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Potent inactivation-dependent inhibition of adult and neonatal $Na_V1.5$ channels by lidocaine and levobupivacaine

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Abstract

Background: Cardiotoxic effects of local anesthetics (LAs) involve inhibition of $Na_V1.5$ voltage-gated Na^+ channels. Metastatic breast and colon cancer cells also express $Na_V1.5$, predominantly the neonatal splice variant ($nNa_V1.5$) and their inhibition by LAs reduces invasion and migration. It may be advantageous to target cancer cells while sparing cardiac function through selective blockade of $nNa_V1.5$ and/or by preferentially affecting inactivated $Na_V1.5$, which predominate in cancer cells. We tested the hypotheses that lidocaine and levobupivacaine differentially affect 1) adult ($aNa_V1.5$) and $nNa_V1.5$ and 2) the resting and inactivated states of $Na_V1.5$.

Methods: The whole-cell voltage-clamp technique was used to evaluate the actions of lidocaine and levobupivacaine on recombinant $Na_V1.5$ channels expressed in HEK-293 cells. Cells were transiently transfected with cDNAs encoding either $aNa_V1.5$ or $nNa_V1.5$. Voltage protocols were applied to determine depolarising potentials that either activated or inactivated 50% of maximum conductance (V½ activation and V½ inactivation, respectively).

Results: Lidocaine and levobupivacaine potently inhibited $aNa_V1.5$ (IC_{50} mean (SD): 20 (22) and 1 (0.6) µM, respectively) and $nNa_V1.5$ (IC_{50} mean (SD): 17 (10) and 3 (1.6) µM, respectively) at a holding potential of -80 mV. IC_{50}s differed significantly between lidocaine and levobupivacaine with no influence of splice variant. Levobupivacaine induced a statistically significant depolarising shift in the $V_{½}$ activation for $aNa_V1.5$ (mean (SD) from -32 (4.6) mV to -26 (8.1) mV), but had no effect on the voltage-dependence of activation of $nNa_V1.5$. Lidocaine had no effect on $V_{½}$ activation of either variant, but caused a significantly greater depression of

maximum current mediated by nNa_V1.5 compared to aNa_V1.5. Similar statistically significant shifts in the $V_{½}$ inactivation (approximately -10 mV) occurred for both LAs and Na_V1.5 variants. Levobupivacaine (1 μM) caused a significantly greater slowing of recovery from inactivation of both variants than did lidocaine (10 μM). Both LAs caused approximately 50% tonic inhibition of aNa_V1.5 or nNa_V1.5 when holding at -80 mV. Neither LA caused tonic block at a holding potential of either -90 or -120 mV, voltages at which there was little steady-state inactivation. Higher concentrations of either lidocaine (300 μM) or levobupivacaine (100 μM) caused significantly more tonic block at -120 mV.

Conclusions: These data demonstrate that low concentrations of the LAs exhibit inactivation-dependent block of Na_V1.5, which may provide a rationale for their use to safely inhibit migration and invasion by metastatic cancer cells without cardiotoxicity.

Key Points Summary:

Question: Can lidocaine and/or levobupivacaine be used to preferentially target the cardiac Na_V1.5 variant expressed by metastatic colon and breast cancer cells?

Findings: Low concentrations of both local anesthetics preferentially inhibit inactivated Na_V1.5 with minor differences in their effects on the adult and neonatal splice variants.

Meaning: Na_V1.5 channels on cancer cells may be preferentially targeted by low concentrations of lidocaine and levobupivacaine by virtue of prevalent inactivation in these cells.

Introduction

Voltage-activated Na^+ channels (VASCs) are targets for local anaesthetics (LAs). There are 10 subtypes of human VASCs; those expressed by sensory neurons mediate the beneficial analgesic effects of LAs [1]. However, LAs are non-selective, inhibiting all VASCs including the cardiac isoform, $Na_V1.5$, an action that contributes to their cardiotoxicity [2,3]. Furthermore, at high concentrations they interact with additional ion channels in the heart and central nervous system.

In addition to their classical actions LAs have additional effects. Several retrospective studies suggest that LAs are beneficial during tumour excision, reducing recurrence and metastases in patients who received regional anesthesia [4-7]. These effects might be indirect, through the reduced requirement for general anesthesia and opioids, factors that may adversely affect the stress response, the immune system and natural killer cells [8]. LAs might also directly affect cancer cells.

$Na_V1.5$ channels are not only expressed in the heart, they are also expressed by breast and colon cancer cells in which their activity contributes to migration and invasion [9-12]. In keeping with this, inhibition of $Na_V1.5$ by LAs attenuates colon cancer invasion [9,10] and xenograft studies demonstrate that VASC inhibitors reduce cancer progression *in vivo* [13,14].

Intravenous administration of LAs, during surgical tumour excision, might be advantageous to optimise their putative direct beneficial effects on circulating cancer cells [8]. Lidocaine and levobupivacaine are commonly used to provide analgesia during surgical tumour excision. However, blood concentrations during their intravenous administration reach the low micromolar range well below concentrations usually examined in studies of interactions of LAs with VASCs [15,16].

Metastatic breast and colon cancer cells predominantly express the neonatal splice variant of $Na_V1.5$ ($nNa_V1.5$), which contains amino acids encoded by exon 6a in place of those encoded by exon 6b in the $aNa_V1.5$ adult variant [10,12]. Therefore, it may be advantageous to target cancer cells while sparing adult cardiac function through selectively blocking $nNa_V1.5$. Alternatively, preferential block of the inactivated state of $Na_V1.5$, which predominates in colon cancer cells due to their depolarized resting potential [10], may provide an approach for their selective blockade by LAs.

We used the whole-cell voltage-clamp technique to evaluate the actions of micromolar concentrations of lidocaine and levobupivacaine on recombinant $nNa_V1.5$ and $aNa_V1.5$ channels expressed in HEK-293 cells. We tested the hypotheses that the LAs differentially affect 1) $aNa_V1.5$ and $nNa_V1.5$ and 2) the resting and inactivated states of these $Na_V1.5$ variants.

Materials and methods

Cell culture and transfection

Human embryonic kidney (HEK-293) cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin (Invitrogen, Paisley, UK). Confluent cells were sub-cultured every 3-4 days and plated onto 35 mm dishes at low density for electrophysiological experiments. All cells were maintained in an incubator at 37^{o}C and 5 % CO_2.

HEK-293 cells were transfected using the calcium phosphate precipitation method with human SCN5A (adult or neonatal) in pcDNA3.1 vector at 1 µg/dish, along with cDNA for green fluorescent protein (GFP) at 0.1 µg/dish [10]. GFP was used to identify transfected cells.

Electrophysiology

Currents were recorded from HEK-293 cells transiently expressing a$Na_v1.5$ or n$Na_v1.5$ using the whole-cell voltage-clamp technique. Currents were recorded using an Axopatch 200B patch-clamp amplifier, low-pass filtered at 2 KHz, digitised by Digidata 1320A interface, sampled at 4 KHz and acquired using pClamp8 software (Molecular Devices, CA, USA). Pipettes were formed from borosilicate glass capillaries and had resistances between 1.5 and 3 MΩ when filled with intracellular solution, containing (in mM) the following: 130 CsCl, 15 NaCl, 2 $MgCl_2$, 10 EGTA and 10 HEPES. The extracellular solution contained (in mM) the following: 140 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 glucose and 10 HEPES. Series resistance compensation was ≥ 85 %, in order to minimise voltage errors, which were only accepted if below 4 mV, taking into consideration the peak current and the access

resistance. A holding potential of –80 mV was used in all protocols unless stated otherwise. All solutions were bath applied and recordings were made under continuous perfusion of bath solutions at room temperature. No corrections were made for liquid junction potential compensation.

Data analysis

Current amplitudes were measured using pClamp10 (Molecular devices, CA, USA). Plots of the voltage-dependence of activation were derived from current-voltage relationships. The driving force at each holding potential (up to +20 mV) was determined from the theoretical Na^+ equilibrium potential, in order to calculate Na^+ conductance. Conductances were normalised to the respective peak values in each cell. For the voltage-dependence of inactivation, the amplitude of the available current was normalised to peak amplitude values for each cell. Activation and inactivation data were fitted with Boltzmann functions, as described previously [10]. Concentration-response data were fitted with a logistic function [10]. Average parameters were determined from fits to data acquired from individual cells. For recovery from inactivation, currents were normalised to peak values and data were fitted using the sum of two exponential functions. Time constants were determined from data obtained from individual cells. Weighted time constants (τ_w) were calculated as follows:

$$\tau_w = \left(\tau_f \times A_f\right) + \left(\tau_s \times A_s\right)$$

where τ_f and τ_s are the fast and slow time constants, respectively, and A_f and A_s are the relative amplitudes of the fast and slow components, respectively. Non-linear regression fitting and graphing were performed using GraphPad Prism software (La Jolla, CA, USA).

Statistical analysis

All data are presented as mean (SD). Comparisons of current density values, $V_{½}$ activation, $V_{½}$ inactivation and their slope values before and after LA application were performed using the paired t-test (two-tailed). Pairwise comparisons between low and high concentrations of LA at hyperpolarised potentials were performed using the 2 sample t-test (two-tailed). Comparisons involving the extent of inhibition by LAs at different holding potentials (-80, -90 and -120 mV) were performed using a one-way ANOVA. Subsequent multiple pairwise comparisons between each holding potential were corrected using the Tukey method. All comparisons interrogating the influence of both LA and splice variants were performed using a two-way ANOVA. In all cases, an interaction between the two factors (LA and splice variant) was also tested. Statistically significant influences of either factor or a significant interaction between the factors were further analysed with pairwise comparisons corrected with the Bonferroni method. These pairwise comparisons were performed interrogating the difference between each LA to control within each splice variant, and also across the two splice variants. Comparisons of IC_{50} values were performed on log transformed data to preserve normality. P-values < 0.05 were considered statistically significant for t-tests. For multiple comparisons, corrected by the Bonferroni method, the alpha value was 0.05. Adjusted p-values are reported throughout the study. Statistical analyses were performed using IBM SPSS 22 (Armonk, NY, USA).

The number of biological replicates for this study were chosen on the basis of our previous work with similar *in vitro* experiments [10] enabling detection of differences ≥10% in our parameters of interest. We considered differences <10% unlikely to be physiologically meaningful.

Results

Concentration-dependence of lidocaine and levobupivacaine inhibition of $Na_V1.5$ variants

We established the potency of lidocaine and levobupivacaine as inhibitors of $aNa_V1.5$ and $nNa_V1.5$ (Figure 1A & B). Peak current amplitudes recorded in the presence of LAs were normalised to control and plotted as a concentration-response relationship (Figure 1C & D). Supplementary table 1 contains the fit parameters. We used a two-way ANOVA to analyse differences in mean IC_{50} and Hill coefficient values between the LAs and splice variants. There was no significant influence of splice variant on IC_{50} ($F_{1,20} = 1.8$; $p = 0.2$). However, the LAs did have different potencies ($F_{1,20} = 41$; $p < 0.0001$). A simple pairwise comparison with a Bonferroni correction revealed levobupivacaine to be more potent than lidocaine at either $aNa_V1.5$ or $nNa_V1.5$ ($p < 0.0001$ and $p = 0.001$, respectively; Supp. table 1). In general, levobupivacaine was ~10-fold more potent than lidocaine. There were no differences in the Hill coefficients between either LA or splice variants ($F_{1,20} = 0.08$; $p = 0.78$; $F_{1,20} = 1.5$; $p = 0.24$, respectively, two-way ANOVA). In subsequent experiments, we used 10 µM lidocaine and 1 µM levobupivacaine (approximately equally effective concentrations; Fig. 1).

Lidocaine and levobupivacaine affect the voltage-dependence of $Na_V1.5$ activation

Current-voltage relationships were established in the absence or presence of lidocaine (10 µM) or levobupivacaine (1 µM). A representative example of the currents mediated by $nNa_V1.5$ is shown in Figure 2A. Peak current amplitudes were expressed as current densities, averaged and plotted against voltage (Fig. 2B & C).

Statistically significant (paired t-test) reductions in $nNa_V1.5$ current density caused by the presence of LAs are indicated with an asterisk (Fig. 2B & C). Data for $aNa_V1.5$ are similar and therefore not shown.

Plots of the voltage-dependence of activation were fitted with Boltzmann functions (Fig. 2D - G). The fit parameters are summarised in Supplementary table 2. Lidocaine (10 µM) did not affect the $V_{½}$ activation for either variant (versus control; paired t-test; Supp. table 2). By contrast, levobupivacaine (1 µM) significantly shifted the $V_{½}$ activation of $aNa_V1.5$, but not $nNa_V1.5$ (versus control; paired t-test; Supp. table 2). In light of this differential effect of levobupivacaine, we compared the change in the $V_{½}$ activation ($\Delta V_{½}$) using a two-way ANOVA (Fig. 2H). We found a significant effect of the variant on $\Delta V_{½}$ ($F_{1,33} = 10$; $p = 0.003$), but no significant influence of the LA used ($F_{1,33} = 0.10$; $p = 0.32$), and also no significant interaction between the splice variant and LA used ($F_{1,33} = 2.7$; $p = 0.11$). A pairwise comparison using a Bonferroni correction revealed that levobupivacaine caused a significantly larger shift in $V_{½}$ of activation in $aNa_V1.5$, than $nNa_V1.5$ (Fig. 2H).

The LAs altered the shape of the activation curves, both significantly increasing the slope parameters for both $aNa_V1.5$ and $nNa_V1.5$ (versus control; Supp. table 2). We analysed the extent of change (Δ slope) with a two-way ANOVA (Fig. 2I). We found no significant influence of either LA ($F_{1,33} = 1.5$; $p = 0.23$) or splice variant ($F_{1,33} = 1.8$; $p = 0.19$), suggesting that the degree of change in the slope parameter is similar (Fig. 2I).

Lidocaine and levobupivacaine show a preference for the inactivated state of both $Na_V1.5$ splice variants

We examined the influence of lidocaine (10 µM) and levobupivacaine (1 µM) on inactivation of currents. We applied 100 ms prepulses from -140 mV to -10 mV, followed by an activating step to 0 mV to examine the proportion of channels available for activation. The plots of voltage-dependence of inactivation and the associated Boltzmann fits are shown in Figure 3A to 3D, with fitting parameters summarised in Supplementary table 2. Both LAs caused a statistically significant hyperpolarizing shift in the $V_{½}$ of inactivation for both variants (paired t-test; Supp. table 2). The magnitude of $\Delta V_{½}$ was neither dependent on the LA ($F_{1,33} = 0.05$; $p = 0.83$) nor the splice variant ($F_{1,33} = 0.35$; $p = 0.56$; two-way ANOVA; Fig. 3E). Both LAs significantly increased the slope for $nNa_V1.5$ (paired t-test; Supp. table 2). For $aNa_V1.5$, only lidocaine significantly increased the slope (Supp. table 2).

The maximum available current at -140 mV appears less depressed for $aNa_V1.5$ in the presence of lidocaine (Fig. 3A). Indeed, a comparison of the maximum inhibition (from the Boltzmann fits) revealed a significant (paired t-test) reduction for levobupivacaine on both variants and lidocaine on only $nNa_V1.5$ (Supp. table 2). We analysed the maximum inhibition using a two-way ANOVA (Fig. 3F). We found no significant influence of either LA ($F_{1,33} = 0.11$; $p = 0.75$) or splice variant ($F_{1,33} = 0.26$; $p = 0.62$), but a significant interaction between the two ($F_{1,33} = 8.7$; $p = 0.006$). A simple effects paired comparison with a Bonferroni correction revealed that levobupivacaine caused a greater reduction in the maximal available current than lidocaine in $aNa_V1.5$ ($p = 0.03$), but not $nNa_V1.5$. Also, lidocaine caused a greater inhibition of the maximal available current mediated by $nNa_V1.5$ than that mediated by $aNa_V1.5$ ($p = 0.009$; Fig. 3F).

Inhibition by lidocaine and levobupivacaine is dependent on the inactivated state

Low concentrations of lidocaine or levobupivacaine shifted the $V_{½}$ of inactivation to more hyperpolarised potentials, consistent with a potent action of these LAs to stabilise the inactivated state (Fig. 3). These experiments were performed with a holding potential of -80 mV at which there was approximately 40% steady-state inactivation. To test the hypothesis that inhibition by LAs requires inactivation we investigated tonic inhibition at -80, -90 and -120 mV. Currents were first recorded in control conditions. Stimulation was subsequently stopped and cells were exposed to lidocaine (10 µM) or levobupivacaine (1 µM) for 120 s. The extent of reduction of the first current following LA exposure revealed the magnitude of tonic inhibition. Representative examples of tonic inhibition by lidocaine for $nNa_V1.5$ are shown in Figure 4A. Robust inhibition was observed at -80 mV. However, negligible inhibition occurred at -90 mV and -120 mV (Fig. 4B & C). There were significant differences (one-way ANOVA) in the inhibition at the three holding potentials for $aNa_V1.5$ ($F_{2,12}$ = 17; $p < 0.0001$ for lidocaine, $F_{2,9}$ = 26; $p < 0.0001$ for levobupivacaine; Fig. 4B) and $nNa_V1.5$ ($F_{2,8}$ = 23; $p = 0.001$ for lidocaine, $F_{2,9}$ = 15; $p = 0.001$ for levobupivacaine; Fig. 4C). Using a *post-hoc* Tukey's comparison, we found a significant reduction in tonic inhibition for $aNa_V1.5$ between -80 mV and -90 mV ($p = 0.001$ for lidocaine; $p = 0.001$ for levobupivacaine), and between -80 mV and -120 mV ($p = 0.001$ for lidocaine; $p < 0.0001$ for levobupivacaine). A similar relationship was observed for $nNa_V1.5$ (-80 mV vs -90 mV, $p = 0.001$ for lidocaine; $p = 0.001$ for levobupivacaine; -80 mV vs -120 mV, $p = 0.01$ for lidocaine; $p = 0.001$ for levobupivacaine; Fig. 4C).

Inhibition of resting $Na_V1.5$ by lidocaine and levobupivacaine occurs at high concentrations

Neither lidocaine (10 µM) nor levobupivacaine (1 µM) had a significant impact on $Na_V1.5$ at potentials more hyperpolarised than -90 mV in cells that were held at -120

mV between sweeps (Fig. 5A – D). The best fit parameters are summarised in Supplementary table 3. For both variants at a holding potential of -120 mV, we observed a small shift in $V_{½}$ of inactivation at low concentrations of either LA, with no other significant changes to the fitting parameters (Supp. table 3). Most notably, there was no depression of the maximum available current, by contrast to the inhibition observed at -80 mV (Fig. 3).

The efficacy of the LAs to depress the maximum amplitude was restored (paired t-test versus control; Supp. table 3) at hyperpolarized potentials by a higher concentration of either lidocaine (300 µM) or levobupivacaine (100 µM). There were no significant differences (two-way ANOVA) in the extent of this inhibition by either LA or splice variant (Fig. 5E). The $\Delta V_{½}$ was generally similar to that of lower concentrations of LAs, except in the case of lidocaine (300 µM) on a$Na_V1.5$, which caused a greater $\Delta V_{½}$ than in the presence of 10 µM lidocaine ($p = 0.009$; t-test; data not shown). There was a significant influence of splice variant on $\Delta V_{½}$ (two-way ANOVA, $F_{1,13} = 12$; $p = 0.004$; Fig. 5F). Paired comparisons using a Bonferroni correction revealed significant differences between a$Na_V1.5$ and n$Na_V1.5$ in the presence of either lidocaine ($p = 0.02$) or levobupivacaine ($p = 0.048$; Fig. 5F). Lidocaine significantly changed the slope of the Boltzmann fit, while levobupivacaine did not (paired t-test versus control; Supp. table 3).

We extended our investigation to evaluate tonic block of a$Na_V1.5$ at a holding potential of -120 mV. We compared the extent of tonic block in the presence of low concentrations of either LA (data from Fig. 4) with that observed in the presence of high concentrations of lidocaine (300 µM; Fig. 5G) and levobupivacaine (100 µM; Fig. 5H). Using a t-test, we found a significant increase in tonic inhibition in the presence of the higher concentration of either lidocaine ($p < 0.0001$; Fig. 5G) or

levobupivacaine ($p < 0.0001$; Fig. 5H). Taken together, our data indicate that the inactivated state is not required for inhibition of $Na_V1.5$ currents by high concentrations of LAs, but is necessary for inhibition at lower concentrations.

Low concentrations of lidocaine and levobupivacaine cause differential slowing of $Na_V1.5$ recovery from inactivation

Finally we investigated the rate of recovery from inactivation in the presence and absence of lidocaine (10 µM) or levobupivacaine (1 µM). After inhibition had plateaued at -80 mV the holding potential was stepped to -120 mV for 2 ms – 30 s, to sample recovery from inactivation (Fig. 6A, inset). The recovery data for the $aNa_V1.5$ (Fig. 6A) and $nNa_V1.5$ (Fig. 6B) were fitted with double exponential functions. The best fit parameters are summarised in Supplementary table 4, and used to calculate τ_w values (Fig. 6C & Supp. table 4). The identity of the splice variant did not influence τ_w (two-way ANOVA, $F_{1,28} = 0.48$; $p = 0.50$). However, the identity of the LA did influence τ_w ($F_{2,28} = 11.8$; $p < 0.0001$). In the absence of LA, the mean τ_w values for recovery from inactivation did not differ. However, the τ_w of recovery in the presence of levobupivacaine was significantly slower than controls for both splice variants ($p = 0.01$ for $aNa_V1.5$; $p = 0.003$ for $nNa_V1.5$; *post hoc* Bonferroni correction; Fig. 6C; Supp. table 4). The τ_w for lidocaine did not significantly differ from controls. The slowed recovery from inactivation in the presence of levobupivacaine can be mainly attributed to an increase in the slow time constant (Supp. table 4). An increase in the slow time constant was not seen in the presence of lidocaine. Taken together, our data indicate that low concentrations of LA alter the rate of recovery from inactivation, with levobupivacaine having a larger impact than lidocaine.

Discussion

Lidocaine and levobupivacaine cause potent inhibition of nNa_V1.5 and aNa_V1.5, an effect that was lost at holding potentials below -80 mV. Inhibition was half maximal in the low micromolar range, which can be safely achieved in the blood [15,16]. There was no difference in the potency of block of either variant by the LAs, suggesting that upregulation of neonatal Na_V1.5 on breast [17] and colon cancer cells is unlikely to provide a strategy for selectively targeting their inhibition, consistent with our previous findings with ropivacaine [10].

Human cardiac myocytes have a membrane potential of approximately -90 mV [18], at which high micromolar concentrations of lidocaine or levobupivacaine were required to inhibit either Na_V1.5 variant. The enhanced sensitivity to LA block, in cells with membrane potentials more depolarized than -90 mV, coincides with the appearance of prevalent inactivation, in agreement with previous observations with lidocaine, bupivacaine (the racemic mixture of the S- and R-enantiomers) and ropivacaine [3,10,19,20]. Indeed, the large increase in native cardiac VASC affinity for lidocaine caused by inactivation has been well established [21,22]. Importantly, the inactivated state of Na_V1.5 predominates at the resting membrane potential of colon cancer cells (approximately -40 mV) [10,23]. Consistent with our previous observation with ropivacaine [10], both LAs shifted the peak window current (representing the membrane potential of peak steady state current) to potentials below -40 mV (Supp. fig. 1). Therefore, inactivation state-dependent high potency block provides a potential strategy for targeting Na_V1.5 on cancer cells while sparing cardiac myocytes, in which VASCs recover from inactivation during repolarisation even at high stimulation frequencies [18].

Our findings support previous observations of large state-dependent block by LAs which overshadows any VASC subtype specificity of these and related drugs [24,25]. There were differences observed for both $Na_V1.5$ variants with regard to their responses to LAs. Levobupivacaine caused a greater depolarizing shift in the V½ of activation of $aNa_V1.5$ compared to $nNa_V1.5$. In this regard it is relevant that the high affinity block of $Na_V1.5$ by LAs has been termed voltage-sensor block due a proposed stabilization of S4 voltage sensors in domains III and IV [2]. The $nNa_V1.5$ variant has several alternative amino acids in the S4 of domain I, including a lysine in place of the aspartate at the equivalent position in $aNa_V1.5$. This substitution leads to a depolarizing shift in the V½ of activation [10,17]. This lysine may stabilize the voltage sensors in a manner similar to that of LA stabilisation in $aNa_V1.5$, thereby masking this effect in $nNa_V1.5$.

There are developmental changes in block by lidocaine on neonatal and adult rat cardiac myocytes. Native neonatal VASCs are more sensitive to inhibition by lidocaine [26]. However, this developmental effect appears to be species specific [27]. Our observations for $nNa_V1.5$ and $aNa_V1.5$ suggest that any developmental changes in lidocaine sensitivity of human myocardial VASCs is unlikely to be explained by alternative $Na_V1.5$ splicing.

Molecules that bind to the LA site are thought to inhibit function through a similar mechanism [1,28]. Structural modelling suggests that these drugs inhibit current through steric interactions with the Na^+ binding sites close to the LA binding site [29]. As expected, in our study inhibition by the two LAs was similar; however, reversal of $Na_V1.5$ inactivation-dependent inhibition by levobupivacaine was slower than that of lidocaine. In this paradigm reversal of inhibition from slow inactivation likely represents a combination of recovery from slow inactivation and LA unbinding [21,30,31].

In the absence of LAs, both variants exhibited similar rates and contributions of fast and slow components of recovery from inactivation. Levobupivacaine reduced the slow rate of recovery from inactivation. Lidocaine, by contrast, did not affect either the fast or slow rates, but did somewhat increase the contribution of the slow component. The slower rate of slow recovery from inactivation in the presence of levobupivacaine could be caused by slow unbinding. Levobupivacaine is more hydrophobic than lidocaine due to its propylpiperidine group, and likely contributes to its higher potency. Additionally, highly hydrophobic inhibitors may be able to enter and exit the binding site via lipid fenestrations within the membrane spanning domains of VASCs and may remain trapped within hydrophobic pockets [28,32]. This may result in a longer lasting reduction in Na^+ entry by levobupivacaine than lidocaine.

Previous studies with bupivacaine on guinea-pig heart showed that bupivacaine fails to unbind in diastole and causes cardiotoxicity [33]. It is unclear to what extent VASC inhibition is involved in the cardiotoxic effects of LAs, although slowed recovery from inactivation is likely to be a contributing factor [34]. The S-enantiomer, levobupivacaine, appears less toxic than the R-enantiomer, according to preclinical data, and may therefore cause less slowing of recovery from inactivation [35]. However, our results demonstrate that levobupivacaine causes a greater slowing of recovery of $Na_V1.5$ inactivation than lidocaine and this may contribute to its cardiotoxicity.

In this study, we used recombinant $Na_V1.5$ isoforms, which avoids confounding influences of multiple VASCs and simplifies interpretation. However, in cardiac myocytes and cancer cells additional factors may influence the function of VASC and/or the actions of LAs.

The findings from this study will inform future *in vitro* investigations into the effectiveness of LAs in inhibiting colorectal cancer cell invasion, for instance, in Matrigel invasion assays. Furthermore, there is considerable interest in the potential of LAs to reduce recurrence or metastases following surgical tumour excision. In light of the direct inhibitory effects on metastatic cancer cell invasion it might be advantageous to administer LAs intravenously or directly onto tumours prior to excision [10]. Indeed, the activity of $Na_V1.5$ influences expression of multiple genes associated with the metastatic potential of colon cancer cells, and blockade by lidocaine, and the associated reduction in Na^+ entry, reduces the expression of these genes [9,11]. However, *in vivo*, LAs are mostly bound to serum proteins and this limits the free concentration available to inhibit $Na_V1.5$. Nevertheless, since low concentrations of lidocaine and levobupivacaine selectively interact with inactivated $Na_V1.5$, our study can inform the selection of an appropriate dose which maximises beneficial effects on cancer progression, while minimising the likelihood of cardiac toxicity.

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Figure legends:

Figure 1. Concentration-dependence of lidocaine and levobupivacaine mediated inhibition of $Na_V1.5$ currents. Traces show representative examples of voltage-activated n$Na_V1.5$ currents evoked by a step to 0 mV from a holding potential of -80 mV, in the presence of increasing concentrations of lidocaine (A) or levobupivacaine (B). Examples of a$Na_V1.5$ are similar and therefore not shown. Concentration-response relationship for lidocaine (C) and levobupivacaine (D) were derived from normalised peak amplitudes (with respect to control) in the presence of either LA for a$Na_V1.5$ (closed circles) and n$Na_V1.5$ (open circles). The fit to the data points represents the logistics function. Mean best fit parameters (and statistical analysis) are summarised in Supplementary table 1.

Figure 2. Current-voltage relationship of $Na_V1.5$. (A) Representative example of a family of control n$Na_V1.5$ currents evoked by a series of voltage steps (from holding potential of -80 mV). The voltage protocol is shown as an inset. Peak amplitudes in the absence or presence of 10 µM lidocaine (B) or 1 µM levobupivacaine (C) were

normalised to cell capacitance and plotted against the step voltage as mean current density values. Control current density values at each step potential were compared to those in the presence of LA using a paired t-test (two-tailed). Asterisks indicate the voltages at which the presence of LA caused a statistically significant reduction in current density values. Data for $aNa_V1.5$ are similar and therefore not shown. Peak current amplitudes evoked by step voltages between -80 mV and +20 mV were converted into conductance values (see Methods) and plotted for both $aNa_V1.5$ (D & F) and $nNa_V1.5$ (E & G) in the absence (closed circles) or presence (open circles) of LA (10 µM lidocaine, D & E; 1 µM levobupivacaine, F & G). The solid line indicates the Boltzmann function fitted to the points. The best fit parameters (and their statistical analyses) are summarised in Supplementary table 2. Mean $\Delta V_{½}$ of activation (H) and Δ slope (I). The data were analysed using a two-way ANOVA with LA and splice variant as the factors. Asterisk indicate statistically significant difference following the Bonferroni correction. Statistical parameters are given in the text.

Figure 3. Voltage-dependence of inactivation of $Na_V1.5$. Peak current amplitudes (evoked by step voltages to 0 mV) following a pre-pulse step to between -140 mV and -10 mV are normalised to maximum (see Methods) and plotted for both $aNa_V1.5$ (A & C) and $nNa_V1.5$ (B & D) in the absence (closed circles) or presence (open circles) of LA (10 µM lidocaine, A & B; 1 µM levobupivacaine, C & D). The solid line indicates the Boltzmann function fit to the points. The best fit parameters (and their statistical analyses) are summarised in Supplementary table 2. (E & F) Mean $\Delta V_{½}$ of activation (E) and extent of inhibition of maximum available current (F). The data were analysed using a two-way ANOVA with LA and splice variant as the factors. Asterisk indicate statistically significant difference following the Bonferroni correction. Statistical parameters are given in the text.

Figure 4. Lidocaine and levobupivacaine inhibition of $Na_V1.5$ is dependent on the inactivated state. (A) Representative examples show $nNa_V1.5$ currents (evoked by step to 0 mV from indicated holding potential) under control conditions (black trace) and immediately following 2 min exposure to 10 µM lidocaine (grey trace) in the absence of any stimulation. The extent of inhibition is plotted as mean % inhibition in the presence of lidocaine (10 µM) or levobupivacaine (1 µM) for both $aNa_V1.5$ (B) and $nNa_V1.5$ (C). The change in the mean % inhibition was analysed using a one-way ANOVA (details in the text). Asterisks indicate statistical significance ($p < 0.05$) from *post hoc* pairwise comparisons (with Tukey corrections) versus mean % inhibition at -80 mV.

Figure 5. Concentration-dependence of lidocaine and levobupivacaine stabilisation of the inactivated state of $Na_V1.5$ at -120 mV. (A to D) Voltage-dependence of inactivation relationships and their associated Boltzmann fits were constructed as described in Figure 4, with the exception that the holding membrane potential was -120 mV. Data show voltage-dependence of inactivation in the absence (closed circles) or in the presence of low concentrations of LA (10 µM lidocaine A & B; 1 µM levobupivacaine C & D; open circles) and high concentrations of LA (300 µM lidocaine A & B; 100 µM levobupivacaine C & D; open squares) for $aNa_V1.5$ (A & C) and $nNa_V1.5$ (B & D). The best fit parameters of the Boltzmann function (and their

statistical analyses) are summarised in Supplementary table 3. (E & F) Mean $\Delta V_{½}$ of activation (E) and extent of inhibition of maximum available current (G). The data were analysed using a two-way ANOVA with LA and splice variant as the factors. Asterisk indicate statistically significant difference following the Bonferroni correction. Statistical parameters are given in the text. Graphs show mean % inhibition of lidocaine (G) and levobupivacaine (H) following 2 min exposure to low (10 µM for lidocaine, 1 µM for levobupivacaine) and high concentrations (300 µM for lidocaine, 100 µM for levobupivacaine) without stimulation, with the holding membrane potential at -120 mV. The low concentration data is reproduced here from Figure 4 B & C for comparison. Asterisk indicate a statistically significant (t-test) increase in tonic inhibition with the higher concentration of LA.

Figure 6. Lidocaine and levobupivacaine influence recovery from inactivation of $Na_V1.5$. (A) Inset, the voltage command protocol used to test recovery from inactivation. (A & B) Graphs of peak current amplitudes evoked by a step voltage to 0 mV following recovery were normalised to maximal current amplitudes and plotted against the recovery time. Mean fractional recovery in control (closed circles), 10 µM lidocaine (open circles) and 1 µM levobupivacaine (open squares) are plotted for $aNa_V1.5$ (A) and $nNa_V1.5$ (B). The lines fitted to the points represent a double exponential function. The best fit parameters from the double exponential function (and their statistical analyses) are summarised in Supplementary table 4. From these parameters the weighted time constant (τ_w) was calculated. Mean τ_w are plotted in (C). We compared mean τ_w using a two-way ANOVA across treatment (control, lidocaine, levobupivacaine) and splice variant (details of statistics in the text). Asterisks indicate a statistically significant change in τ_w versus control following the Bonferroni correction.

Figure 1

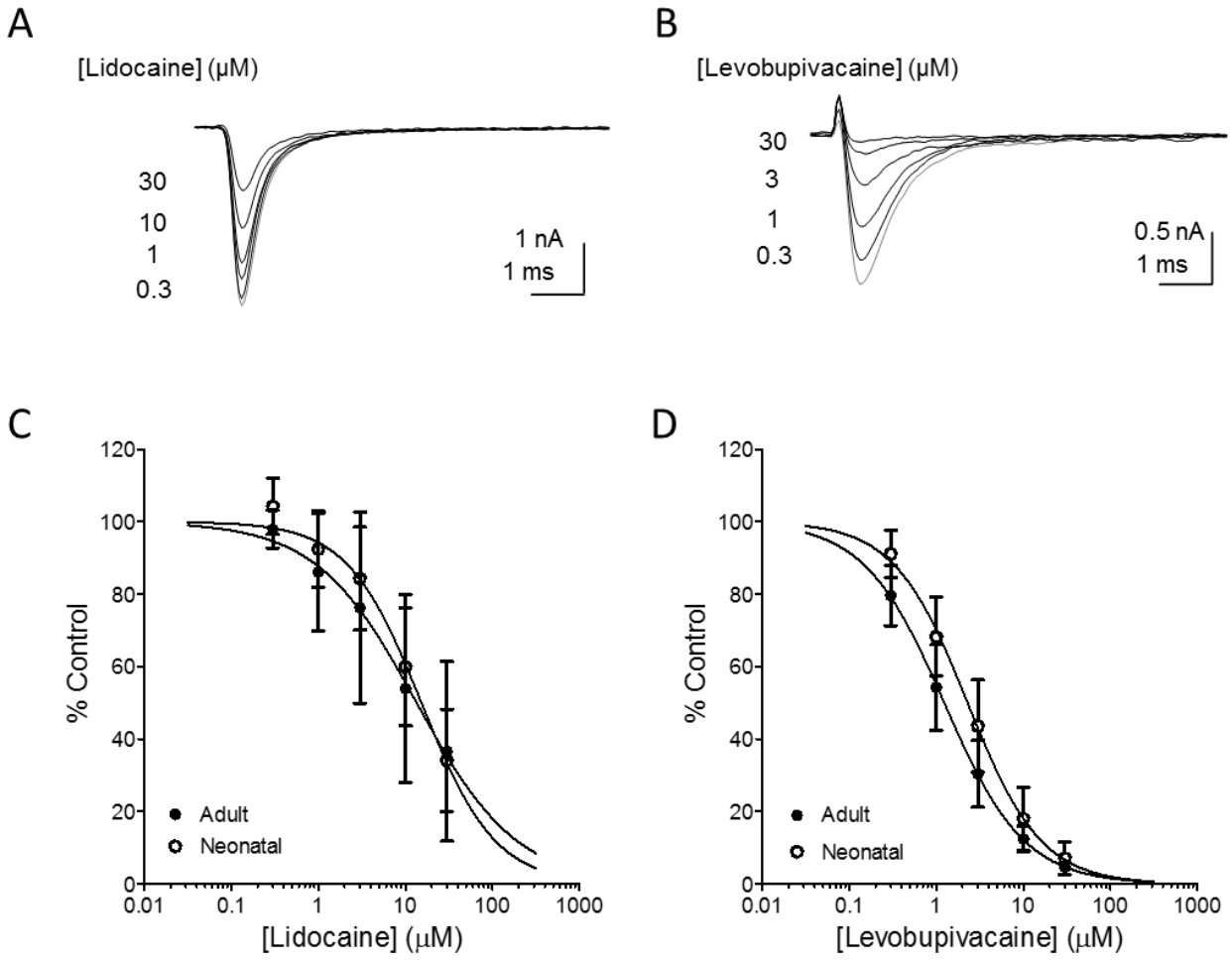

A
[Lidocaine] (μM)
30
10
1
0.3
1 nA
1 ms
B
[Levobupivacaine] (μM)
30
3
1
0.3
0.5 nA
1 ms
C
% Control
Adult
Neonatal
[Lidocaine] (μM)
D
% Control
Adult
Neonatal
[Levobupivacaine] (μM)

Figure 2

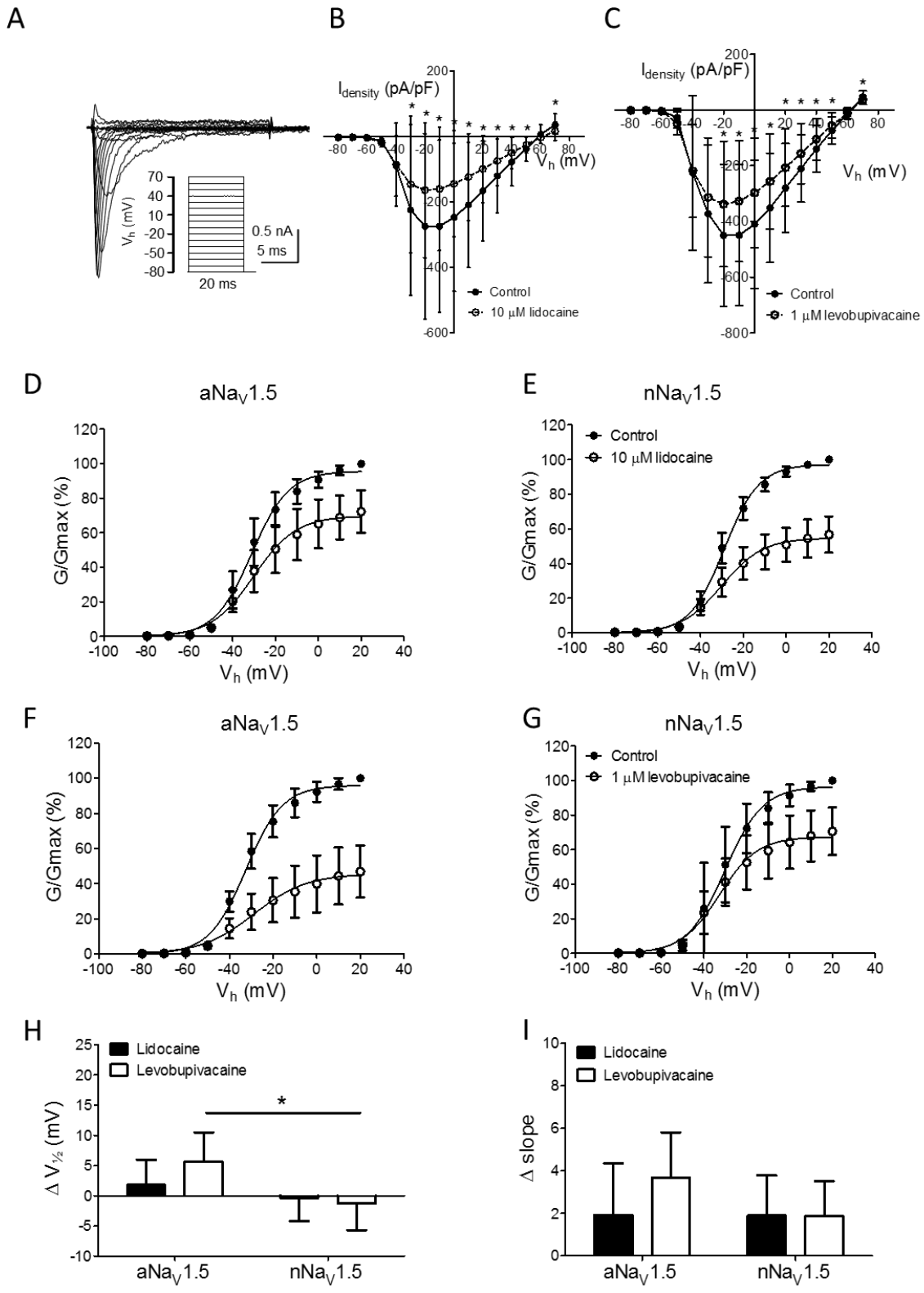

A
B
C
$I_{density}$ (pA/pF)
V_h (mV)
Control
10 μM lidocaine
1 μM levobupivacaine
0.5 nA
5 ms
20 ms
D
E
F
G
aNa$_V$1.5
nNa$_V$1.5
G/Gmax (%)
H
I
Lidocaine
Levobupivacaine
Δ $V_{½}$ (mV)
Δ slope

Figure 3

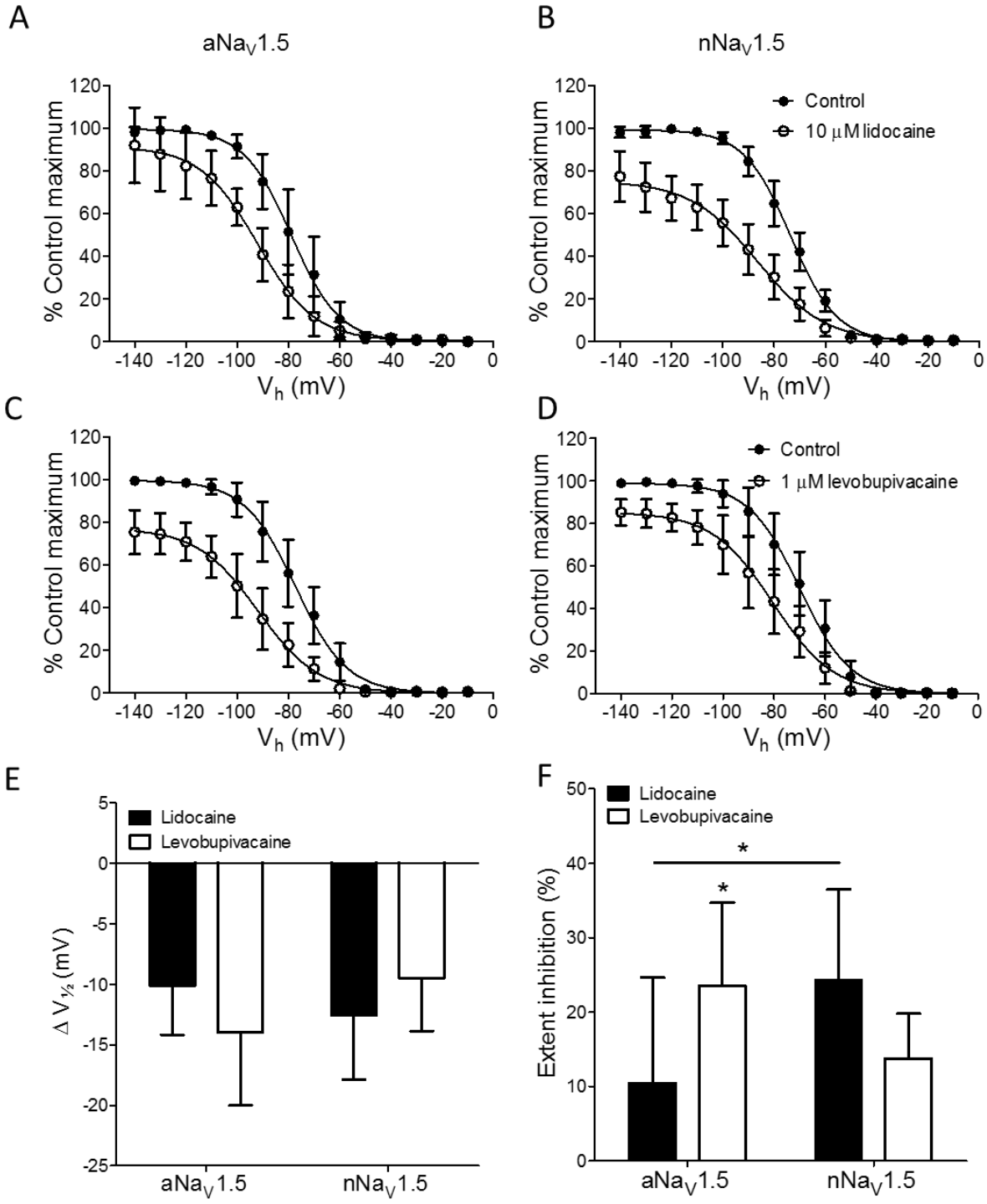

A
aNaV1.5
% Control maximum
Vh (mV)
B
nNaV1.5
Control
10 μM lidocaine
C
1 μM levobupivacaine
D
E
Lidocaine
Levobupivacaine
ΔV½ (mV)
F
Extent inhibition (%)
*

Figure 4

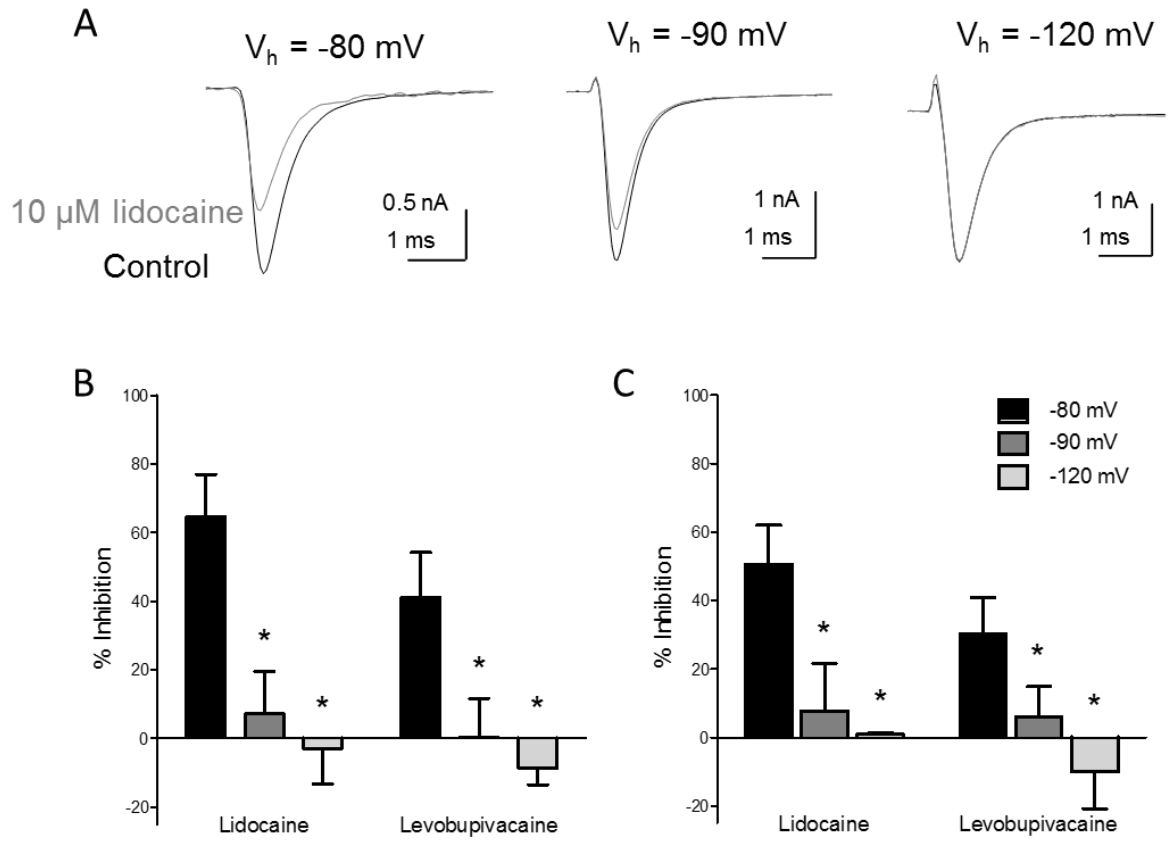

A
V_h = -80 mV
V_h = -90 mV
V_h = -120 mV
10 μM lidocaine
Control
0.5 nA
1 ms
1 nA
1 ms
1 nA
1 ms
B
C
% Inhibition
100
80
60
40
20
0
-20
-80 mV
-90 mV
-120 mV
Lidocaine
Levobupivacaine

Figure 5

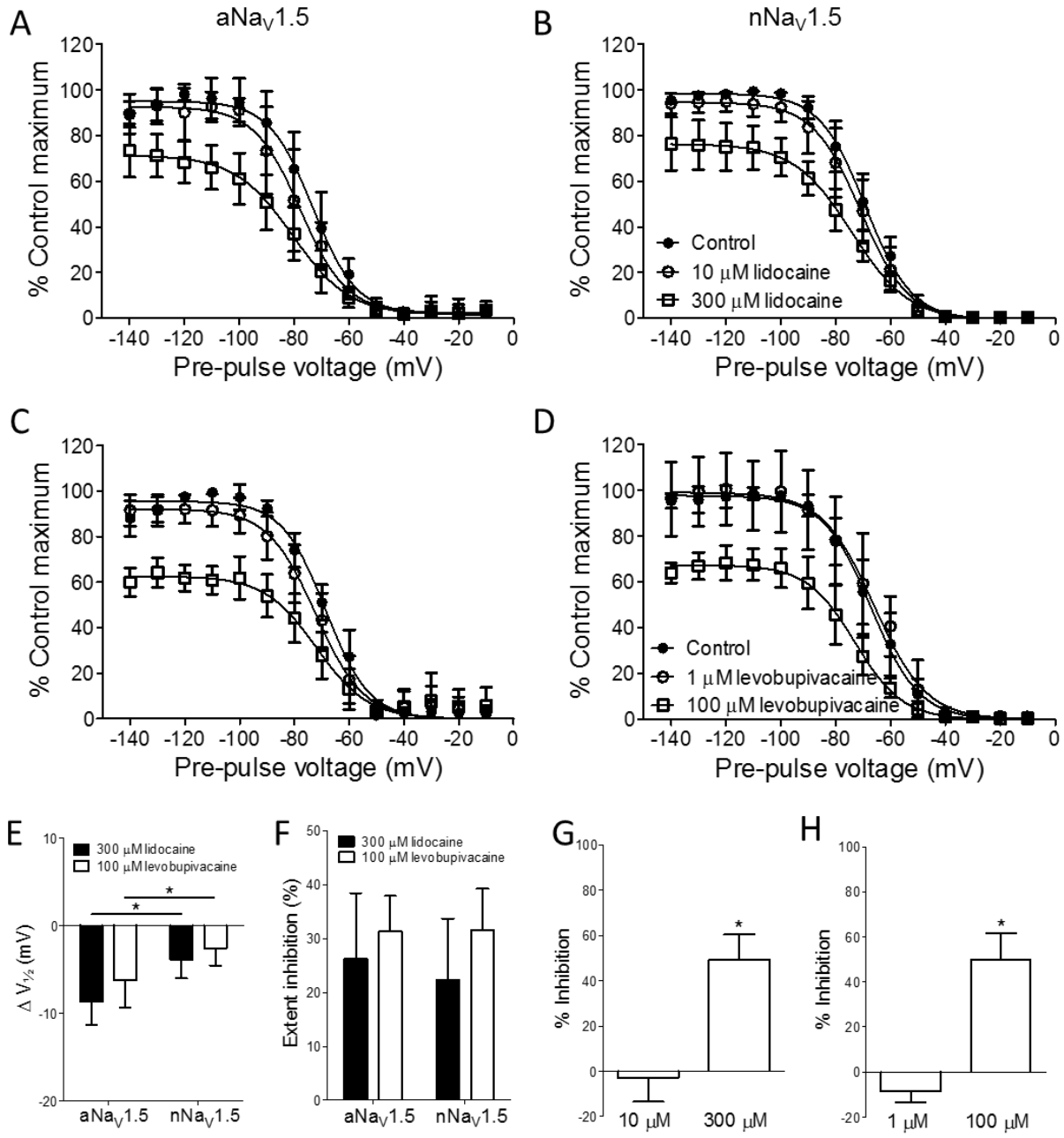
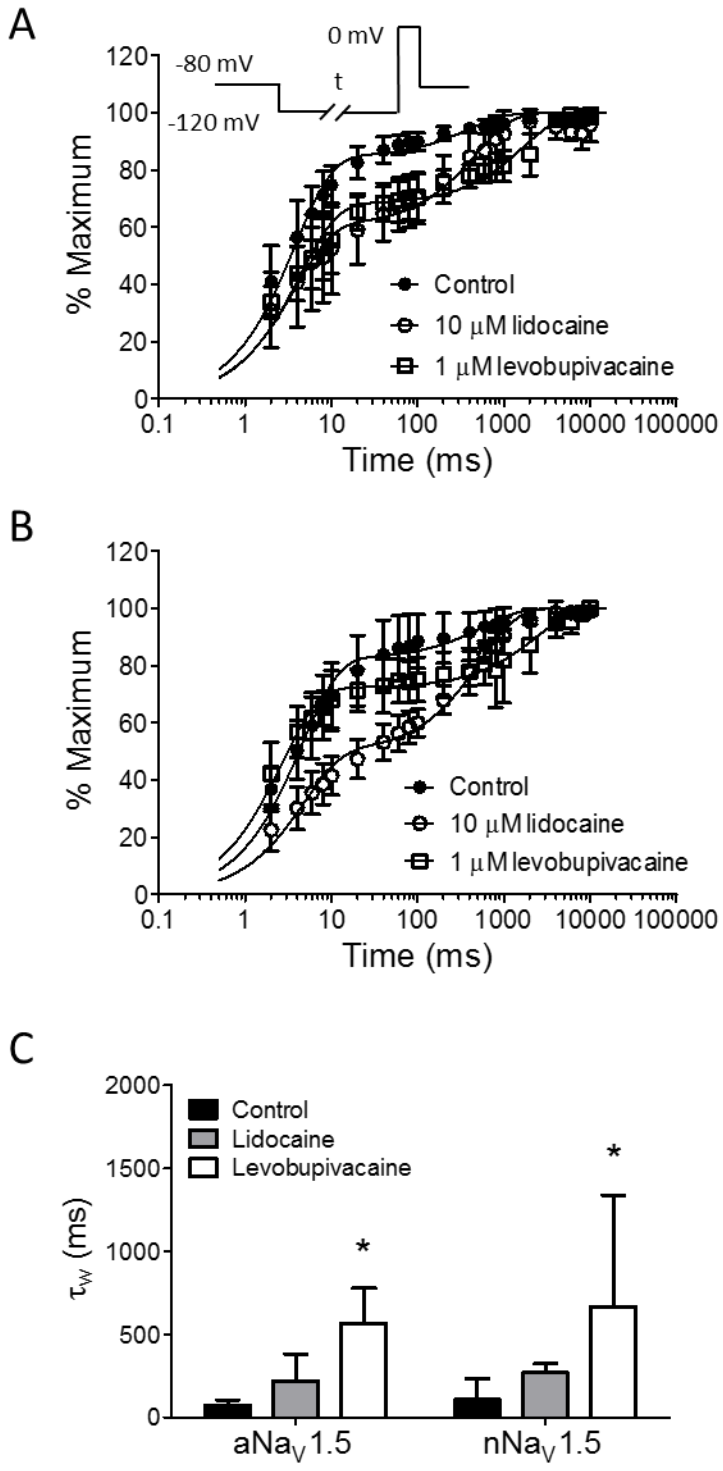

A
aNaV1.5
% Control maximum
Pre-pulse voltage (mV)
B
nNaV1.5
Control
10 μM lidocaine
300 μM lidocaine
C
D
Control
1 μM levobupivacaine
100 μM levobupivacaine
E
300 μM lidocaine
100 μM levobupivacaine
Δ V½ (mV)
aNaV1.5
nNaV1.5
F
Extent inhibition (%)
G
% Inhibition
10 μM
300 μM
H
1 μM
100 μM

Figure 6


A
0 mV
-80 mV
t
-120 mV
% Maximum
Control
10 μM lidocaine
1 μM levobupivacaine
Time (ms)
B
% Maximum
Control
10 μM lidocaine
1 μM levobupivacaine
Time (ms)
C
Control
Lidocaine
Levobupivacaine
τW (ms)
aNaV1.5
nNaV1.5