Non-genomic effects of sex hormones on CLC-1 may contribute to gender differences in myotonia congenita

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Running Title: Hormones and CLC-1

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Title:

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Abstract (150)

Myotonia congenita is caused by mutations in the voltage-gated chloride channel ClC-1. It is more severe in men than women and often worsens during pregnancy, but the basis for these gender differences is not known. We show here that both testosterone and progesterone rapidly and reversibly inhibit wild-type ClC-1 channels expressed in Xenopus oocytes by causing a prominent rightward shift in the voltage dependence of their open probability. In contrast, 17ß-estradiol at similar concentrations causes only a small shift. Progesterone and testosterone also profoundly inhibit ClC-1 channels containing the mutation F297S associated with dominantly inherited myotonia congenita. The effects of sex hormones are likely to be non-genomic because of their speed of onset and reversibility. These results suggest a possible mechanism to explain how the severity of myotonia congenita can be modulated by sex hormones.

Key words: CLC-1, hormone, non-genomic, myotonia congenita, chloride channel

Introduction

Myotonia congenita (MC) is caused by mutations in *CLCN1*, which encodes the skeletal muscle chloride channel ClC-1[1]. Mutations lead to a reduction of chloride current, which causes muscle stiffness and delayed muscle relaxation in affected patients. The severity of the phenotype shows marked intra- and inter-familial differences, even amongst individuals with identical mutations. Clinical observations suggest that hormonal factors may influence the phenotype. The most striking phenomenon is worsening of myotonia during pregnancy[2]. Indeed some female patients, particularly those with dominant mutations, may only experience symptoms while pregnant[3]. In addition our experience and that of others is that women tend to be less severely affected than men[2, 4]. It is also recognized that male carriers of recessive mutations are more likely to have subclinical (EMG) myotonia compared to females[5, 6]. Although hormones have long been suspected to influence disease severity, the underlying mechanism remains unclear.

It is increasingly evident that steroid hormones can exert rapid, non-genomic effects on many organ systems, in addition to conventional genomic actions mediated by nuclear receptors[7]. Non-genomic effects of sex hormones including testosterone, progesterone and 17ß-estradiol have been implicated in gender differences in many disorders including cardiovascular and bone disease, migraine, breast cancer and malaria[7-9]. We therefore asked whether non-genomic effects of sex hormones could contribute to the influence of gender and pregnancy on the severity of MC, by examining their actions on human ClC-1 channels expressed in Xenopus laevis oocytes.

Materials and Methods

Preparation of oocytes and site-directed mutagenesis was carried out as described previously[10]. Testosterone, progesterone and 17ß-estradiol were obtained from Sigma-Aldrich and dissolved in ethanol solution at 100 mM stock concentration. Stage V and VI oocytes were selected and injected with 2.5 ng wild-type (WT) or F297S mutant RNA. Oocytes were incubated for 60 hours at 10-15°C and then kept at 4°C. Pre-treatment of oocytes with pertussis toxin (PTx) was carried out by incubating oocytes injected with ClC-1 cRNA for 16 hours in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6) containing 2 µg/ml PTx (Sigma-Aldrich)[11]. Two-electrode voltage-clamp recordings were performed at room temperature (20-22 $^{\circ}$C) 3-5 days after RNA injection (GeneClamp 500B and DigiData 1200 Interface, Axon Instruments). Recording electrodes were filled with 3 M KCl and had a tip resistance between 2-5 MΩ. Oocytes were kept at -35mV holding potential. Tail currents were measured at -80 mV following voltage steps between +80 to -160mV. To test the effect of hormones on ClC-1, oocytes were perfused for 10 min with ND96 to establish a baseline, then exposed to ND96 containing 100 µM testosterone, progesterone or 17ß-estradiol for 10 min, and finally with ND96 for a 10 min wash-out period. Data acquisition, analysis and fitting were done with pCLAMP 9 (Axon Instruments) and Origin 6 (Microcal). All data are shown as mean ± standard error. Leak current was subtracted and tail currents were normalised to the peak current at +80 mV. Tail currents at -80mV were fitted with a Boltzmann distribution: $I(V)=I_o+(I_{max}-I_o)/(1+\exp((V-V½max)/dV))$, where I_{max} is the (extrapolated) current at maximal activation, I_o is a constant offset, V½max is the voltage at which half of the channels are open and dV

represents the slope factor. To test for reproducibility results were confirmed in at least two different batches of oocytes. Statistical analysis was done using Student's paired or unpaired t-tests.

Results

Application of 100 μM testosterone and progesterone resulted in a significant shift of the open probability curve to more positive voltages (Fig 1). The V½max shifted from -54.4 ± 1.7 mV to -24.6 ± 1.2 mV with testosterone ($n = 6$, $p << 0.01$) and from -57.0 ± 2.5 mV to -15.01 ± 6.64 mV with progesterone ($n = 7$, $p = 0.009$). The effects of testosterone and progesterone were similar ($p = 0.34$), rapid in onset and reversible, with most cells achieving ≥90% of the maximal change in current within 2 minutes (Fig 2a). None of the hormones had non-specific effects on endogenous chloride channels in water-injected control oocytes. Conversely, chloride currents in ClC-1 injected oocytes were unaffected by the vehicle, ethanol, applied without hormones (data not shown).

Non-genomic effects of hormones in Xenopus oocytes involve both membrane-located androgen and progesterone receptors, and newly-identified PTx-sensitive G-protein coupled receptors[12]. PTx (250 ng/mL applied by overnight incubation) had no significant effect on the inhibition mediated by the hormones (Fig 3). It is thus unlikely that the rapid inhibition of ClC-1 currents by progesterone and testosterone in Xenopus oocytes involves this class of G protein-coupled receptors.

In contrast to testosterone and progesterone, exposure of ClC-1 to 100 μM 17ß-estradiol resulted in only a small (albeit statistically significant) shift of V½max to more positive

voltages (average shift 7.2 ± 1.8 mV, n = 6, p = 0.01; Fig 1c). This V½max shift was significantly smaller than that caused by testosterone ($p << 0.01$) or progesterone ($p = 0.02$). The concentration-dependence of the effects of testosterone and progesterone was similar (Fig 2b), unlike that of 17ß-estradiol. The hormones could not be reliably tested at concentrations > 100 μM because of precipitation from the perfusion solution.

Given the link between disease severity and pregnancy, we asked whether the effects of hormones were also present in oocytes expressing mutant channels. The F297S mutation, which is associated with dominantly inherited MC, has an open probability curve shifted to more positive voltages compared to WT channels[10]. Exposure to testosterone resulted in a similar shift of the open probability curve as seen with WT channels, changing from a baseline of -8.8 ± 3.1 mV to +22.6 ± 7.0 mV (n = 5, $p << 0.01$). This shift was not significantly different from that seen in WT channels ($p = 0.77$; Fig 3). (A similar shift was caused by progesterone in an oocyte expressing F297S mutant channels.) This result suggests that the right-shift conferred by sex hormones is additive with that caused by the disease-associated mutation, and is consistent with more severe symptoms being observed in the presence of elevated testosterone and progesterone.

Discussion

We have shown that ClC-1 channels are inhibited by testosterone and progesterone via a rapid, most likely non-genomic sex hormone signalling pathway. This provides a candidate mechanistic explanation for the clinical observation that symptoms due to loss of function of ClC-1 channels are exacerbated when testosterone or progesterone are elevated. The main caveat is that the hormone concentrations used here are higher than

physiological levels: progesterone levels during late pregnancy are approximately 0.2μM [13]. This may reflect a limitation of oocytes as an experimental tool, because prolonged incubation in low concentrations of sex hormones is impractical [14]. The relative specificity of the effect for testosterone and progesterone, but not 17ß-estradiol suggests that the inhibition of chloride channels is not simply a non-specific steroid hormone effect on membranes [15]. If smaller but qualitatively similar actions occur at physiologically relevant concentrations, they could contribute to gender and pregnancy-related differences of symptoms in MC. Because oocytes do not lend themselves to studies of prolonged incubation in sex hormones, we were unable to determine whether lower concentrations have a qualitatively similar, slowly-evolving action on ClC-1.

Non-genomic effects of hormones on ion channels in muscle fibers have been investigated in cardiac muscle[16] and smooth muscle[17] but have hitherto received relatively little attention in skeletal muscle[18, 19]. This is the first demonstration of a non-genomic effect on a cloned skeletal muscle chloride channel. Interestingly, the steroid hormone 1α,25(OH)(2) vitamin D(3) has been shown to potentiate chloride currents in osteoclasts through a non-genomic pathway[20], although the underlying channels are not known. Our results suggest that the interaction between sex hormones and CLC-1 channels does not involve a PTx–sensitive signalling pathway, but instead may rely on classic androgen and progesterone receptors located in the membranes of oocytes. (Because the PTx incubation protocol used here has been shown to be highly effective in blocking G-protein pathways [21], and the same batch of PTx was effective in a separate study on G-protein-dependent signalling in HEK cells, we consider it unlikely that G protein signalling was unaffected in the oocytes.) An alternative,

intriguing possibility, is that the sex hormones may bind directly to the ClC-1 channels. Further work will be needed to determine the concentration-dependence and signalling pathway used by sex hormones to target CLC-1 channels in skeletal muscle.

Understanding the mechanism of hormonal regulation of CLC-1 may help to develop new forms of treatment for patients who suffer from myotonia. Analogous actions of neurosteroids on $GABA_A$ receptors have been proposed to underlie catamenial seizure predisposition and premenstrual dysphoric symptoms[22, 23]. It remains to be determined whether other members of the ClC family of chloride channels expressed in the CNS contribute to the hormonal influences observed in migraine and epilepsy.

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Figure Legends

Fig 1 – Changes in the open probability of WT CLC-1 channels from baseline (black triangles) to depolarised voltages after hormone application (open triangles) and return following washout (grey triangles). A) 100 μM Testosterone; B) 100 μM Progesterone; C) 100 μM 17β-estradiol. D) and E) Whole cell current traces from WT at baseline (D) and with 100 μM Testosterone (E) in a representative cell. F) Voltage clamp protocol with the point at which tail current was sampled indicated by an arrow.

Fig 2 – A) Rapid onset and washout of hormonal effect. A representative cell showing changes in current amplitude in response to a single voltage step at 15 second intervals. Testosterone was applied twice for approximately 10 min as indicated by the bars above the data points. B) Dose responses for hormonal effect on V½ max

Fig 3 – Comparison of effects on V½ max of wild-type (WT) and F297S mutant CLC-1 channels for Testosterone (TT), Progesterone (PG) and 17β-estradiol (ES). Preincuabtion with Pertussis toxin (PTx) caused non-specific toxicity leading to increased cell death and increased non-specific leak, which is reflected in smaller sample size and larger error bars. Numbers represent numbers of oocytes recorded from.

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Figure 1

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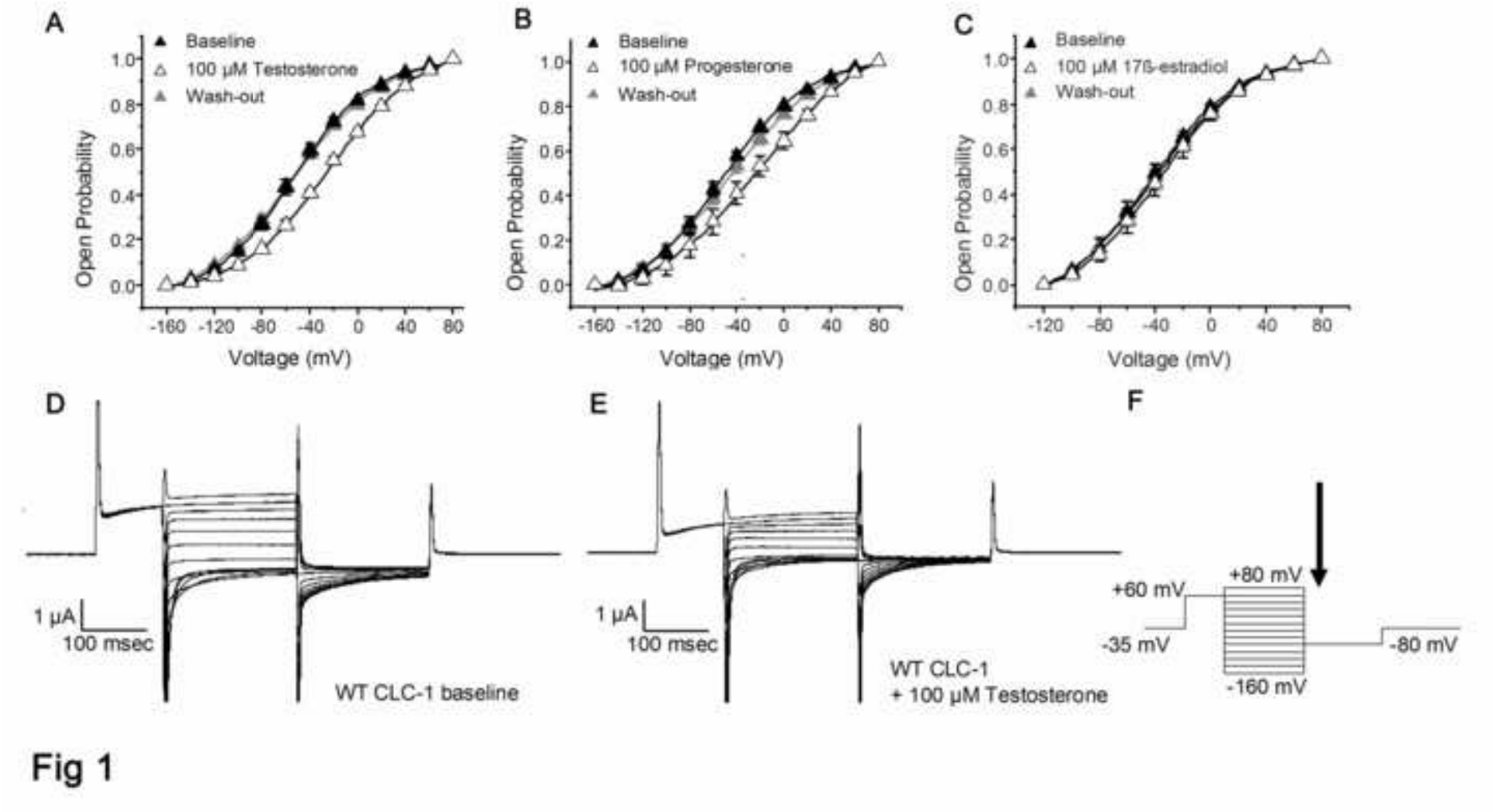

Fig 1

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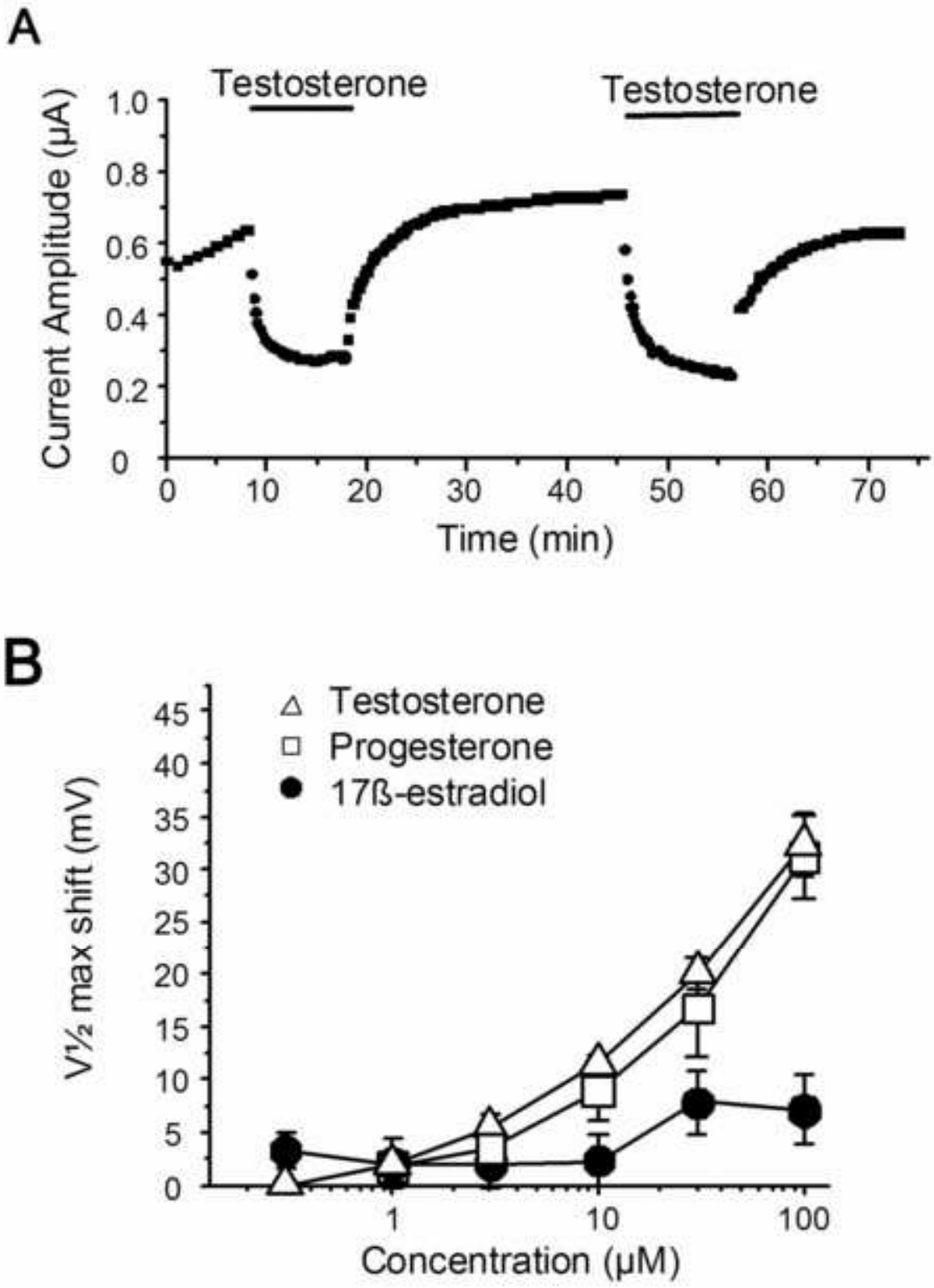

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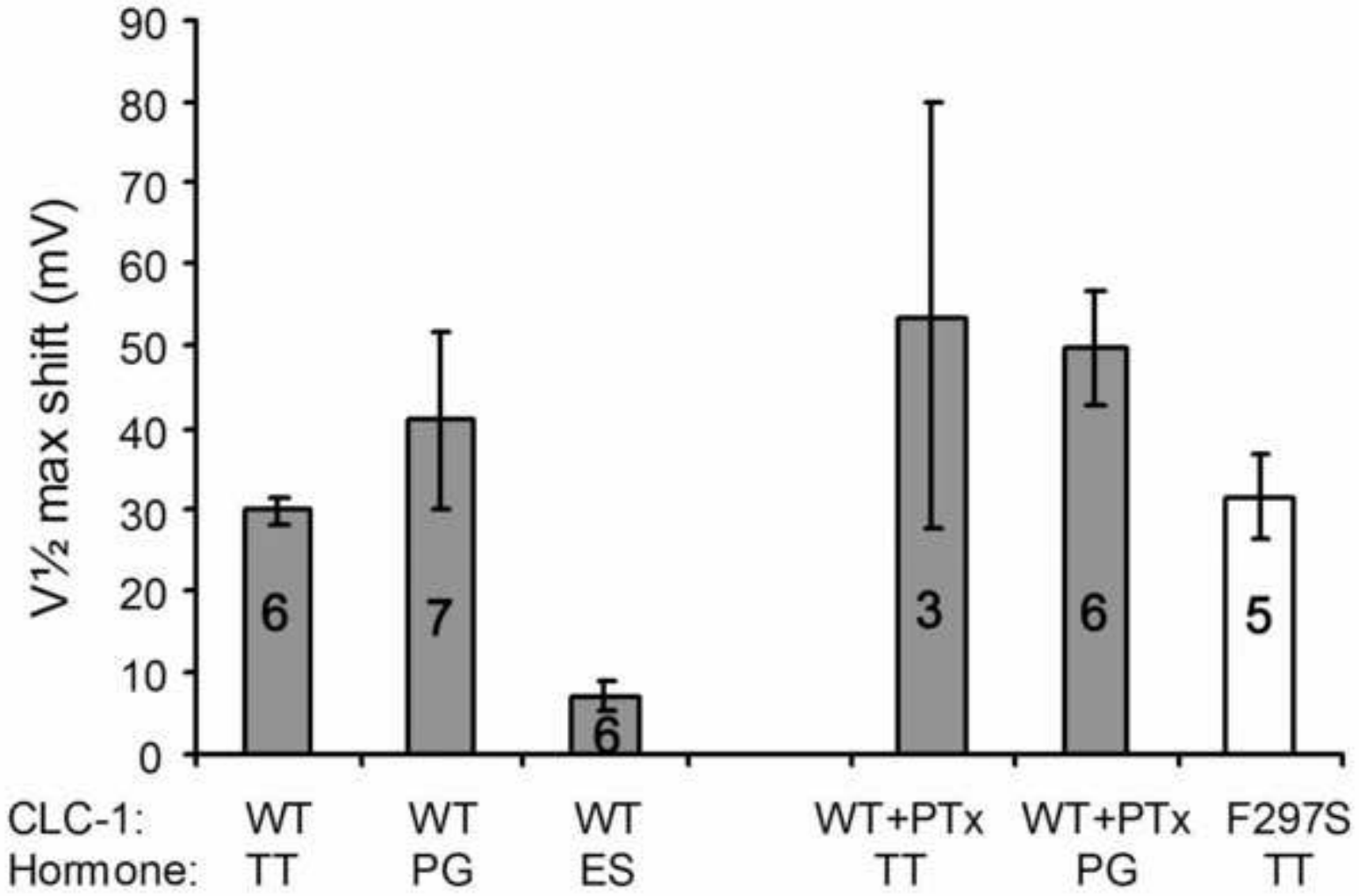

Fig 3