Title:

Human labor pain is influenced by the voltage-gated potassium channel $K_V6.4$ subunit

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One Sentence Summary:

Human labor pain is influenced by *KCNG4*/K_V6.4 ion channel, which modulates the excitability of sensory neurons.

Abstract:

We sought Mendelian genetic influences on labor pain by studying healthy women who neither requested nor used drug-based analgesia during their first labor; a discovery cohort of 116 were exome sequenced, and an additional 80 had targeted sequencing. Thirty-three of these 196 women underwent comprehensive sensory and psychometric tests, which revealed higher experimental pain thresholds, particularly to deep somatic pressure, when compared to matched controls. We found an excess of heterozygotes carrying the rare allele SNP rs140124801 p.Val419Met in *KCNG4* (encoding the voltage-gated potassium channel subunit K_V6.4); 6 versus an expected 1.57, $P < 0.001$. We show that the rare variant K_V6.4-Met419 fails to traffic to the plasma membrane and, unlike K_V6.4, does not modulate the voltage-dependence of K_V2.1 inactivation. *In vivo*, we observed *Kcng4* (K_V6.4) to be present in 40% of retrolabelled mouse uterine sensory neurons, all of which expressed *Kcnb1* (K_V2.1), and over 90% of which expressed the nociceptor markers *Trpv1* and *Scn10a*. Moreover, the voltage-dependence of inactivation for K_V2.1-mediated currents is more depolarized when K_V6.4-Met419 is overexpressed in mouse sensory neurons compared to when K_V6.4 is overexpressed and hence expression of K_V6.4-Met419 produces less excitable sensory neurons. Lastly, we show that K_V6.4-Met419 has a dominant–negative effect on wild type K_V6.4, consistent with the reduction of labor pain observed in the individuals of our cohort who were heterozygotes for the *KCNG4* SNP rs140124801 allele. K_V6.4 impacts human labor pain by modulating the function of nociceptors that innervate the uterus.

Introduction

All eutherians (placental mammals) experience contraction of the uterus and discomfort during parturition. Whilst this discomfort is universal in eutherians, it appears to be most marked in humans (1-3). The severity of labor pain is considered a consequence of positive sexual selection in modern humans (with females seeking the cleverest mate), which has led to the human brain (and head) being three times the relative size of our nearest primate relatives (4-6). Despite neoteny (birth of offspring in a relatively immature state), this imposes a need to deliver a large neonatal head through the birth canal causing labor pain (7). While labor pain is clearly linked to uterine contractions and cervical distension, the generation of this visceral signal and the sensory afferents involved are poorly understood (8-10).

Although there are well-established ethnic, social and cultural factors that influence the experience and expression of pain during labour (11, 12), broader genetic influences on labor pain may also exist. For example, women with the very rare Mendelian disorder Congenital Insensitivity to Pain due to bi-allelic non-functional mutations in SCN9A (OMIM: 243000) do not report labor pain or require analgesics during labor (13). SCN9A encodes for the voltage-gated sodium channel NaV1.7, expressed selectively in nociceptive and autonomic neurons. Mutations in SCN9A have well-documented roles in causing

extremely painful or painless phenotypes. The painlessness conferred by loss of function SCN9A mutations is clearly maladaptive and can be associated with severe injury during human parturition (14).

As the generation and transmission of action potentials in nociceptors is dependent upon ion channels that control action potential electrogenesis (*15*), we hypothesised that further ion channel variants exist that cause a less extreme form of painlessness in humans. Hence, we sought to discover rare, functional gene changes in healthy women who neither requested nor used drug-based analgesia available to them during their first labor. Psychometrics and quantitative sensory testing, performed in them, revealed modestly increased pain thresholds and tolerance when compared to controls. We next assessed the allele frequencies of all (genome-wide) protein changing single nucleotide polymorphisms (SNPs) in these women compared to population frequencies. We found that the voltage-gated potassium channel (K_V) modifier *KCNG4* (K_V6.4) SNP rs140124801 rare allele c.1255G>A p.(Val419Met) was over-represented. We demonstrate effects of this rare K_V6.4-Met419 variant on sensory neuron excitability, and hence reveal a mechanism through which labor pain may be attenuated in humans.

Results

Identifying women who did not require analgesics during labor as nulliparous parturients: the test cohort

1029 potential cases were identified from 8 maternity units in the United Kingdom over a three-year period. Each potential case was invited to contact researchers, independent of knowledge of their genotype. 383 women responded and were screened via telephone (Fig. 1, Study A). Key inclusion criteria were: healthy women who experienced term (beyond 37-week gestation) spontaneous vaginal delivery as nulliparous parturients without any use or request for any form of systemic or regional analgesia (spinal or epidural). We excluded women who had major disease or co-morbidities that are known to influence labor pain or pain in general. Two hundred and thirty-one women met the full eligibility criteria (Table S1), returned written consent, and donated either 10 ml of blood (collected at their local hospital) or 2 ml of saliva sent via post, from which DNA was extracted (Study A).

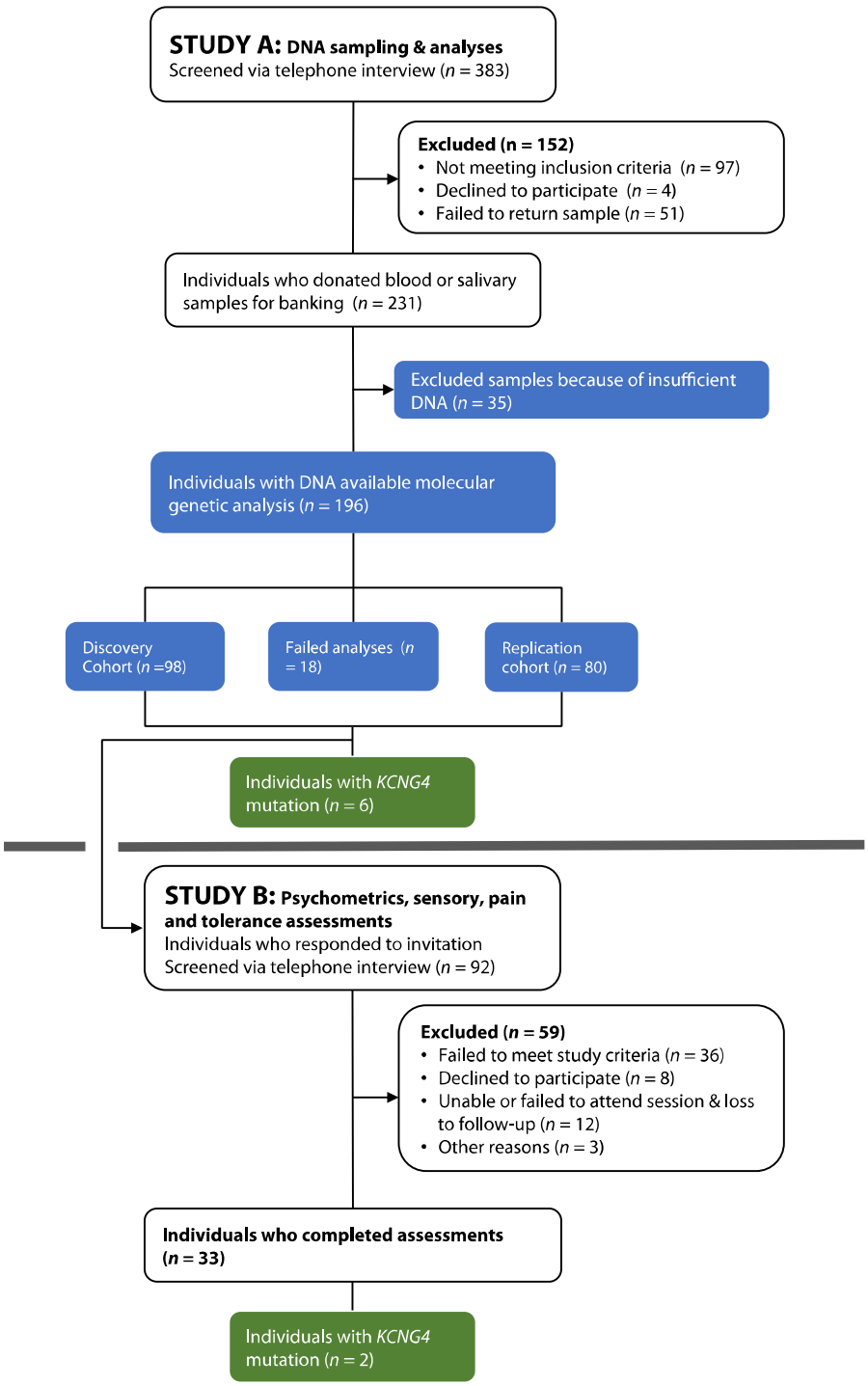

Fig. 1 Flow-chart illustrating recruitment and screening of participants for genetic sampling (Study A) and the subset of those participants who underwent psychometric, sensory and pain (threshold and tolerance) assessments (Study B). Blue rectangles indicate handling, processing and analyses of DNA samples that were donated by participants. Green rectangles indicated number of individuals assessed or DNA analysed with KCNG4 mutation. n, number of samples or individuals.

Of the women who donated DNA, 33 consented for a subsequent study of psychometrics and quantitative sensory testing. These women comprised the subset of the test cohort for Study B (Fig. 1).

For a control cohort for Study B, we recruited 33 women who were matched in age at delivery of the firstborn and location of maternity service but who used analgesics during labor and delivery of their firstborn. There were no significant differences in the means of newborn weight or head circumference between test and control cohorts (Table 1).

Variables (at delivery of first-born)	**Test cohort**			**Control cohort**			P unadjusted	P adjusted *	CI5	CI95
	n	mean	SD	*n*	mean	SD				
Age (years)	33	32.73	4.03	33	31.94	3.98	0.42		-2.76	1.18
Head circumference of newborn (cm)	+22	33.97	0.99	+24	34.46	0.97	0.10		-0.09	1.08
Weight of newborn (g)	32	3382	458.5	33	3384	419.2	0.98		-215.62	219.70
Variables (at research visit)	**Test cohort**			**Control cohort**			P unadjusted	P adjusted *	CI5	CI95
	n	mean	SD	*n*	mean	SD				
Age (years)	33	35.45	4.20	33	36.45	4.11	0.33		-1.04	3.04
Upper arm diameter at assessment (cm)	33	29.14	3.60	33	29.23	3.63	0.92		-1.69	1.87
Sensory and pain thresholds										
Cold detection (°C)	33	30.36	0.97	33	30.35	0.95	0.90		-0.37	0.30
Warm detection (°C)	33	34.53	1.03	33	34.97	0.87	0.014	0.08	0.15	0.98
Cuff pressure detection (mmHg)	33	28.86	8.21	33	27.10	8.38	0.345		-5.33	0.00001
Cold pain (°C)	33	11.56	8.26	33	16.88	9.03	0.021	0.12	1.03	10.07
Heat pain (°C)	33	44.19	2.75	33	42.36	3.40	0.011	0.060	-3.00	-0.37
Cuff pressure pain (mmHg)	33	158.02	51.65	33	113.03	42.96	0.00028	0.0017	-68.35	-21.63
Pain tolerance (cold immersion)										
Pre-immersion hand temperature (°C)	28	30.51	2.05	33	30.82	1.66	0.52		-0.68	1.26
Post-immersion hand temperature (°C)	++27	18.05	4.87	33	20.51	3.54	0.050	0.097	0.00002	4.60
Latency to hand withdrawal (s)	++29	80.35	73.44	33	44.11	55.73	0.026	0.051	-42.00	-0.06996
Peak pain occurrence (0-100mm)	++29	78.29	28.89	33	79.04	28.99	0.99		-5.30	5.10
Peak pain intensity (0-100mm)	++29	56.24	16.11	33	65.82	13.20	0.02	0.048	1.50	17.30
SFMPQ (sensory)	30	8.43	3.97	33	10.97	4.00	0.014	0.04	0.52	4.55
SFMPQ (affective)	30	1.00	1.34	33	1.24	1.35	0.41		-0.00006	0.99995

Table. 1 Summary of key characteristics of test and control cohorts in Study B. n, number of participants; SD, standard deviation; * Sidak's correction; CI5-CI95, 5-95% confidence interval; +missing clinical record; ++equipment failure or unavailable; SFMPQ, short-form McGill's Pain Questionnaire.

Cognitive and emotional function is normal in the test cohort

Psychometrics, comprising validated questionnaires and computerized cognitive assessments, were employed to quantify mood, beliefs and personality traits that can influence pain in experimental or clinical settings. The questionnaires included were: Hospital Anxiety and Depression Scale (HADS) (*16*), Pain Catastrophizing Scale (PCS) (*17*), Multidimensional Health Locus of Control Scale (MHLC) (*18, 19*) and Life Orientation Test-Revised (LOTR) (*20*). Computerized cognitive assessments were implemented in CANTAB® (Cambridge Cognition, UK) (*21*); details provided in Table S2. These tasks rely on frontal-limbic brain regions (*22-25*), which also exert descending control of nociceptive inputs (*26, 27*). There were no significant differences in psychological or cognitive measures between control and test cohorts (Table S3).

Experimental pain thresholds and tolerance are increased in the test cohort

Next, we quantified detection and pain thresholds to cold, heat and mechanical pressure. Thermal stimuli were delivered using a contact-thermode applied to the forearm. Mechanical pressure was exerted via compression of upper arm by a sphygmomanometer cuff. There were no significant differences in the detection thresholds of cold, heat or cuff pressure in the test and control cohorts to suggest sensory deficits or impairments pertaining to those stimuli in the test cohort (Table 1, Fig. 2A).

The test cohort had increased pain thresholds to all stimuli compared to controls. Most striking was an increase of pressure pain threshold ($P < 0.0003$, uncorrected; $P < 0.05$, Sidak's correction) (Table 1); the range of outcomes for individual women in the test and control cohorts is detailed in Fig. 2A. During testing for tolerance to pain from the immersion of hand in cold water (3°C) (*28*), when compared to controls, the test cohort showed increased hand withdrawal latency, lower post-immersion skin temperatures (Fig. 2B), and lower peak intensity of pain on the 100 mm Visual Analogue Scale (VAS) on later assessment. The Short-Form McGill Pain questionnaire (*29*) revealed significantly lower scores for the sensory descriptors for the test group. There was no between-group difference in scores related to the affective aspects of the experimentally induced pain experienced. These results suggest that the test cohort of women showed greater tolerance to pain when compared to controls.

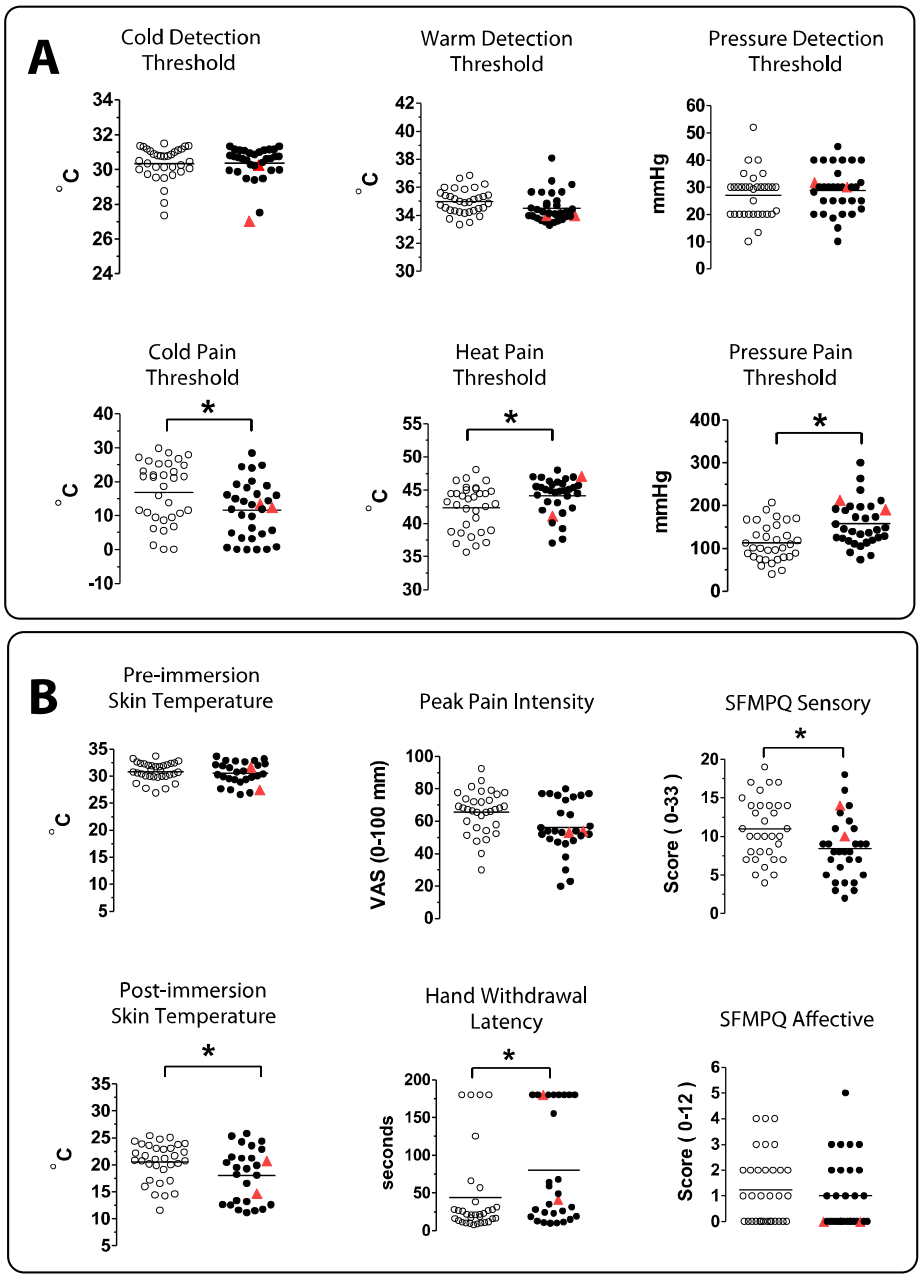

Fig. 2. Sensory detection, pain threshold and tolerance assessments (A) Sensory thresholds for detection and pain for heat, cold and cuff pressure. (B) Testing of pain tolerance to hand immersion in cold water. Left-sided graphs: skin temperatures pre- and post-immersion. Middle graphs: withdrawal latency and ratings of peak pain experienced during hand immersion. Bottom graphs: ratings of the sensory and affective qualities of pain experienced with the SFMPQ. Clear circles indicate individuals in control cohort, and filled circles indicate those in the test cohort. The two individuals with KV6.4 p.Val419Met are indicated by red triangles. Horizontal lines represent the mean for each cohort. * P<0.05, *** P<0.001 (Sidak adjusted P-values)

The rare allele of rs140124801 in *KCNG4* is over-represented in the test cohort

In 196 of the 231 women who did not require analgesics during their first labor, we obtained sufficient high-quality DNA for molecular genetic analysis (Fig. 1). The chronologically first 116 such women (by date of banking DNA) constituted a discovery cohort (Fig. 3A); the next 80 women constituted our replication cohort. Those in the discovery cohort each had exome sequencing, from which we used the bam and bam.bai files for genome wide SNP allele frequency assessment using the fSNPd programme (*30*). This was successful in 98 of 116 cases. The replication cohort of 80 were assessed only for SNP rs140124801 alleles using Sanger sequencing of genomic DNA.

Our discovery cohort analysis identified one ion channel SNP where the allele frequency was altered compared to reference. The rare allele of rs140124801 in *KCNG4* was over-represented. The rare allele frequency was 2.04% versus an expected 0.5% (q = 0.0138, FDR corrected). For the replication cohort, all 80 were Sanger sequenced to determine if they possessed the rare SNP rs140124801 allele, and a further two heterozygotes found. For the total cohort of 178 women not requiring analgesia during their first delivery, there were six heterozygotes carrying the rs140124801 rare allele compared to an expected 1.57 (Chi^2 two tail with Yates correction = 6.699, P = 0.0096; Fig. 3A).

The rare allele of rs140124801 in *KCNG4* introduces a p.Val419Met change in the highly conserved K^+ selectivity filter consensus sequence

The rare allele of rs140124801 in *KCNG4* causes the mis-sense change p.Val419Met in the voltage-gated potassium channel $K_V6.4$ protein (from here on referred to as $K_V6.4$-Met419; Fig. 3A-B). Voltage-gated potassium channels are tetrameric complexes with each subunit having six transmembrane domains (S1-S6). $K_V6.4$ is a member of the electrically silent group of K_V subunits, which cannot form functional plasma membrane-expressed homotetramers, but instead act as modulators of K_V2 subunits (*31*). Indeed, $K_V6.4$ is known to heterotetramerise with $K_V2.1$ in a 1:3 stoichiometry (*32*). Valine 419 is located in the pore forming S5-S6 linker and is part of the highly conserved K^+ selectivity filter consensus sequence (T**V**GYG) (Fig. 3C), in which the equivalent position is always occupied by a branched chain amino acid. Whilst originally thought to be relatively rigid, this structure is also involved in open-pore or C-type inactivation, as subtle rearrangements block the conductive path of K^+ ions (*33*). The molecular mechanisms underlying $K_V2.1$ channel inactivation remain unclear, but interference with the predominant U-type inactivation from closed states (*34*) by the $K_V2.1$-E352Q mutation (*35*) or, in the case of $K_V6.4/K_V2.1$ heterotetrameric channels, treatment with 4-aminopyridine (*36*), unveiled C-type inactivation. $K_V6.4/K_V2.1$ heterotetramers display a hyperpolarising shift in the voltage-dependence of inactivation compared to the $K_V2.1$ homotetramers, which has been linked to differences in the voltage sensing S4 segment (*37*), but additional contributions from the S5-S6 linker have not been excluded. It seemed likely that rs140124801 might affect K^+-selectivity and/or inactivation. We therefore studied the electrophysiological properties of $K_V6.4$-Met419 in complex with $K_V2.1$ compared to the most frequent *KCNG4* allele that possesses a Valine at position 419 ($K_V6.4$) in complex with $K_V2.1$.

The p.Val419Met change in $K_V6.4$ impairs function of $K_V2.1$ heterotetramers

We used HEK293 cells as a heterologous expression system that does not express significant

endogenous KV currents (Fig. S2E). As expected, over-expression of KV6.4 or KV6.4-Met419 alone did not produce measurable K+ currents (Fig. S2E). However, in cells expressing KV2.1 alone, outward currents were observed that were activated by potentials more positive than −40 mV and displayed a slow inactivation (Fig. S2A). Co-expression of KV2.1 with wild-type KV6.4 produced outward currents with similar kinetics and amplitude (Fig. S2D), but we observed a small shift in the voltage of half-maximal activation (V0.5 act) to more negative potentials. This shift was not observed when KV6.4-Met419 was co-expressed with KV2.1 (Fig. S2D). Importantly, the current amplitude was similar to the other two groups showing that expression of KV6.4-Met419 does not negatively regulate maximal current flux, a factor that would impact sensory neuron excitability (Fig. S2E). The slope factors of the Boltzmann fits did not significantly differ between the 3 groups (KV2.1: $k = 9.5 \pm 0.8$, $n = 13$; KV2.1 + KV6.4: $k = 15.9 \pm 1.7$, $n = 14$; KV2.1 + KV6.4-Met419: $k = 11.0 \pm 0.8$, $n = 13$; one-way ANOVA, $P > 0.05$). Furthermore, the reversal potential was not significantly different between the groups (Fig. S2F).

Similar to previous reports (37), co-expression of KV6.4 resulted in a large hyperpolarising shift in the voltage-dependence of inactivation by ~30 mV compared to KV2.1 homomeric currents (Fig. 3D, E & G). This hyperpolarising shift was not observed when KV2.1 was co-expressed with KV6.4-Met419 (Fig. 3F & G). There was however no significant difference in the slope factor of inactivation curves between the three groups (KV2.1: $k = 9.8 \pm 1.4$, $n = 9$; KV2.1 + KV6.4: $k = 13.6 \pm 2.4$, $n = 12$; KV2.1 + KV6.4-Met419: $k = 12.2 \pm 1.2$, $n = 15$; Kruskal-Wallis, $P > 0.7$), or in their time courses of recovery from inactivation (Fig. S2G). These data suggest a loss of KV6.4 function as a result of the p.Val419Met mutation.

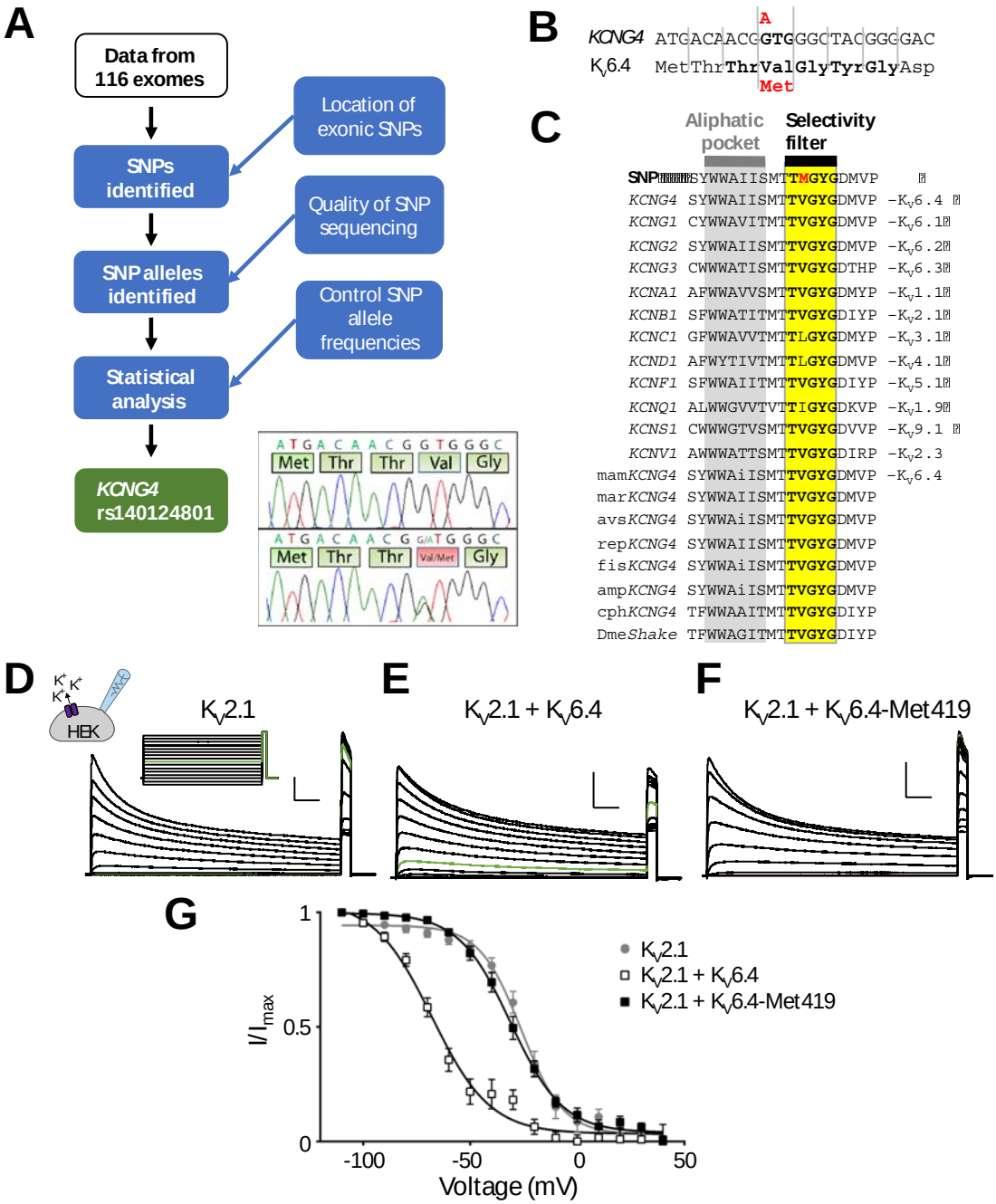

Fig. 3 Molecular genetics of *KCNG4* SNP rs140124801, and analysis of $K_V2.1$ inactivation currents (A) Summary of the genetic analysis. The resultant finding is of the SNP rs140124801 in *KCNG4*. Inset: electrophoretograms showing the alleles. (B) The nucleotide sequence of the SNP rs140124801 (NM_1.NM_172347.2) showing the altered GTG codon (in bold), and the rare allele (in red). Amino acids 416 to 423 of $K_V6.4$ (NP_758857.1) are shown below their nucleotide codons. The selectivity filter is in bold, and the wild type Valine-419 shown above Methionine-419. (C) Evolutionary conservation of human $K_V6.4$ positions 408 to 426: rs140124801 alleles, representative proteins of each human K_V class, and of $K_V6.4$ in vertebrates. Invariant amino acids are capitalized. The selectivity filter TVGYG in yellow, and conserved aliphatic region in grey, see Supplemental Figure S1B and C. Representative current recordings to determine $K_V2.1$ (D), $K_V2.1/K_V6.4$ (E), $K_V2.1/K_V6.4$-Met419 (F) steady-state inactivation properties. The applied voltage protocol is illustrated above (D). Vertical scale bar is 10 nA, horizontal scale bar is 0.5 s. Green traces indicate currents recorded during the −40 mV conditioning step. (G) Voltage-dependence of steady-state inactivation of $K_V2.1$ (grey filled circles, n = 9), $K_V2.1/K_V6.4$ (white squares, n = 12), and $K_V2.1/K_V6.4$-Met419 (black squares, n = 15). Solid lines represent the Boltzmann fitted curves.

K_V6.4-Met419 does not traffic with K_V2.1 to the plasma membrane

As discussed above, K_V6.4 forms heterotetramers with K_V2.1 with altered biophysical properties compared to homotetrameric K_V2.1 channels (Fig. 3D-G, Fig. S2 and (*38*)). In addition, K_V6.4 is retained in the endoplasmic reticulum in the absence of K_V2.1, requiring the expression of K_V2.1 for trafficking to the cell membrane (*39*). We thus tested whether the p.Val419Met alteration might affect the trafficking of K_V6.4. For this, K_V6.4 was cloned into a pcDNA3 based vector containing a CMV-poliolRESmCherry expression cassette, tagged with HA and the p.Val419Met alteration introduced. K_V2.1 had been previously cloned into the pCAGGS-IRES2-nucEGFP which displays nuclear GFP signal upon transfection. To assess membrane localization, HEK293 cells were co-transfected with both K_V2.1 and K_V6.4, stained for HA-tagged K_V6.4, with co-expressing cells identified by both mCherry and nuclear GFP signal. K_V6.4 was retained within the cytoplasm in the absence of K_V2.1 expression but displayed a striking shift to the cell membrane upon co-transfection with K_V2.1 (Fig. 4A). There was no appreciable difference in the localisation of K_V6.4-Met419 in the absence of K_V2.1, but in the presence of K_V2.1 and in contrast to the wild-type protein, K_V6.4-Met419 was retained intracellularly and showed no membrane localization (Fig. 4A). Importantly, expression of K_V6.4-Met419 in HEK293 cells showed only a modest reduction in steady-state stability compared with wild-type K_V6.4, and this was not affected by co-expression with K_V2.1 (Fig. 4B-C).

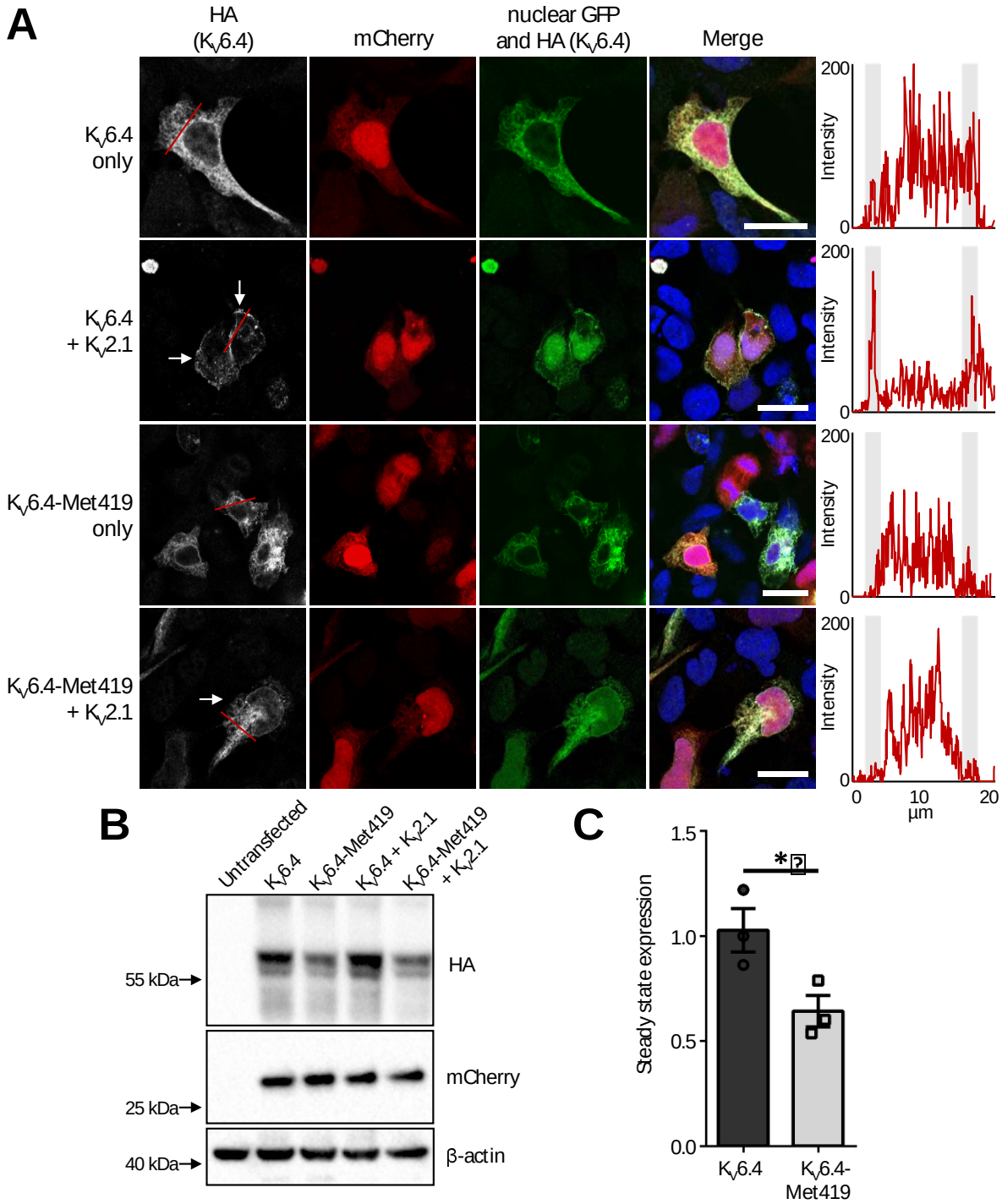

Fig. 4 p.Val419Met blocks $K_V6.4$ from reaching the plasma membrane independent of changes in steady-state expression

(A) Immunofluorescence analysis of $K_V6.4$ localisation. In the absence of $K_V2.1$, $K_V6.4$ was retained in the cytoplasm (white channel, top panel), and was trafficked to the cell membrane in the presence of $K_V2.1$ (white channel, 2nd panel down, white arrow). In contrast, HA-tagged $K_V6.4$-Met419 did not localise to the cell membrane in either the absence or presence of $K_V2.1$ expression (white channels in the 3rd and 4th panel down, white arrow). Expression of $K_V2.1$ is demonstrated by presence or absence of green nuclei, expression of $K_V6.4$ is displayed directly by HA tag in the white channel and expression of the IRES vector expressing $K_V6.4$ is displayed by presence of mCherry signal in the red channel. Graphs adjacent to each panel display the intensity of $K_V6.4$ HA signal along the red line in each respective white channel; note membrane localised peaks only in $K_V6.4$

when co-expressed with $K_V2.1$. (B) HA-tagged $K_V6.4$ was transiently expressed in the presence or absence of $K_V2.1$. There was a modest reduction in steady state stability for $K_V6.4$-Met419 compared with wild-type $K_V6.4$. (C) Stability assessed by densitometry of HA compared with mCherry as a control of transfection efficiency. Unpaired *t*-test ($P = 0.04$).

K_V6.4 is expressed in nociceptors that innervate the uterus

Altered K_V function produces dramatic effects upon sensory neuron excitability; K_V7 openers (*40*) and K_V2 inhibitors (*41*) decrease and increase sensory neuron excitability respectively. We hypothesised that expression of K_V6.4-Met419 within sensory neurons innervating the uterus would alter neuronal excitability and contribute to the impaired nociception. We first investigated the expression of *Kcng4* and *Kcnb1* in mouse uterine sensory neurons using single-cell qRT-PCR of sensory neurons retrogradely labelled with fast blue from the uterus (Fig. 5A). Sensory innervation of the mouse uterus possesses two distinct peak densities within thoracolumbar (TL) and lumbosacral (LS) spinal segments (*42*). As such, fast blue-positive uterine sensory neurons were collected from dorsal root ganglia (DRG) isolated from vertebrae levels T12-L2 and L5-S2. These had an average cell diameter of 31.0 ± 0.7 µm (n = 89), which is in broad agreement with studies investigating sensory neurons innervating the uterus and other visceral organs including the distal colon (*42, 43*). Most uterine neurons expressed *Kcnb1* (TL: 82% [36/44] and LS: 66% [30/45]) and *Kcng4* mRNA was detected in a subset of uterine neurons from both spinal pathways (TL: 43% [19/44] and LS: 24% [11/45]; Fig. 5B). Importantly, all but one LS neuron co-expressed *Kcng4* with *Kcnb1*, suggesting that these two K_V subunits are predominantly present in the same uterine sensory neuron subset. We also assessed the mRNA expression of the nociceptor markers transient receptor potential vanilloid 1 (*Trpv1*) and voltage-gated sodium channel type 10 (S*cn10a*). In *Kcng4*-positive uterine sensory neurons *Trpv1* mRNA was present in 100 % of TL and 91 % of LS neurons, and *Scn10a* in 95 % of TL and 91 % of LS neurons, suggesting that K_V6.4 is primarily expressed in a nociceptive population of neurons (Fig. 5B).

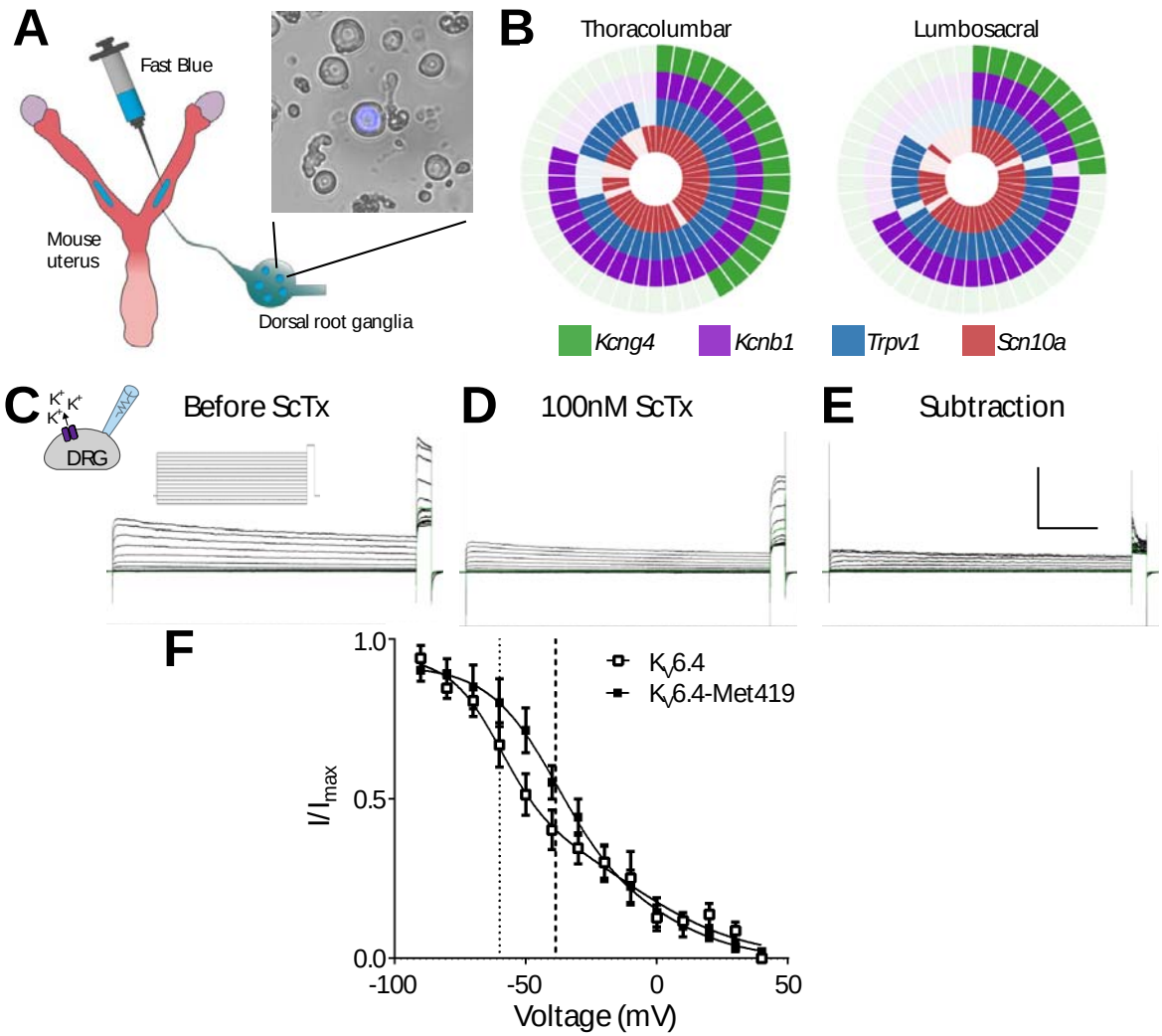

Fig. 5 *KCNG4* is coexpressed with *KCNB1* in mouse uterine sensory neurons and $K_V6.4$, but not $K_V6.4$-Met419, shifts the voltage dependence of inactivation of the Stromatoxin-1-sensitive current in mouse sensory neurons

(A) Uterine sensory neurons were retrogradely labelled using Fast Blue and harvested following dissociation. (B) Co-expression analysis of thoracolumbar (T12-L2) and lumbosacral (L5-S2) uterine sensory neurons expressing transcripts for *Kcng4*, *Kcnb1*, *Trpv1* and *Scn10a*. Each segment in the wheel-diagram is representative of a single cell, with a colored segment signifying positive expression. (C) Representative current recordings to determine the voltage dependence of steady-state inactivation of the Stromatoxin-1 (ScTx)-sensitive I_K elicited by the *inset* voltage protocol in the absence (C) and presence (D) of 100nM ScTx. Green traces indicate currents recorded during the −40 mV conditioning step. (E) The ScTx-sensitive I_K was obtained by subtraction of D from C. Vertical scale bar is 6 nA, horizontal scale bar is 1 s. (F) Inactivation curves for the ScTx-sensitive I_K for neurons transfected with either wild-type $K_V6.4$ or $K_V6.4$-Met419. Both datasets were fit with a sum of two Boltzmann functions. The midpoints of both the 2nd components of these fits are plotted as either light dashed (wild-type $K_V6.4$) or heavy dashed ($K_V6.4$-Met419) lines.

K_V6.4–Met419 causes loss of modulatory function of K_V2.1 in DRG sensory neurons

Given the high co-expression of *Kcng4* with *Kcnb1* in uterine sensory neurons, we next characterized the effect of K_V6.4 and K_V6.4–Met419 on sensory neuronal function. We recorded outward delayed rectifier K^+ currents (I_K) and investigated the effect of transient transfection of either K_V6.4 or K_V6.4-Met419 on the Stromatoxin-1(ScTx)-sensitive I_K; ScTx is a gating modifier of K_V2.1, K_V2.2 and K_V4.2 which effectively blocks these channels (*44*), as well as K_V2.1 heterotetramers formed with silent K_V subunits (*45, 46*). Through subtraction of I_K in the presence of ScTx from the total I_K in the absence of ScTx, we isolated the ScTx-sensitive I_K, which is predominantly dependent on K_V2 channels (Fig. 5C-F). A diverse and heterogenous population of K_V2 and silent K_V subunits is expressed in sensory neurons (*43, 47, 48*) and previous studies suggest that silent K_V subunits only heterotetramerise with K_V2 subunits and not K_V1, K_V3 and K_V4 subunits (*38*). As such, we predicted that wild-type K_V6.4 heterotetramerisation with K_V2.1 in sensory neurons would produce functional channels, but with a hyperpolarised shift in the voltage-dependence of inactivation compared to homotetrameric K_V2.1 channels, as we (Fig. 3D-G) and others have observed previously in HEK293 cells (*38*). By contrast, we hypothesized that the K_V6.4-Met419 subunit would be unable to evoke such a hyperpolarising shift in the voltage-dependence of inactivation.

By transfecting mouse sensory neurons with either wild-type K_V6.4 or K_V6.4-Met419, we attempted to bias available K_V2.1 into heterotetramers with K_V6.4 subunits, thus increasing the probability of recording the contribution of K_V2.1/K_V6.4 heterotetramers to ScTx-sensitive I_K. In both K_V6.4 and K_V6.4-Met419 experiments, addition of ScTx led to a maximum reduction in the outward K^+ current at a 20 mV step potential, which did not differ significantly (K_V6.4, 52.7 ± 3.8 %; K_V6.4-Met419, 45.1 ± 7.7 %; Student's t-test, P = 0.37; Fig. 5C-E). The voltage-dependence of ScTx-sensitive I_K activation was similar for neurons transfected with wild-type K_V6.4 or K_V6.4-Met419 subunit ($V_{1/2}$ = −5.4 ± 1.8 mV *vs.* −9.8 ± 1.1 mV, and k = 8.6 ± 1.5 *vs.* 8.9 ± 0.9, respectively; Fig. S3). As observed previously (*48*), the voltage-dependence of ScTx-sensitive I_K inactivation, for both wild-type K_V6.4 and K_V6.4-Met419 experiments, was multifactorial and fitted with a sum of two Boltzmann functions. In neurons transfected with wild-type K_V6.4, the midpoint of the first component was −9.1 ± 32.9 mV, which likely correlates with homotetrameric K_V2.1 currents. The second component possessed a midpoint of inactivation of −59.9 ± 4.9 mV (n = 8); a current that is likely a function of heterotetrameric K_V2/silent K_V channels or differentially phosphorylated K_V2 channels and in line with what others have reported for the second component of I_K in DRG neurons in the presence of ScTx (*48*). Importantly, expression of K_V6.4-Met419 led to a significant depolarising shift in the second component of the voltage-dependence of inactivation (−38.6 ± 4.2 mV, n = 7, unpaired t-test, P = 0.006, Fig. 5F), whilst the first component, attributed to homotetrameric K_V2.1 I_K, remained unchanged (4.9 ± 25.7 mV, unpaired t-test, P = 0.75). The functional consequences on neuronal excitability of such a shift in the availability of K_V2 channels towards more depolarised potentials would likely increase the after-hyperpolarisation duration and intra-action potential refractory period, leading to a reduction in action potential conduction probability: sensory neuronal characteristics to which K_V2 is known to contribute (*41*). We thus postulate that during

sustained activation, uterine primary afferent input into the pain pathway is likely to be reduced in women carrying the rare *KCNG4* SNP rs140124801 allele.

Heterozygous K_V6.4-Met419 acts as a dominant negative mutation to abolish wild-type function.

The SNP rs140124801 minor allele identified in those healthy women not requiring analgesia during their first labor was always in a heterozygote state. We asked if this heterozygous state has as much of an effect on K_V2.1 as the homozygous state used in our sub-cellular localisation and electrophysiology studies, or if the effect size was in-between homozygous K_V6.4 and homozygous K_V6.4-Met419. Indeed, our findings of reduced labor pain are compatible with the minor allele of rs140124801 having a dominant-negative effect, or a reduced dosage effect, but incompatible if acting as a recessive. K_V2.1 was co-transfected into HEK293 cells with equimolar concentration of wild-type K_V6.4 and K_V6.4-Met419, stained for HA-K_V6.4 and the membrane marker Na^+/K^+ ATPase. We found significant co-localisation of wild-type K_V6.4 with Na^+/K^+ ATPase at the plasma membrane, but no evidence of trafficking to the cell membrane for either homozygote K_V6.4-Met419, nor when wild-type K_V6.4 and K_V6.4-Met419 were co-transfected (Fig. 6A-B).

Similarly, co-transfection of equimolar wild-type K_V6.4 and K_V6.4-Met419 with K_V2.1 produces electrophysiological properties comparable to transfection of K_V2.1 only, i.e. the co-expression of the minor allele variant prevented the hyperpolarising shift of the voltage-dependence of inactivation produced by the major allele variant (Fig. 6C). We therefore conclude that the K_V6.4-Met419 variant acts as a dominant negative subunit and significantly affects the function of K_V6.4 (and hence in turn K_V2.1) in the heterozygote state identified in our cohort of women who did not require analgesia during their first labor.

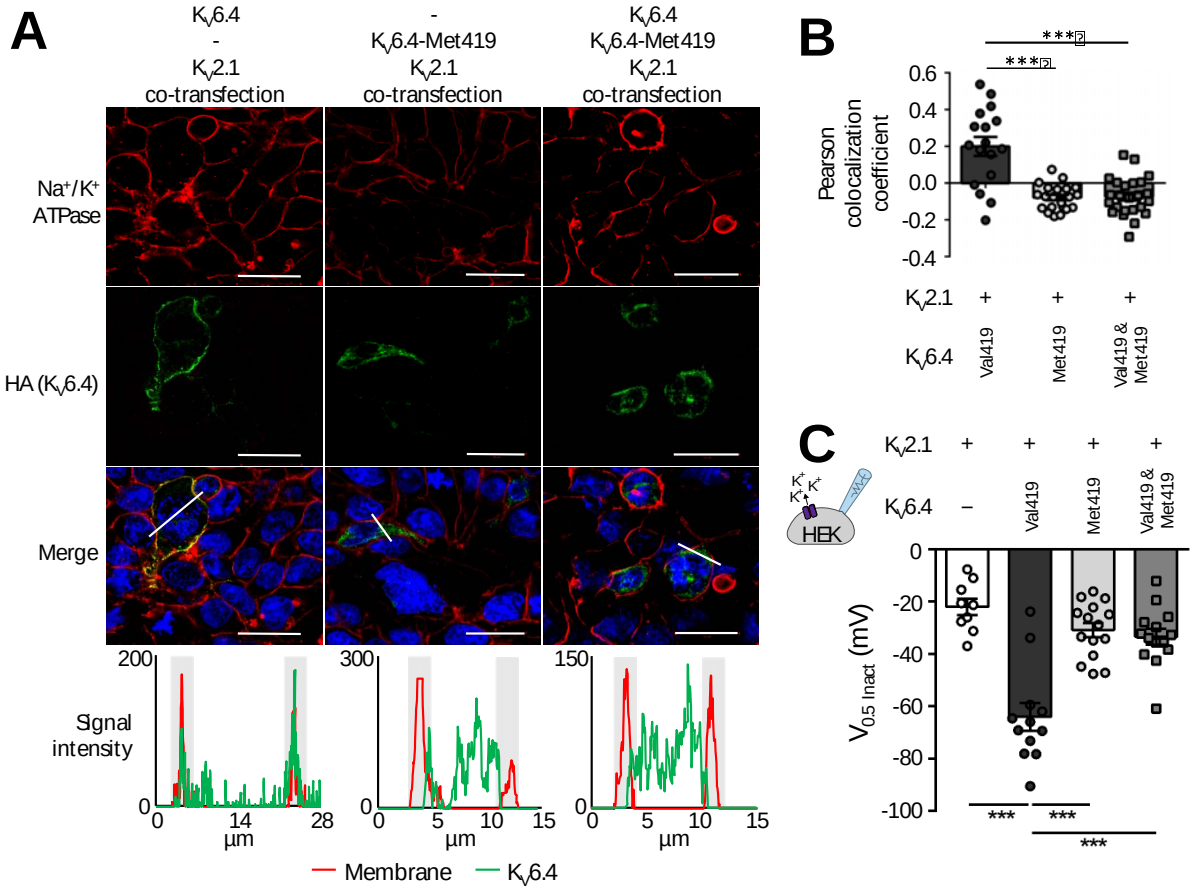

Fig. 6 Sub-cellular localisation and electrophysiology studies of the dominant-negative effect of human $K_V6.4$-Met419 (A) HEK293 and HeLa cells (separate experiments) were transfected with $K_V2.1$ and either wild-type $K_V6.4$, Kv6.4-Met419 or equimolar concentrations of $K_V6.4$ /$K_V6.4$-Met419. Cell membranes were stained with Na^+/K^+ATPase (red channel) and HA-tagged $K_V6.4$ (green channel). HA-tagged $K_V6.4$ wild-type localised to the cell membrane, showing significant co-localisation with Na^+/K^+ATPase. Both $K_V6.4$-Met419 and $K_V6.4/K_V6.4$-Met419 co-expression showed cytoplasmic retention of $K_V6.4$ and no evidence of co-localisation with Na^+/K^+ATPase. Graphs below each pane display the profile of signal for membrane and $K_V6.4$.HA along the plane of the white line in the merged image. Note red and green signal co-localise in the wild-type $K_V6.4$ experiment and are distinct in the $K_V6.4$-Met419 and heterozygote experiment. (B) Quantification of Pearson's co-localisation co-efficient between $K_V6.4$.HA and Na^+/K^+ATPase in each experimental condition. For each condition < 17 cells were counted from three independent experiments. (C) Voltage of half-maximal inactivation from inactivation protocols shown in Fig. 3D-G. Co-expression of both $K_V6.4$ and $K_V6.4$-Met419 with $K_V2.1$ failed to evoke a shift in the voltage-dependence of inactivation. Bars indicate mean values, error bars indicate SEM, n = 9-15, *** $P < 0.001$. Statistics in B and C represent one-way ANOVA with Bonferroni's multiple comparisons test.

Discussion

Labor pain is a complex experience with many biopsychosocial determinants, of which visceral nociception is fundamental and necessary. Although the cellular and molecular substrates for visceral nociception are ill defined in humans, we hypothesised that ion channels, which are important regulators of sensory neuron excitability, could influence visceral nociception. We sought those by investigating the less extreme but nonetheless unusual behavioral phenotype of reduced pain during labor: healthy nulliparous women who chose and were able to undergo spontaneous vaginal delivery of term labor without any analgesia, and who hence comprised our test cohort. Our first finding was that these women do not differ significantly in terms of sensing of innocuous warmth, cool or somatic pressure, nor do their cognitive and emotional functions differ from controls. However, they demonstrated increased experimental pain and tolerance thresholds. Blinding was not feasible in our experiments and social desirability bias (*49*) may explain our findings of increased threshold and tolerance of pain in the test cohort. However, such bias might be expected to also significantly lower scores for self-reported pain related traits, particularly pain catastrophizing (*17*) in the test cohort, but that was not observed. Pain is a complex experience, with sensory-discriminatory and affective-motivational aspects (*50*). We found that the test cohort had significantly lower SFMPQ scores that pertained to the sensory, but not the affective qualities of the pain that was experienced during cold tolerance testing. These observations supported the validity of studying this group of women to discover predisposing genetic changes in sensory neurons (nociceptors) that influence labor pain: a phenotype that could confidently have been expected to be highly heterogeneous.

Our genetic approach was not to discover very rare Mendelian mutations that could cause profound changes to visceral nociception, but instead to seek modulatory changes which were likely to be more common and to be already known SNPs. Within our cohort of 196 women, 33 had extensive psychometric and sensory testing compared to a control cohort and as a group, had increased pain thresholds to all stimuli. We detected a single SNP, rs140124801 in the gene *KCNG4* where the rare allele had a significant over-representation in our cohort 196 women who neither requested nor used drug-based analgesia during their first labor. The rare allele of SNP rs140124801 causes a mis-sense change p.Val419Met in K_V6.4, a silent K_V subunit that forms heterotetramers with K_V2 channels and modulates their function (*37*). We, and others, show that wild-type K_V6.4 traffics to the plasma membrane only when co-expressed with K_V2.1 (*39*). In contrast, we found that the rare allele product K_V6.4-Met419 failed to traffic to the plasma membrane when co-expressed with K_V2.1. Moreover, K_V6.4-Met419 failed to induce the hyperpolarising shift in the voltage-dependence of K_V2.1 inactivation that is observed with wild-type K_V6.4.

For K_V6.4 to modulate labor pain it needs to be expressed in an appropriate part of the sensory nervous system. We focused on uterine sensory neurons, but this does not negate the possibility that *Kcng4*/K_V6.4 also exerts influence elsewhere in the nervous system. We observed K_V6.4 expression in Trpv1 and Na_V1.8-positive mouse uterine sensory neurons, consistent with the observation that sensory neurons innervating deep tissues display comparatively high Trpv1 expression (*51*). Results from unbiased single-cell RNA-sequencing of mouse DRG obtained from cervical to lumbar levels reveal no

specific coexpression of $K_V6.4$ in nociceptive Trpv1/Scn10a expressing neurons (*52*). However, single-cell RNA-sequencing of colonic sensory neurons identified that $K_V6.4$ does co-localises with Trpv1 and $Na_V1.8$ (*43*), consistent with our findings here that $K_V6.4$, Trpv1 and $Na_V1.8$ are coexpressed in uterine sensory neurons from T12-L2 and L5-S2 DRG. Taken together, these data suggest that $K_V6.4$ might be a marker for a dedicated type of visceral nociceptor. Due to the restricted expression of *Kcng4* in a particular sensory neuron type, expression of $K_V6.4$-Met419 is expected to reduce excitability specifically for this class of sensory neurons which may contribute to higher experimental pain thresholds.

For the rare allele rs140124801 to modulate labor pain it needs to cause a significant change in $K_V6.4$-influenced neuronal activity, and to do so in the heterozygote state. Our electrophysiology and cell trafficking studies showed that the mutant $K_V6.4$-Met419, as opposed to wild type $K_V6.4$, had no effect on $K_V2.1$ function, nor was it trafficked to the plasma membrane. Transfection of wild-type $K_V6.4$ into mouse sensory neurons produced a more hyperpolarised voltage-dependence of inactivation for the predicted heterotetrameric K_V2/silent K_V channel component of I_K than when $K_V6.4$-Met419 was transfected, further supporting the hypothesis that the loss-of-function $K_V6.4$-Met419 results in more $K_V2.1$ activity at positive voltages. $K_V2.1$ is known to contribute to the after-hyperpolarisation duration, intra-action potential refractory period, and thus regulate neuronal excitability (*41*). Hence a $K_V6.4$-Met419-induced deficit in $K_V2.1$ function would likely result in fewer action potentials and less pain during periods of sustained nociceptor activity, such as that occurring with uterine contractions during labor (Fig. 7). Critically, we observed that $K_V6.4$-Met419 has a dominant negative effect on wild-type $K_V6.4$, with regard to modulation of the voltage-dependence of inactivation for $K_V2.1$. This result explains the reduction in labor pain seen in individuals in our cohort who were heterozygotes for the SNP rs140124801 rare allele.

There is a growing understanding of the distinctions between the neural pathways for pain from visceral and somatic tissues: each have evolved nociceptors that sense damage in different physical environments (*53*). Our findings suggest a key role for $K_V6.4$ in specifically regulating nociceptor excitability, and hence pain, in normal labour. If druggable, this could provide a potential target for modulating labour pain without the maternal and neonatal side effects inherent in other analgesic interventions in this setting. These data also raise the question of whether $K_V6.4$ has roles in other painful visceral disorders, both within and outside the female genital tract. One closely related context would be primary dysmenorrhea, which is characterized by severe pain associated with uterine contraction during menstruation (*54*). Exploration of these issues will require the development of selective $K_V6.4$ pharmacological agents to fully probe the role of $K_V6.4$ in visceral pain.

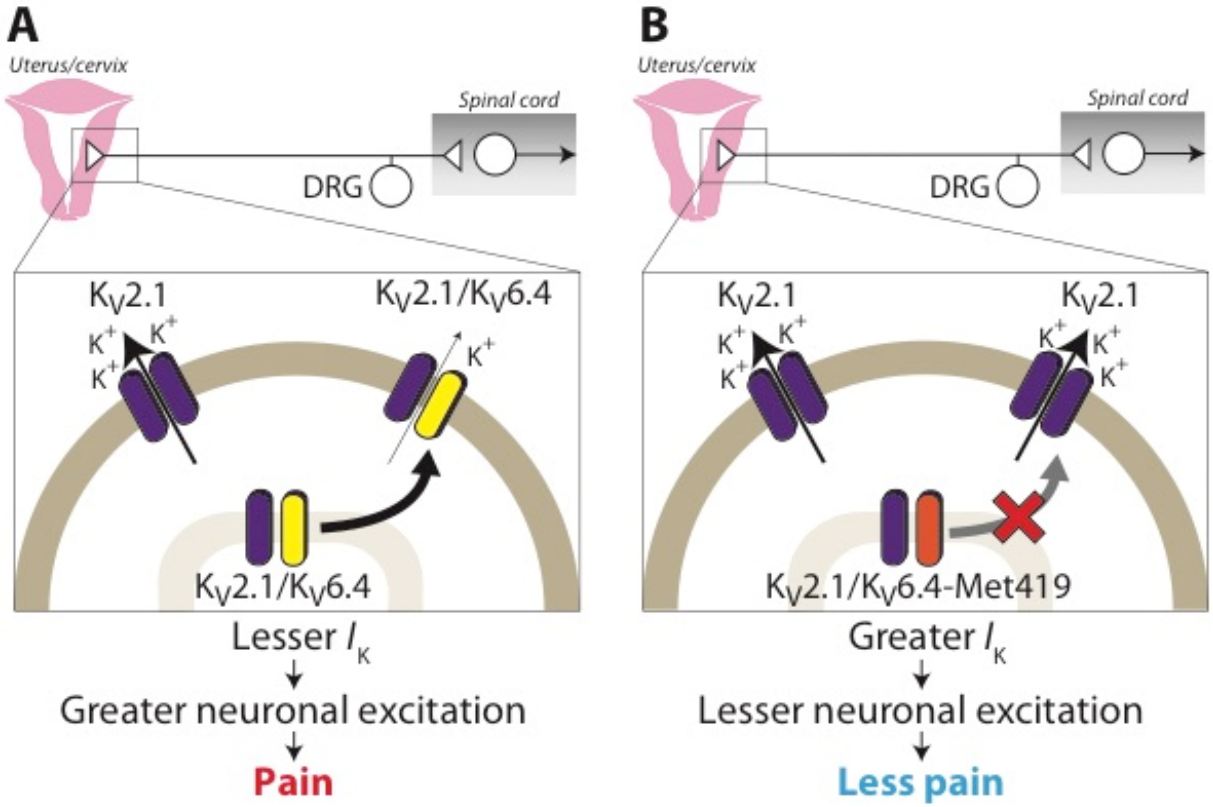

Fig. 7 Schematic of the mechanisms underlying reduced labor pain as a result of carrying the rare allele SNP rs140124801 p.Val419Met in *KCNG4* (encoding voltage-gated potassium channel subunit $K_V6.4$) (A) Visceral nociceptors capable of transducing labour pain possess a combination of homomeric $K_V2.1$ channels and heteromeric $K_V2.1/K_V6.4$ channels. (B) Due to their steady-state inactivation properties, $K_V2.1/K_V6.4$ heteromers have reduced availability at more depolarised membrane potentials compared to $K_V2.1$ homomers. (C) By contrast, $K_V2.1/K_V6.4$-Met419 heteromers fail to traffic from the cytoplasm to the plasma membrane and do not contribute to total I_{Kv2} leading to greater $K_V2.1$ homomer-mediated current at depolarized membrane potentials and thus reducing neuronal excitability and perceived pain.

Materials and Methods (628 words)

For detailed Materials and Methods, see Supplementary Materials

Study Design

The purpose of this study was to identify possible genetic contributions affecting the levels of pain experienced by women during their first labor. We screened >1000 cases and collected a cohort of 231 healthy women who had given birth without any use or request for analgesics. A DNA sample was collected from each (196 provided DNA samples of sufficient quality for further analysis). 33 of this cohort consented for comprehensive psychometric and quantitative sensory testing (QST) and were compared to 33 matched controls who used analgesics during labor and delivery of their firstborn. Psychometric evaluation focused on emotional function, cognitive abilities and personality traits that may influence the experience of pain. QST comprised ascertaining somatic sensory detection, pain and tolerance thresholds of thermal and mechanical stimuli in controlled experimental settings.

The chronologically first 116 DNA samples collected were designated a discovery cohort, and underwent full exome sequencing (98 successfully). We mined the data for rare allele SNPs that were statistically under or over represented (by Chi^2 test with two tails and Yates correction) in the discovery cohort after false discovery rate (FDR) correction and identified a p.Val419Met alteration in *KCNG4*, which was then sequenced by Sanger Sequencing methodology in the 80 women replication cohort. This data led us to investigate the potential contribution of the *KCNG4* encoded protein $K_V6.4$ to the phenotype observed.

$K_V6.4$ was cloned, tagged and the p.Val419Met introduced by site-directed-mutagenesis with pathogenicity assessed by eletrophysiological characterization and assessment of intracellular localization in HEK293 cells. The finding of the alteration behaving as a dominant negative, loss of function mutation led us to investigate the physiological role of $K_V6.4$ in uterine sensory neurons. Using retrograde labelling we identified uterine sensory neurons (89 were collected) and performed single-cell qRT-PCR to identify $K_V6.4$ expression alongside binding partner $K_V2.1$ and nociceptor marker genes. Finally, having identified $K_V6.4$ expression alongside $K_V2.1$ in a significant proportion of uterine sensory neurons, we further characterized the effects of expressing the $K_V6.4$ minor allele in mouse sensory neurons by whole-cell patch-clamp recording (n = 15), confirming our initial findings of $K_V6.4$ p.Val419Met as a loss of function mutation.

Statistical Analyses

For psychometric and quantitative sensory testing, statistical analyses were performed with R Studio (Version 1.1.442). The mean and standard deviation were generated for each outcome variable for test and control cohorts. Shapiro-Wilk tests and F-tests were used assess data normality and differences in variances. Differences between the means of each outcome variable in test and control cohorts were assessed using tests for two independent samples, using Student's *t*-test, Welch's *t*-test or Mann-Whitney U tests when the relevant assumptions were met. The level of statistical significance was adjusted using Sidak's correction. The correction applied to multiple outcomes associated with each

domain of assessments: questionnaire-based, CANTAB, sensory detection, pain thresholds and tolerance.

For statistical assessment of the genetic data collected in this study, enrichment of amino acid altering SNPs was assessed by exome sequencing , with exome vcf, bam and bam.bai files iteratively analyzed to extract data on all SNPs in or near to exons, including the depth and quality of the sequence data, and the alleles detected (*30*). For each SNP the allele frequencies were compared to normal values derived from the 1000 genomes project and exome variant server, and deviations assessed for significance using a Chi^2 test with two tails and Yates correction. The resulting *P* values were subject separately to a FDR correction, as approximately 100,000 SNPs were assessed in our fSNPd method.

Further statistical tests used to assess differences between $K_V6.4$ and $K_V6.4$-Met419 throughout the manuscript are unpaired *t*-tests and ANOVA with Bonferroni's multiple comparison post-hoc test, as described in the relevant figure legends and Supplementary Methods. Differences between groups were considered significant at a P value < 0.05, and were tested using GraphPad (Prism5.0, California, USA).

Acknowledgments

The authors would like to thank the mothers who participated in this study. We acknowledge the support from the National Institute of Health Research who facilitated identification of potential participants. We thank midwives and other clinicians who assisted with case ascertainment and invited potential participants on our behalf. We would like to acknowledge Ingrid Scholtes and staff at the Cambridge NIHR Clinical Investigation Ward who cared for our participants during their visit.

Funding: MCL, DW, DM and CGW acknowledge funding from Addenbrooke's Charitable Trust and the NIHR Cambridge Biomedical Research Centre. MN was funded by the Wellcome Trust (200183/Z/15/Z); JH and ESS by a Rosetrees Postdoctoral Grant (A1296) and the BBSRC (BB/R006210/1), VBL and FR by the Wellcome Trust (106262/Z/14/Z and 106263/Z/14/Z) and a joint MRC programme within the Metabolic Diseases Unit (MRC_MC_UU_12012/3). EF, GI and CB were funded by the Cambridge NIHR Biomedical Research Centre Integrative Genomics theme. We thank Professor Naomichi Matsumoto and Professor Hirotomo Saitsu for the kind use of their KCNG4 vector. We would like to thank Walid Khaled for the kind loan of the Nucleofector key to electrophysiological experiments.

Author contributions: MCL, MSN, FR, DW, DKM, ESS and CGW made substantial contributions to the conception and design of this work. MCL, GI, CB and CGW made substantial contributions to the acquisition of clinical data. MSN, JRFH, VBL and PE made substantial contributions to the acquisition of cell and molecular biology data. MCL, MSN, VBL, KS, FR, EVF, PE, ESS and CGW made substantial contributions to the analysis and interpretation of data for the work. All authors were responsible for the drafting of the work or revising it, and giving final approval of the version to be published, and agree to be accountable for all aspects of the work.

Competing interests: The authors have no competing interests pertinent to this publication.

Material requests correspondence: ESS or CGW

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