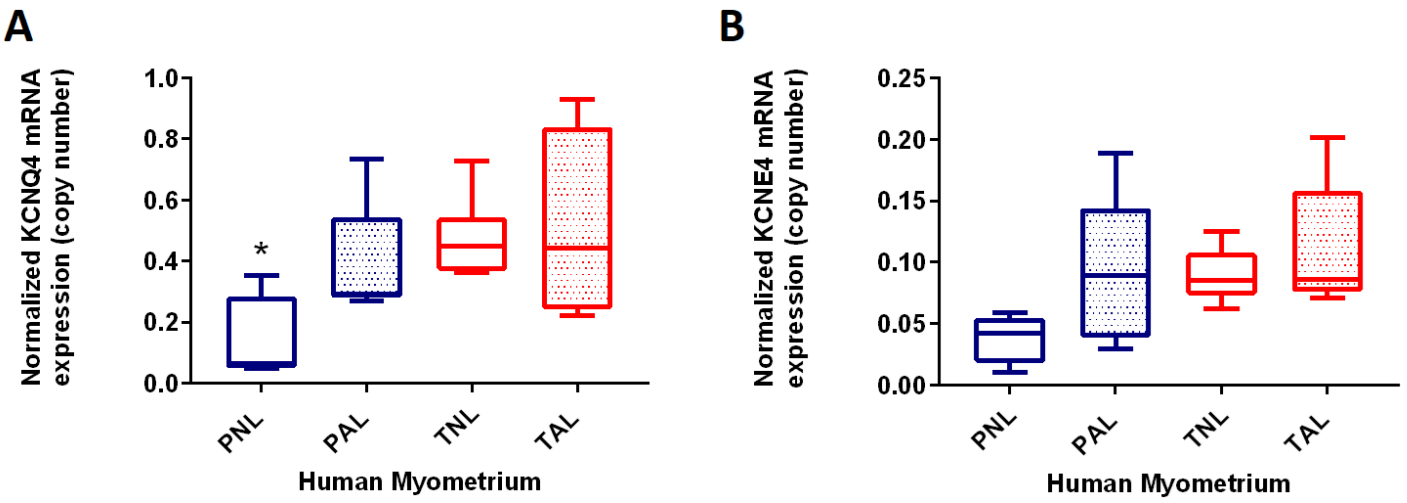


Figure 2

Normalized mRNA expression profile of KCNQ4 (A) and KCNE4 (B) genes in pregnant human myometrium taken at preterm gestations (<37 weeks) not in labor (PNL) and in active labour (PAL) and at term gestations prior to term (not in labor, TNL) and after during active labor (TAL). Normalized (to GAPDH) mRNA copy number expressed as median and quartile ranges. Statistical significance was determined using Kruskal Wallis and Dunn's multi-comparison post-test. *P<0.05 PNL *versus* TNL. PNL, N= 4; PAL, N=5; TNL n=6; TAL n=6.

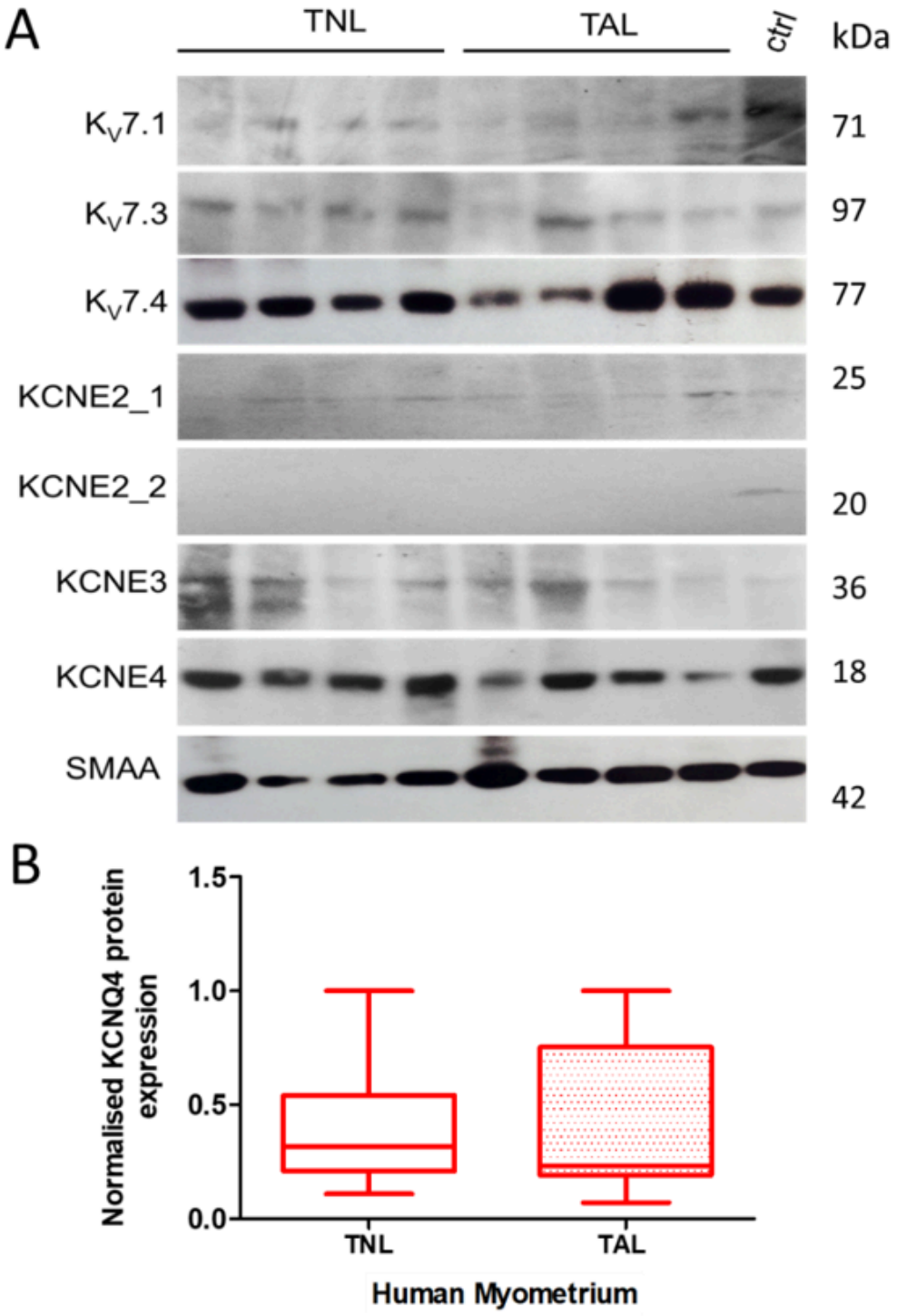


Figure 3

(A). Representative Western blot of protein expression in pregnant human myometrium taken at the time of cesarean section from women at term not in labor (TNL, n=4) and in labor (TAL, n=4) and control (mouse brain, heart, or aorta). Proteins include those encoded by KCNQ1, 3, 4 (K_V7.1, Kv7.3 and KV7.4 respectively), KCNE 1-4 and smooth muscle alpha actin (SMAA, housekeeper). (**B)**. Kv7.4

expression normalized to SMAA expressed as median and interquartile range, no significant difference between $K_V7.4$ expression in TNL v TAL samples.

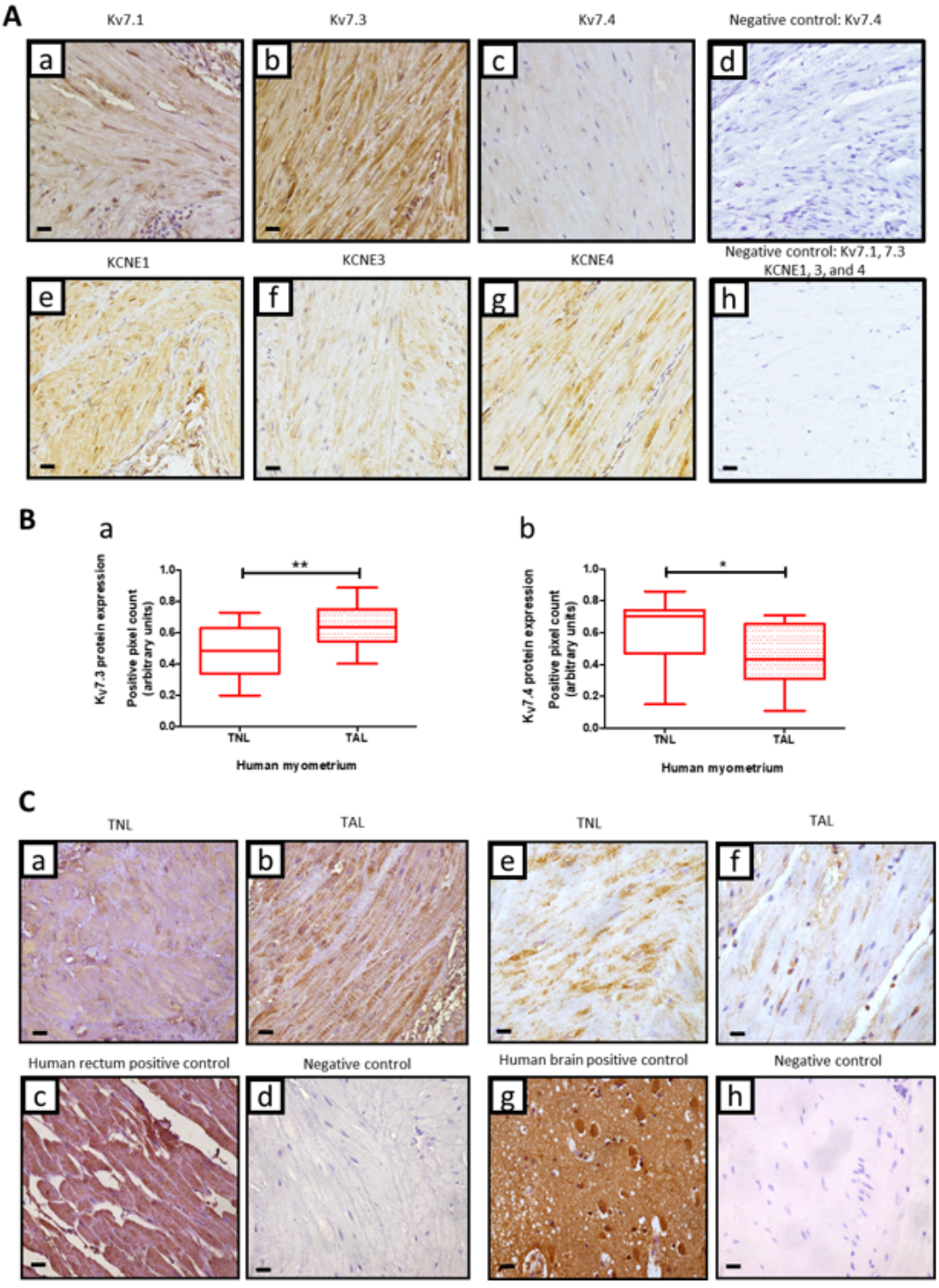


Figure 4

K_V7 and KCNE isoform immunostaining in pregnant human myometrium taken at the time of cesarean section from women at term not in labor (TNL) and in labor (TAL). (**A**). Representative (of n=14)

immunostaining in TNL myometrium a. K_V7.1, b K_V7.3, c K_V7.4, d. negative control for K_V7.4, no primary antibody; e. kcne1, f kcne3, g, kcne4, h negative control for K_V7.1 7.3 and all KCNE isoforms, (no primary antibody). (**B**). Mean positivity and (**C**). representative immunostaining for K_V7.3 (**Ca**, **Cb**) and K_V7.4 (**Ce**, **Cf**) in pregnant human myometrium TNL (n=14) versus TAL (n=18). Box plots are presented as median [IQR], * p <0.05 and ** p <0.01. (**Cc, Cg**) positive controls from human rectum and brain; (**Cd**, **Ch**) negative controls for K_V7.3 and K_V7.4, respectively. Positive staining, shown in brown, was mainly localised to intracellular areas within smooth muscle cells, with some staining around vessels. Black bars are scale bars representing 100 μM. All photomicrographs are taken at x 400 magnification.

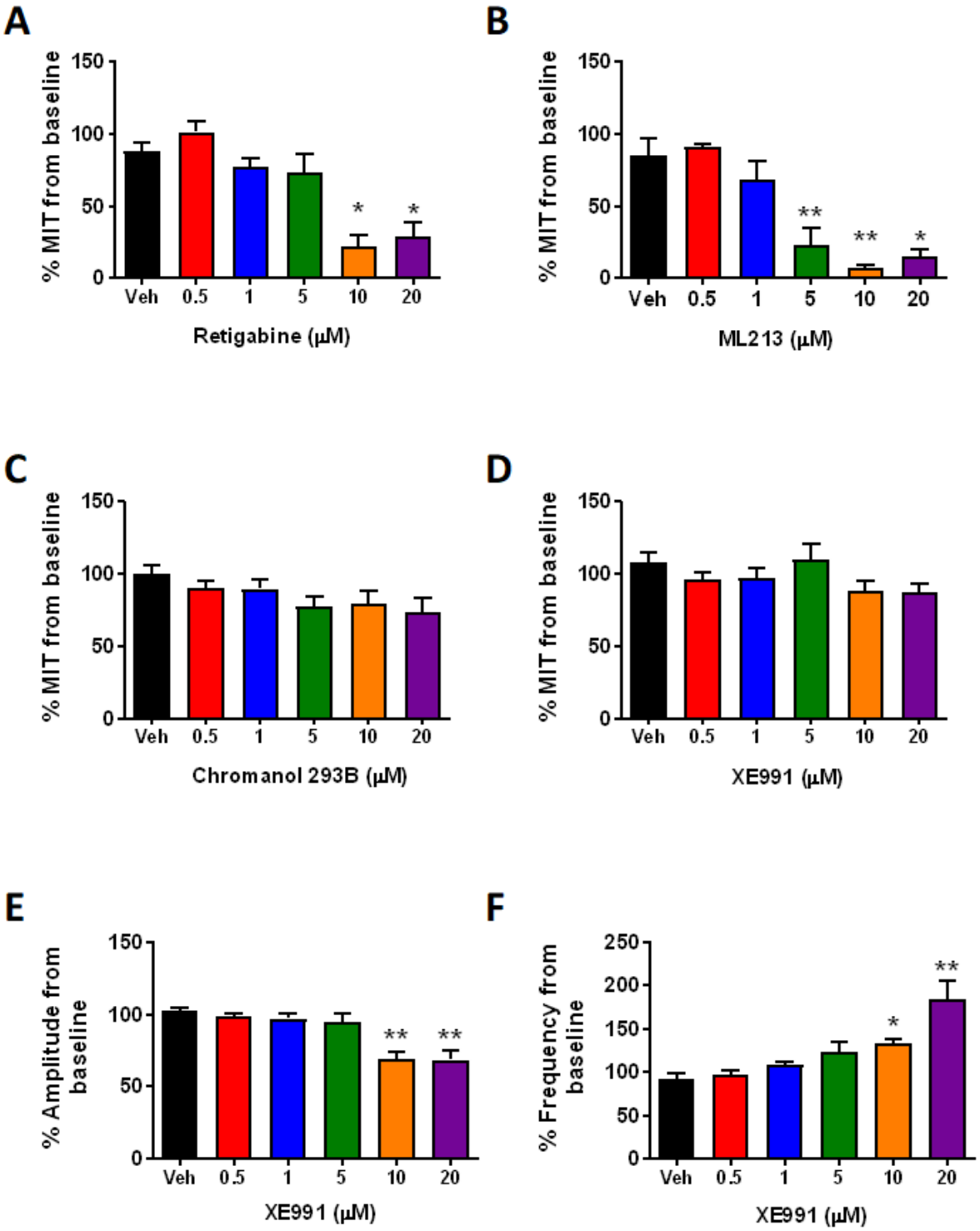


Figure 5

Effect of K_V7.2-5 activators (retigabine and ML213) and the pan K_V7 inhibitor XE991 and chromanol (Kv7.1 inhibitor) on spontaneous contractility of myometrium from women at term prior to labor onset (TNL). Single doses of activator (0.5 – 20 μM) were added to spontaneously contracting myometrium tissue in an organ bath (**A**) retigabine; (**B**) ML213 and mean integral tension (MIT) recorded over a 60-minute period for each dose compared to vehicle control (10 μl DMSO). Impact of single concentrations

(0.5 – 20 μM) of Kv7.1 inhibitor chromanol (C) on MIT and pan K_V7 activator XE991 (D-F) on MIT, contractile amplitude and frequency versus vehicle control. Mean ± SEM data are expressed as a percentage MIT of the preceding baseline prior to the addition of K_V7 modulator. *p<0.05, **p<0.01.

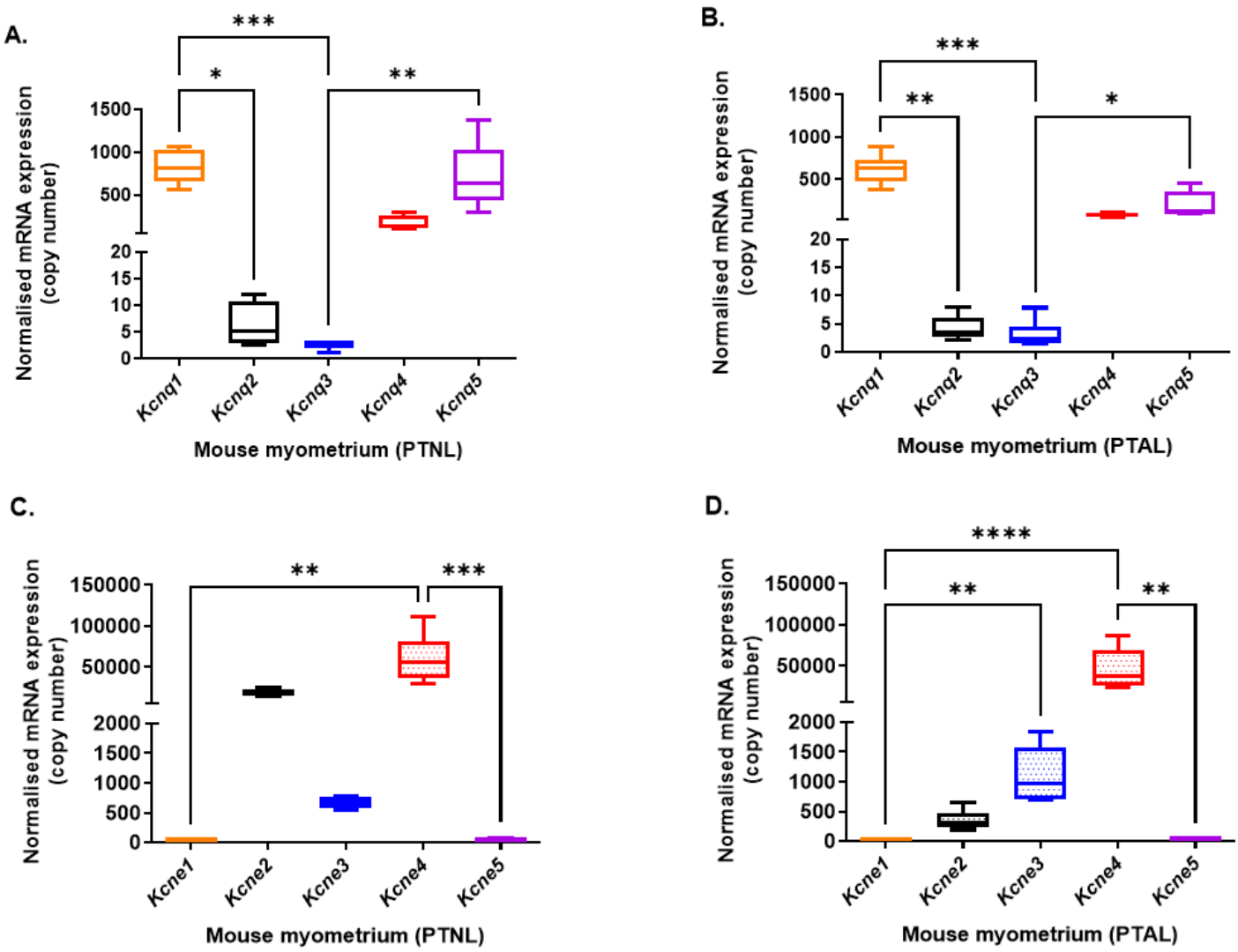


Figure 6

Normalized mRNA expression profile of KCNQ and KCNE genes in preterm [not in labor (PTNL, n=4-6) and in labor (PTAL, n=5-6), day 16] pregnant mouse myometrium. (**A**). *Kcnq1-5*mRNA normalized (GeNorm) copy number expression profile in myometrium from PTNL; (**B**). *Kcnq1-5* mRNA normalized copy number expression profile in myometrium from PTAL; (**C**). *Kcne1-5* mRNA normalized copy number expression profile in myometrium from PTNL mice; (**D**). *Kcne1-5*mRNA normalized copy number expression profile in myometrium from PTAL mice. Data expressed as median [IQR]. Statistical significance was determined using Kruskal Wallis and Dunn's multi-comparison post-test. * P<0.05; ** P<0.01; *** P<0.001; **** P< 0.0001.

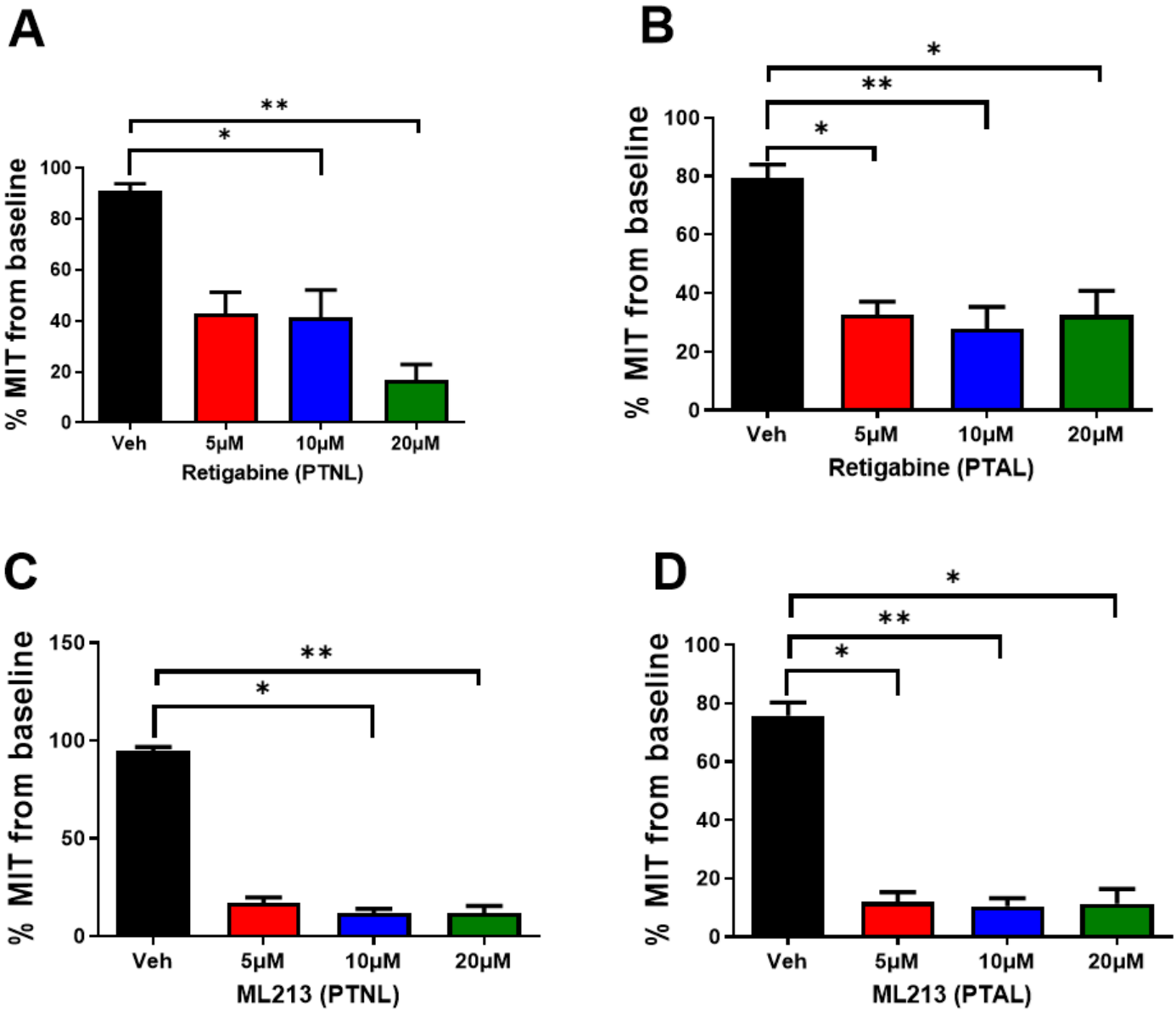


Figure 7

Effect of K_V7 activators (retigabine and ML213) on spontaneous contractility of myometrium from preterm non laboring mice (PTNL, DMSO controls) and preterm laboring mice (PTAL, RU486 treated) on day 16 post conception. Single concentrations of either retigabine and ML213 (5, 10 and 20 μM) were added to spontaneously contracting myometrium tissues (n=6 for each concentration). Mean integral tension (MIT) recorded over a 20-minute period for each concentration compared to vehicle control (DMSO; 10 μl) in myometrium from PTNL (**A, C**) and PTAL (**B, D**) mice. Data are expressed as a percentage MIT of the preceding baseline prior to the addition of K_V7 activator and are presented as mean ± SEM (* $p<0.05$; ** $p<0.01$).

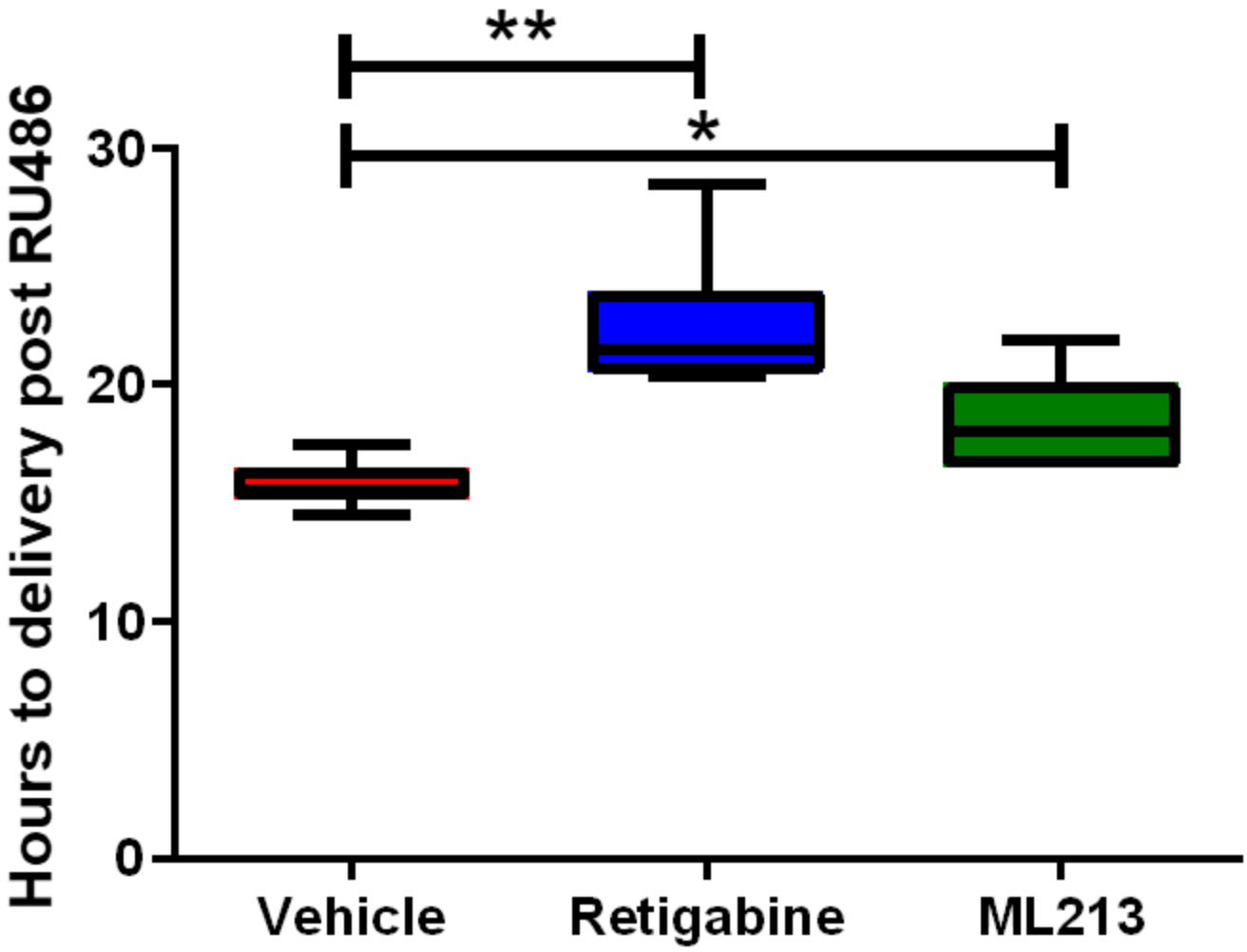


Figure 8

Effect of repeat gavage of 20 mg/kg retigabine or ML213 (compared to vehicle) on the onset of preterm labor in RU486 treated mice. Mice were treated with six-hourly doses (or until delivery occurred) of retigabine (n=6), ML213 (n=6) (20 mg/kg, P.O.) or vehicle (n=9) from 13 hours post RU486 (150 µg) administration. Mice were continually monitored by CCTV for signs of labor. Labor, defined as delivery of first pup, was determined and the hours to delivery post RU486 were calculated. Data are expressed as median [interquartile range]. Data were analyzed using the Kruskal-Wallis test followed by post-hoc Mann-Whitney U tests. (*p<0.05, **p<0.01)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- NatMedKv7SupplementaryMaterialsFinalMarch2025.docx