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A Study on the Hormonal Regulation and Novel Role of Na+ Leak Channel, Non-Selective (NALCN) in Human Myometrial Smooth Muscle Cells

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by
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Dedicated to my parents and my maternal grandparents.

ABSTRACT

A Study on the Hormonal Regulation and Novel Role of Na^+ Leak Channel, Non-Selective (NALCN) in Human Myometrial Smooth Muscle Cells

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Washington University in St. Louis, 2022

Professor Sarah K. England, Chairperson

During pregnancy, the uterus transitions from a quiescent state to an excitable, highly contractile state to deliver the fetus. Two important contributors essential for this transition are progesterone (P4) and estrogen (E2), which promote quiescence or contraction, respectively, by acting on the myometrial smooth muscle cells (MSMCs). While these hormones regulate uterine contractions, it is unclear how they affect electrical activity of MSMCs, which underlies uterine contractile activity. Our lab recently identified Na^+ leak channel, non-selective (NALCN) as a component of the leak current in human MSMCs and showed that mice lacking NALCN in the uterus have dysfunctional labor. In this thesis, we first sought to determine whether P4 and E2 directly regulated NALCN expression and activity. We established that P4, upregulates the expression of NALCN by acting through progesterone response elements in the NALCN promoter while E2 downregulates this channel in humans MSMCs. These expression changes translated functionally as P4 significantly enhanced, and E2 significantly inhibited a NALCN-dependent leak current in human MSMCs. Our findings that NALCN is upregulated by P4, a pro-quiescent hormone: 1) contradicts the traditional concept that Na^+ influx depolarizes the cell to enhance cell excitability and 2) provides a novel role for Na^+ entry through NALCN. Thus, we

further explored if Na^+ influx through NALCN may contribute to promoting quiescence by activating other signaling pathways. We provide evidence that current through NALCN is a source of Na^+ to activate SLO2.1, the Na^+-activated K^+ channel, which contributes to the resting membrane potential (V_m) of MSMCs. Additionally, we observed that SLO2.1 was in proximity with NALCN in human MSMCs and our data suggests these channels form a functional complex to maintain V_m and modulate intracellular Ca^{2+} levels. Overall, our findings identified a new form of regulation of NALCN in human MSMCs and unmasked how NALCN contributes to V_m in order to regulate myometrial excitability.

Chapter 1: Introduction

1.1 Pregnancy and Labor

Human pregnancy is divided into three trimesters each lasting approximately three months. The first trimester represents the first 12 weeks of pregnancy and starts with conception, which is when the sperm fertilizes the egg. It is also a critical time for the development of the fetus' body structure and organs. The second trimester, which lasts from 13 to 28 weeks, is the time when the fetal organs fully develop. Finally, in the third trimester as fetal development continues, the body of the fetus increases in size and weight in preparation for delivery, which occurs at ~40 weeks [1, 2]. In order to adapt to these changes in fetal growth and development, pregnancy requires behavioral, cardiovascular, hormonal, metabolic, renal, hematologic, respiratory, and reproductive adaptations in the mother.

A key adaptation of pregnancy is the remodeling of the uterus. First, there is an increase in uterine size from 40-70 grams in the non-pregnant state to 1100-1200 grams by the end of pregnancy [3]. Uterine growth occurs through both hypertrophy and hyperplasia of smooth muscle cells within the uterus, which help to accommodate the growing fetus for the majority of pregnancy and increases mechanical stretch to prepare for parturition at the end of pregnancy [4]. Second, there is a 3.5-fold increase in blood volume of the mother from non-pregnant to late gestation [5]. For this to occur, the uteroplacental spiral arteries go through a series of changes, including replacing the muscular media and the endothelium. These changes reflect the development and growth of the uteroplacental circulation, which fulfill the metabolic needs of the fetus during gestation. Third, the uterus undergoes autonomic denervation, showing a loss of

adrenergic and cholinergic innervation [6]. Overall, these three major adaptations of the uterus allow for successful growth, maturation and delivery of the fetus.

The growth, remodeling and adaptations that occur during pregnancy culminate with labor; the delivery of the fetus and the placenta. Labor is divided into three stages: 1) onset of labor where the cervix fully dilates, 2) delivery of the fetus, and 3) delivery of the placenta and membranes. Contractions of the uterus occur throughout pregnancy, however at the time of labor, contractions increase in frequency and intensity for successful delivery of the fetus and the placenta [7]. However, we still lack full understanding of the molecular and physiological mechanisms that underlie successful pregnancy and labor.

Many adverse pregnancy outcomes are associated with dysfunctional uterine contractions. For example; uterine tachysystole, defined as more than five contractions in a ten minute period averaged over a 30-minute window [8] occurs in 10-50% of labors and is associated with fetal acidemia [9], decreased fetal oxygen saturation, and deterioration in the fetal heart patterns [10]. Babies from deliveries complicated by tachysystole have higher rates of Neonatal Intensive Care Unit admission and neonatal sepsis [11]. Another type of aberrant uterine activity is dystocia, where the uterus does not efficiently contract during parturition. Dystocia is associated with a higher risk of stillbirth or perinatal death [12] and is the leading indication for primary cesarean delivery in the United States [13]. The last 40 years have seen an exponential increase in the rate of cesarean deliveries: in 2018, 31.9% of infants born in the US were delivered by cesarean section, a high rate that is inversely correlated with the rate necessary for maintaining the lowest infant and maternal mortality [14, 15]. The final type of aberrant uterine activity is preterm labor, or pathological contractions that occur prior to 37 weeks of gestation [16]. About 67% of preterm birth are spontaneous labor, while the remaining are

medically indicated due to maternal or fetal complications, such as preeclampsia or growth restriction [17]. Each year, fifteen million babies are born preterm with global rates ranging from 5% to 18%, with the highest burden in North America and Africa [18]. A significant public health issue; preterm birth is the leading cause of neonatal death and the second cause of childhood death below 5 years old [19]. Those born preterm have higher rates of health and developmental problems that persists into adult life [20]. In the United States, the annual economic burden associated with preterm birth is at least $26.2 billion and rising [21].

Many health agencies, including the Society for Maternal-Fetal Medicine, Centers for Disease Control and Prevention, American College of Obstetricians and Gynecologists and World Health Organization, are focusing their efforts on the reduction of preterm births and cesarean deliveries, in part, by advocating for more research aimed at understanding normal labor progression [22]. Yet, progress has been hindered primarily due to our incomplete understanding of uterine physiology. It is therefore vitally important for maternal-fetal health that we continue to study the mechanisms of uterine quiescence and contractility that govern both normal and pathological labor.

1.2 Uterine Anatomy and Physiology

1.2.1 Uterine Anatomy

The uterus is a “pear-shaped organ” that has a rounded upward component containing the fundus and the body of the uterus and a lower, elongated end forming the cervix. The uterus is comprised of three layers: 1) the serosa, which is a continuation of the peritoneal epithelium and covers the outer surface of the uterus, 2) the endometrium, which covers the inner surface of the uterus and is the site of implantation and placentation, and 3) the myometrium, which is the muscular layer that lies between the serosa and endometrium. The myometrium is divided into

three layers: 1) the stratum submucosum composed of primarily longitudinal smooth muscle fibers, 2) the stratum vasculare, which consists of circular smooth muscle and 3) the stratum subserosum, which contains large longitudinal smooth muscle fibers [23, 24]. The smooth muscle of the stratum submucosum coordinates the endometrial movements (subtle contractions) observed in the menstrual cycle while the myometrial smooth muscle cells (MSMCs) of the stratum subserosum facilitate the excitation-contraction coupling that induces the large uterine contractions that occur during parturition [24, 25].

1.2.2 Regulation of Myometrial Activity

Contractile Activity

In MSMCs, the excitation-contraction coupling cascade begins when intracellular Ca^{2+} ions bind to calmodulin, a Ca^{2+}-dependent cytosolic protein [26]. This complex activates myosin light chain kinase (MLCK) to increase phosphorylation of myosin light chain MLC_{20}, the regulatory subunit of the myosin filament [27]. Myosin thick filaments are composed of two regulatory light chains and two heavy chains. The heavy chains contain a head region containing an ATPase domain, which produces energy required for the contractions and actin-binding domain. Once MLC_{20} is phosphorylated, the ATPase domain hydrolyzes ATP and the angle of the myosin heads are more aligned with the actin filaments. Myosin thick filaments pull the actin filaments towards the center of the cell, shortening the MSMC. Myosin then releases ADP, binds new ATP, and gets released from the actin, in order to start the cycle again [28].

Hormonal Regulation

Uterine contractile status during pregnancy is mediated in part by the actions of two sex steroid hormones, progesterone (P4) and estrogen (E2). Progesterone and E2 signal primarily

through their respective nuclear receptors, a family of ligand-regulated transcription factors [29-31]. Both hormones can freely cross the cell plasma membrane and bind to their respective receptors, and once activated, can regulate the transcription of various genes including those associated with uterine contractile status [31]. Progesterone and E2 can also function at non-genomic levels by activating membrane localized and G-protein coupled steroid receptors [32, 33].

Progesterone's actions are primarily mediated by its binding to two classical nuclear receptor isoforms, PR-A (94 kDa) and PR-B (114 kDa), produced by alternative transcription and translational initiation from a single gene, *PGR* [34, 35]. The PR-A and PR-B isoforms have opposing actions on myometrial contractility: PR-B dominates throughout pregnancy and maintains myometrial quiescence while PR-A is functionally dominant at term and promotes contraction [34]. Progesterone bound to PR-B maintains uterine quiescence by direct and indirect mechanisms. The P4-PR-B complex directly binds to progesterone response elements (PRE) of contractile or proinflammatory genes to inhibit transcription [36]. For example, P4-PR-B directly represses, the oxytocin receptor (*OXTR*), connexin-43 (*GJA1*), proinflammatory gene cyclooxygenase 2 (*PTGS2)*, nuclear factor kappa B subunit (*NFKB1)* and interleukin-6 (*IL6*), all genes which are known to promote contraction (Figure 1.1A) [37-39]. In addition to direct binding to PREs, the P4-PR-B complex blocks the binding of transcription factors that upregulate these genes, while inducing expression of transcription factors that promote quiescence. For example, P4-PR-B inhibits the binding of nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) to promoters of genes such as *PTGS2*, *OXTR*, and *GJA1* (Figure 1.1B) [40-42]. In addition, P4-PR-B increases the expression of three transcriptional regulators: IκBα, a crucial inhibitor of NF-κB activation, ZEB1 and ZEB2 [40, 43-45].

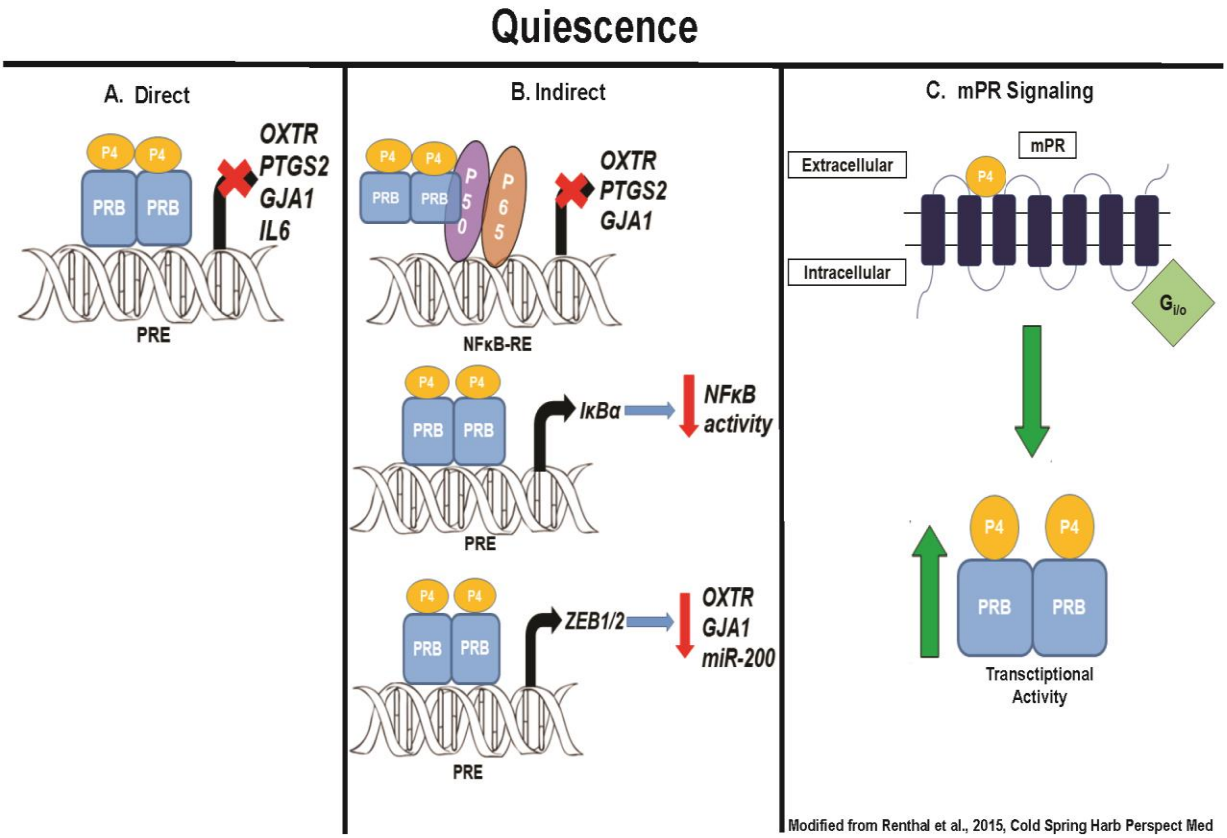

Figure 1.1: Hormonal Regulation of Uterine Quiescence during Pregnancy
A) P4-PR-B complex binds to the PRE promoter region and directly downregulates the transcription of *OXTR*, *PTGS2*, *GJA1* and *IL6*. B) P4-PR-B complex indirectly downregulates the transcription of contractile and pro-inflammatory genes by upregulating *IκBα* and reducing NFκB activity and upregulating *ZEB1/2* expression to decrease expression of *OXTR*, *GJA1* and miR-200. C) The mPRs bound to P4 signal through PR-B to promote P4-PR-B complex transcriptional activity.

The P4-PR-B complex causes rapid induction of IκBα mRNA and protein expression and blocks degradation of IκBα by the proteasome [40, 46]. With the upregulation of IκBα, more NF-κB is sequestered in an inactive state, further inhibiting transcription of *PTGS2*, *OXTR*, and *GJA1* (Figure 1.1B). ZEB1 and ZEB2 downregulate the transcription of *OXTR* and *GJA1* by directly binding to their promoters or indirectly by inhibiting the expression of miR-200 family, microRNAs that upregulate *OXTR* and *GJA1* (Figure 1.1B) [45, 47]. Additionally, the membrane localized progesterone receptors (mPR_α and mPR_β) signal through the P4-PR-B complex to promote uterine quiescence [48]. Specifically, P4 bound to mPRs activates G_i protein alpha subunit and increases PR-B transcriptional activity (Figure 1.1C) [48].

In most mammals including mice, parturition is initiated by a decrease in circulating P4 levels transitioning the myometrial cells from a quiescent to a contractile state. However, humans do not exhibit a decrease in serum P4 at term, but instead become less sensitive to P4, termed a "functional progesterone withdrawal." This occurs through several potential mechanisms: 1) increased expression of specific endogenous PR antagonists, such as protein-associated splicing factor (PSF) [49], 2) decline in the levels of PR-associated co-regulators [50], 3) increased expression of the P4 metabolizing enzyme, 20α-hydroxysteroid dehydrogenase (HSD) [51] and/or 4) increased PR-A expression, leading to an increased PR-A/PR-B ratio (Figure 1.2A) [39, 52, 53].

PR-A, the pro-contractile PR isoform, acts as a dominant negative to repress the genomic and non-genomic pro-quiescent mechanisms of PR-B [54-57]. Pro-inflammatory molecules such as IL-1β and prostaglandin-$F_{2\alpha}$ increase the abundance and stability of PR-A, which contributes to the functional withdrawal observed at term (Figure 1.2B) [52, 58, 59].

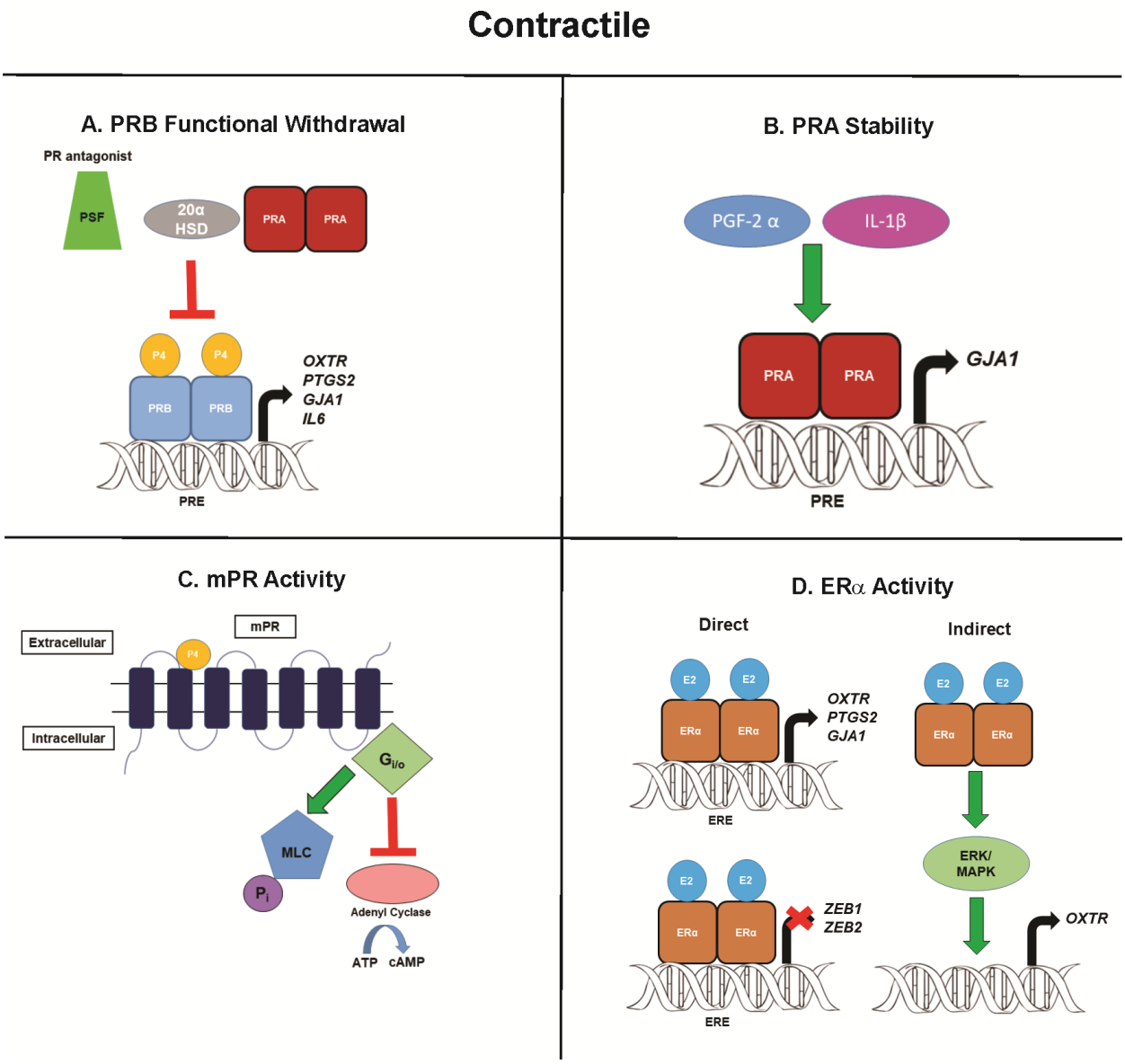

Figure 1.2: Hormonal Regulation during the Contractile Period of Pregnancy
A) The increase in PR antagonists (PSF), 20α HSD and PR-A leads to PR-B functional withdrawal at term. B) The increase in PGF-2α and IL-1β enhances PR-A stability and upregulation of *GJA1*. C) Due to the decline of PR-B function, the crosstalk between mPRs and PR-B is inhibited allowing for mPR and $G_{i/o}$ contractile pathway. D) ERα activity increases leads to: 1) direct upregulation of *OXTR*, *PTGS2* and *GJA1* and downregulation of *ZEB1/2* and 2) indirect upregulation of *OXTR* by activation of ERK/MAPK pathway.

Additionally, increase of 20α HSD decreases local P4, leaving many nuclear PR-A receptors unliganded. At term, unliganded PR-A transcriptionally activates *GJA1* to promote a contractile environment (Figure 1.2 B) [53]. Finally, at a non-genomic level, with the decrease in PR-B, activated mPRs can no longer transactivate PR-B. These events allow P4 to act primarily on the mPRs during labor, to evoke responses such as inhibition of adenylyl cyclase and phosphorylation of myosin light chain (Figure 1.2C). This also sensitizes the myometrium toward a more contractile state at term [48].

Estrogen binding to estrogen receptor α (ERα) promote myometrial contractility as pregnancy progresses to term by signaling through both classical and the non-classical pathways. Classically, E2 bound to ERα results in direct binding to estrogen response elements (EREs) and increases transcription of downstream genes including *OXTR, CX43* and *COX-2* (Figure 1.2 D) [51, 60-62]. E2 also downregulates ZEB1 and ZEB2 expression, which indirectly upregulates pro-inflammatory genes like *COX*-2 (Figure 1.2D) [47]. COX-2 catalyzes the production of prostaglandins to initiate labor [63]. In the non-classical pathway, E2 bound to ERα can also activate the extracellularly regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway to indirectly upregulate the transcription of *OXTR* gene (Figure 1.2D) [64].

The increase in ERα signaling and enhanced uterine stretch caused by the growing fetus also provide an important stimulus for the initiation of labor [65, 66]. These factors stimulate an inflammatory response, invasion of the myometrium by neutrophils and macrophages, and an increase in levels of proinflammatory cytokines in the amniotic fluid [67-70]. The release of chemokines and cytokines by invading immune cells causes activation of many inflammation-associated transcription factors including NF-κB and AP-1 as well as an increase in ERα [71-73]. These key players promote increased expression of myometrial pro-inflammatory genes (e.g. *IL*-

1β and IL-8) and genes of contractile-associated proteins (*OXTR*, *CX43* and *COX2*) [41, 42, 74, 75].

Electrical Basis of Myometrial Contractile Activity

The electrical activity of MSMCs, which underlies uterine contractions, are caused by specific changes in membrane permeability to Ca^{2+}, K^+, Na^+ and Cl^- ions, which result from changes in both expression and activity of various ion channels, ion transporters, pumps and exchangers [76, 77]. A balance of K^+ efflux and Na^+ influx through K^+ leak currents and inward rectifying K^+ channels or Na^+ leak currents, respectively, primarily sets the negative resting membrane potential (V_m) in human MSMCs [76, 78]. In these cells, the V_m has been recorded at ~-75 millivolts (mV) at week 28 of pregnancy, closer to the K^+ equilibrium potential [79]. By the end of pregnancy, the V_m steadily depolarizes (more positive V_m) to ~ -40 mV at term [79].

The MSMC action potential underlies control of uterine contractions and are comprised of three phases: 1) the slow upstroke and depolarization 2) repolarization and 3) the slow pacemaking phase [80-83]. The upstroke is primarily due to Ca^{2+} influx through L-type Ca^{2+} channels, which are activated at ~-40 mV in MSMCs [84, 85]. These channels are slow to both activate and inactivate [86]. This allows the channels to be open for ten to hundred milliseconds, which allows the sustained plateau of depolarization characteristic of MSMCs and uterine contractions [87]. Repolarization is due, in part, to both voltage-dependent and Ca^{2+}-activated K^+ channels [88]. These channels generate a hyperpolarizing outward K^+ current, which inactivates voltage dependent L-type Ca^{2+} channels [88]. Finally, the slow pacemaking phase generates the rhythmic oscillations in the membrane potential and depolarizes the membrane to activate L-type Ca^{2+} channels, supposedley through cationic influx conducted by a non-selective sodium channel [81, 89]. Overall, processes that alter the expression and activity of ion channels,

such as hormones, can effect the V_m and/or the shape and length of the action potentials and subsequently myometrial contractions [90]. This thesis specifically investigates the effect of hormonal regulation on the expression and activity of a non-selective sodium channel in human MSMCs and how this may modulate myometrial excitability.

1.3 Sodium Conducting Channels in the Myometrium

1.3.1 Voltage-gated Sodium Channels in the Myometrium

In MSMCs, Na^+ influx contributes to myometrial cell excitability [77, 91-93]; however, the channels responsible for Na^+ conductance are not completely understood. In other cell types, such as neurons [94], voltage-gated Na^+ channels are responsible for initiating and propagating action potentials. At the V_m, these channels are predominantly closed, but upon sufficient membrane depolarization these channels open, allowing Na^+ influx to drive further depolarization. These voltage-gated Na^+ channels can be blocked by the inhibitor tetrodotoxin (TTX), which is commonly used to study their activity (Fig 1.3). Multiple laboratories have detected TTX-sensitive voltage-gated Na^+ channel currents in MSMCs, but the specific role of these currents in these cells is still unclear [91]. In neurons, these channels produce action potentials that typically last only milliseconds (fast activation and inactivation). In contrast, MSMC action potentials can last hundreds of milliseconds and are more consistent with Ca^{2+}-based action potentials, as described earlier. Since it seems unlikely that voltage-gated Na^+ channels would initiate and propagate action potentials in MSMCS, studies have attempted to determine the function of these channels in MSMCs.

There have been many contrasting views on the identification and role of voltage-gated Na^+ channels in MSMCs. On one side, Amedee *et al.* demonstrated that the Na^+ channels in intact myometrial strips and isolated myometrial cells in short primary culture were TTX-

insensitive [95]. Cytosolic Ca^{2+} concentration and uterine contractile activity did not change in rat myometrial strips after adding TTX [96], and TTX did not dampen the propagation of electrical activity [96, 97]. These functional studies, primarily in rat myometrial cells, suggested that TTX-sensitive voltage-gated Na^+ channels did not contribute to action potentials in MSMCs. In contrast, Ohya *et al.* performed whole-cell voltage-clamp experiments on freshly isolated single rat MSMCs and found TTX-sensitive fast Na^+ currents [98]. Additionally, Yoshino *et al.* identified inward current that included both Na^+ and Ca^{2+} currents (I_{Na} and I_{Ca}) in freshly disassociated MSMCs from non-pregnant, pregnant and post-partum rat uteri [99]. The I_{Na} was voltage dependent, eliminated with either Na^+-free medium or 1 μM TTX and larger in MSMCs from pregnant rats than MSMCs from non-pregnant and postpartum rats [99]. Finally, certain gene candidates have been proposed for individual Na^+ voltage-gated channels; however, no experiments have been reported on whether any of these genes are responsible for currents in myometrial cells (Figure 1.3) [77, 100-102]. Overall, this data suggests that voltage-gated Na^+ channels may play a role in electrical activity in MSMCs during pregnancy, but better tools are needed to elucidate this role.

1.3.2 Transient Receptor Potential Cation Channels in the Myometrium

Another Na^+-conducting pathway in the MSMCs are transient receptor potential canonical channels (TRPCs) including TRPC1, TRPC3, and TRPC6. The TRPC channels are calcium-permeable channels [103-107], which function as a component of the store-operated calcium entry (SOCE) channels to contribute to electromechanical coupling in the myometrium (Figure 1.3) [108, 109]. Specifically, depletion of the Ca^{2+} stores in the sarcoplasmic reticulum activates, oligomerizes and translocates stromal interaction molecule 1 (STIM1) to the sarcoplasmic reticulum-plasma membrane junctional regions [110, 111].

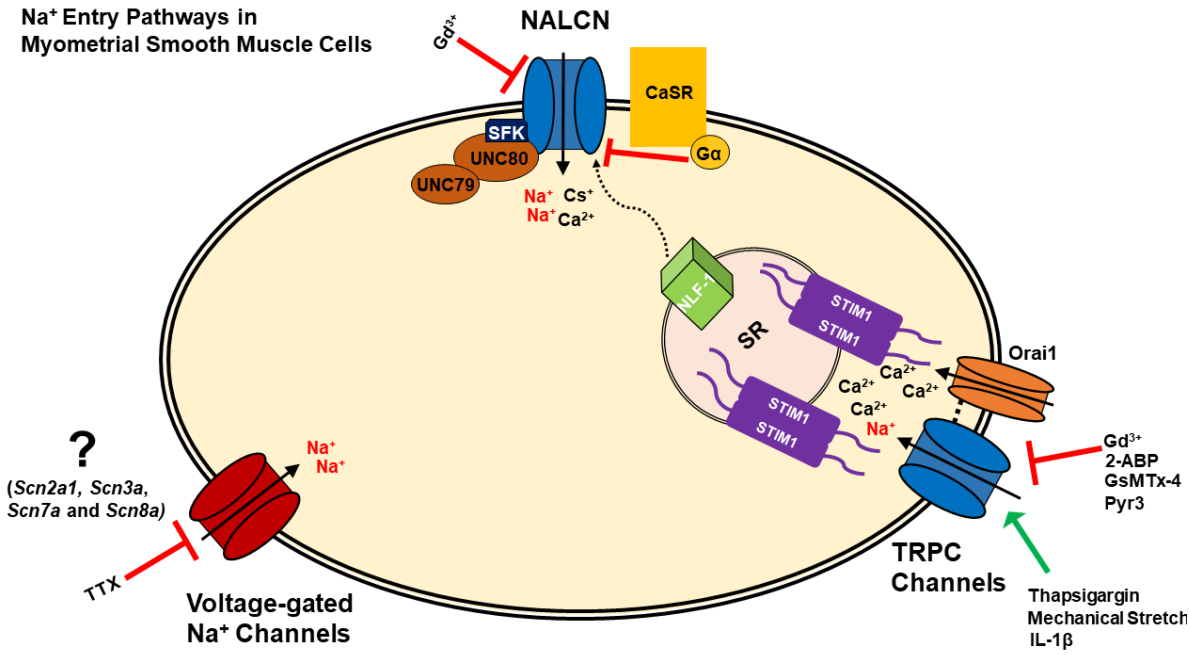

Figure 1.3: Proposed Sodium Entry Pathways in MSMCs
Voltage-gated Na^+ channels conduct Na^+ and are blocked by TTX. Candidate genes have been proposed for these channels but their role in Na^+ entry and myometrial excitability are still unknown. TRPC channels primarily conduct Ca^{2+} but can also conduct Na^+. These channels are stimulated by thapsigargin, IL-1β secretion and mechanical stretch but blocked by Gd^{3+}, 2-ABP, GsMTx-4 and Pyr3. TRPC, STIM1 and Orai1channels comprise a complex that increases influx of Ca^{2+} into the cytoplasm and SR after depletion of the SR-Ca^{2+} stores. NALCN conducts primarily Na^+ but can also conduct Ca^{2+} and Cs^+. In *C. elegans*, NALCN is transported to the plasma membrane with the help of the SR-resident protein NLF-1 [112]. At the plasma membrane, NALCN can interact with SFK and UNC79 through the scaffolding protein, UNC80 and this complex increases NALCN activity as observed in neurons [113]. Finally, NALCN can be inhibited by either CaSR under normal Ca^{2+} concentrations, as shown in neurons [114] or Gd^{3+}. We propose that NLF-1, UNC79, UNC80 and CaSR modulate NALCN similarly in myometrial cells.

This causes an increased conductance of Ca^{2+} through TRPC channels and Orai1, the pore-forming unit of a major channel involved in SOCE [110]. Studies have shown that TRPC1 and STIM1 physically and electrostatically interact at the ezrin/radixin/moesin domain and polybasic tail of STIM1, respectively [115, 116]. The electrostatic interaction allows for the gating of TRPC1 [116]. Finally, knock down of endogenous Orai1 decreases SOCE activity even with TRPC1-STIM1 overexpression [110, 111, 117].

Even though TRPC channels conduct Ca^{2+}, they can also conduct Na^{+} [103-107]. TRPC channels are sensitive to multiple signals, notably mechanical stretch and IL-1β secretion [104], which can stimulate uterine contraction at term (Figure 1.3). Thus, TRPC channel expression and activity may increase at the end of pregnancy to promote uterine contraction [104, 118, 119]. Also, thapsigargin, through activation of TRPC channel activity, induces a rise in intracellular Ca^{2+} [120] (Figure 1.3). TRPCs are inhibited by gadolinium (Gd^{3+}), 2-Aminoethoxydiphenyl borate (2-ABP), *Grammastola spatulata* mechanotoxin #4 (GsMTx4) and pyrazole compound (Pyr3), this inhibition may affect uterine contractility (Figure 1.3) [110, 121, 122]. For example, in the presence of 2-ABP, the stretch-induced spontaneous uterine contraction caused by TRPCs were suppressed, suggesting a possible involvement of TRPC channels in the regulation of contractions in pregnant myometrium [123]. Overall, these studies shed light on complexes that contribute to Na^{+} entry in human MSMCs.

1.4 Na^{+} Leak Channel, Non-Selective (NALCN)

1.4.1 NALCN Channel and its Complex

In many excitable cells, Na^{+} influx contributes to the maintenance of V_m and initiation of action potentials [76]. In neurons, TTX-sensitive voltage gated channels are known to contribute to the action potential [94, 124], so early studies investigated if these channels functioned

similarly in MSMCs. Electrophysiological studies performed in rat myometrium found that substituting Na^+ with either Tris or dimethyldiethanol ammonium chloride prevented action potential generation, whereas blocking Na^+ channels with TTX had no effect [91]. Since the data suggested that the Na^+ channels contributing to excitability were neither voltage-gated nor TTX-sensitive Na^+ channels, other channels need to be considered. One such channel is the sodium leak channel, non-selective (NALCN).

NALCN, originally called Vgcn11, was cloned from the rat in 1999 and showed sequence and size similarity to both voltage-gated Ca^{2+} and Na^+ channels (Ca_v and Na_v) [125]. Homologs of this channel have been identified and characterized in both vertebrates and invertebrates [126]. NALCN, like Na_v and Ca_v, has four homologous repeats (domains I-IV) of six transmembrane (TM) spanning segments (S1-S6) and is part of the 4x6TM family [125, 127]. Lee *et al.* and Lu *et al.* determined that NALCN differs from other channels in the 4X6TM family in three major ways [125, 127]. One, NALCN contains fewer positively charged amino acids in the S4 segment making it voltage-insensitive [125, 127]. Two, ion selectivity is formed by the four S5-S6 pore loops. NALCN has an EEKE motif, a mixture of Ca_v and Na_v motifs, which creates a non-selective and constitutively active pore that conducts Na^+, K^+ Cs^+, and Ca^{2+} (Figure 1.3) [125, 127], but primarily conducts Na^+. Third, the NALCN current was found to be resistant to TTX, but sensitive to gadolinium (Gd^{3+}) (Figure 1.3) [125, 127].

In many cell types, several proteins interact with and/or modulate NALCN: NCA Localization Factor-1 (NLF-1), UNC-80, UNC79, SRC Family Kinase (SFK), and the Ca^{2+} Sensing Regulator (CaSR) (Figure 1.3). NLF-1, first identified in *C. elegans*, is located in the endoplasmic reticulum and promotes membrane localization of all the NCA channels, NALCN homolog [112]. NLF-1 also plays an important role in the superchiasmatic nucleus in regulating

the day/night trafficking of NALCN to the plasma membrane of the pacemaker neuron in *D. melanogaster* [128]. The other ancillary subunits of the NALCN complex interact with NALCN at the plasma membrane. In early studies investigating UNC80 and UNC79, Yeh *et al.* saw that mutants of *unc-79* and *unc-80*, showed fainter locomotion and decreased Ca^{2+} transients at synapses, similar to the phenotypes of mutant *nca(lf)*, in *C. elegans* [129]. In *D. melanogaster*, Lear *et al.* observed that *unc-79* and *unc-80* mutants displayed defects in circadian motor rhythmicity identical to the *na* mutants, NALCN homolog, and the three proteins co-localized within the pacemaker neurons [130]. In mice, NALCN, UNC-80 and UNC79 form a complex in mouse neurons and UNC80 contributes to the stabilization of NALCN at the cell membrane [113]. This allows UNC80 to function as a scaffold for UNC79 and SFK binding, leading to activation of NALCN [113]. Both UNC79 and UNC80 have been identified in human MSMCs but their roles still need to be investigated [92]. Conversely, the Ca^{2+} sensing receptor (CaSR), also localized at the cell membrane, negatively modulates NALCN current in neurons [114, 131, 132]. In the presence of normal extracellular Ca^{2+} levels, CaSR activates a G-protein signaling cascade to decrease NALCN current in neurons [114]. Overall, further investigation is needed to identify the ancillary subunits and their role in the modulation of NALCN in human MSMCs.

1.4.2 Human Mutations of NALCN in Neuronal and Respiratory Function

Since 2013, several mutations of *NALCN* have been identified in children with neurological deficits and respiratory problems. The nine homozygous NALCN mutations found result in infantile hypotonia with psychomotor retardation and characteristic facies (IHPRF) with or without respiratory distress [133-138]. Five of these mutations resulted in a truncated protein by creating premature stop codons in many different locations of the NALCN protein including:

1) between II and III linker, 2) within the S4 II or S4 IV, 3) linker between S5 and S6 of domain II, and 4) N-terminus region [133-137]. The other four mutations were missense and led to milder forms of IHPRF [134, 136]. Bouasse *et al* found that one such NALCN variant, W1287L, lead to loss of NALCN current in neurons and supported the prediction that these recessive mutations in NALCN would cause loss of function [139].

Additionally, many heterozygous, dominant, *de novo* NALCN mutations have been discovered in children having congenital contractures of the limbs and face, hypotonia, and global development delay (CLIFAHDD) [140-144]. Many of these mutations alter amino acid residues in or near the S5 and S6 pore-forming regions [140-144]. First, Chong et al discovered that NALCN mutants decreased the expression of NALCN wild type protein when co-transfected in HEK293 cells, suggesting a dominant-negative effect [140]. Second, Lozic *et al* discovered another *de novo* missense mutation that caused CLIFAHDD but presented with additional phenotypes [143], which included respiratory distress, reversed sleep-wake cycle and high sensitivity to anesthetics. All these phenotypes observed in NALCN in mice were found in its homolog mutants: *na* (*D. melanogaster)* and *nca* (*C. elegans*) [127, 128, 143, 145-147]. Finally, patch clamp studies in HEK293 cells transfected with the dominant NALCN mutants revealed an increased Na^+ background current and inactivation [139]. Both characteristics led to a gain-of-function effect, which they suggested arose from a functional alteration instead of a change in trafficking, since the NALCN mutations actually decreased the number of NALCN on the plasma membrane [139]. Overall, these human mutations demonstrate the crucial role of NALCN in normal neurologic function and development.

1.4.3 The Role of NALCN in Regulating Resting Membrane Potential

Many excitable cells including MSMCs maintain the V_m in a range from -50 to -80 mV. Although V_m is primarily due to the efflux of K^+ ions, the V_m is more depolarized than the K^+ equilibrium potential suggesting conductance of other ions. Due to its voltage-insensitivity and constitutively active pore, NALCN functions at and contributes to regulating the V_m in neurons of vertebrates and invertebrate organisms [127, 128, 148]. Studies by Lu *et al.* showed the V_m in the hippocampal neurons of NALCN knockout mice were more hyperpolarized than the membrane potential of the wild type controls. This suggested that NALCN provided the background sodium inward leak current that balanced the outward K^+ current to regulate and maintain the V_m [127]. Additionally, the lack of Na^+ leak current reduced the spontaneous firing rate and required more depolarizing input to achieve similar firing rates as wild type neurons [127]. Flourakis *et al.* created forebrain-specific NALCN knockout mice and determined that central clock neurons that lacked NALCN in the superchiasmatic nucleus (SCN) were similarly hyperpolarized and silent in firing [128]. Together, these studies identified NALCN as a major contributor of V_m, in neurons of vertebrates and invertebrate organisms. These studies were critical as they laid the foundation to investigate if NALCN could also contribute to the V_m of MSMCs.

1.4.4 The Role of NALCN in Human and Mouse MSMCs

It has been proposed that an important contributor to both the V_m and action potential, is a non-selective cationic leak current that is constitutively active and voltage-insensitive, but such a channel had not been identified [125]. In neurons and gastrointestinal cells, NALCN was characterized as the Gd^{3+}-sensitive Na^+-dependent non-selective leak channel that contributed to

the V_m and the pacemaking phase in action potentials, respectively [127, 149]. Myoshi *et al*. measured a Na^+-dependent, lanthanum and Gd^{3+}-sensitive leak current in rat MSMCs and hypothesized that this current contributed to uterine excitability [150]. Inhibiting this leak current with magnesium, a known cation channel inhibitor, decreased the action potential frequency [150], but these authors did not identify the channel responsible for the current. Thus, our laboratory investigated: 1) whether the myometrial leak current described by Myoshi *et al*. could be carried by NALCN and 2) the roles of NALCN in human and mouse myometrial physiology.

We showed that human MSMCs express NALCN at both the mRNA and protein level and possess an ohmic Na^+-dependent leak current that was attenuated by Gd^{3+} or replacement of Na^+ with NMDG, in whole cell patch recordings [92]. Additionally, NALCN was proposed to contribute to this leak current because shRNA-mediated knockdown of NALCN expression decreased the leak current [92]. Given these results, our group hypothesized that NALCN activity is an underlying contributor to myometrial excitability and successful parturition. Reinl *et al.* investigated this hypothesis in a mouse model [151]. Our laboratory observed that mice lacking NALCN in smooth muscle had dysfunctional firing patterns in the uterus, with shorter burst duration and fewer spikes per burst compared to wild type mice. These changes resulted in abnormal labor, including prolonged, delayed, and dysfunctional labor [151]. This work revealed that NALCN contributes to myometrial leak currents and is important for parturition, establishing the foundation for the work described in this thesis.

Given the importance of ion channels such as NALCN in MSMC excitability, it is not surprising that expression of many of these channels is regulated by E2 and P4 [85, 152-154]. In our mouse pregnancy study, we observed that NALCN mRNA and protein expression decreases mid-pregnancy and increases at term and labor [92], while serum levels of P4 peak during mid-

pregnancy in mice. On the other hand, Soloff *et al.* reported microarray data indicating that NALCN was upregulated by P4 and downregulated by E2 in human MSMCs [152]. Although these data suggest that E2 and P4 regulate NALCN expression, this idea has not been directly tested. Thus, in my thesis, I investigated the mechanisms by which NALCN is regulated by P4 and E2. A better understanding of the interplay between hormones and ion channels in the uterus will allow us to design targeted therapies to treat uterine pathophysiology of pregnancy.

1.4.5 The Role of Na^+-activated K^+ Channel (SLO2.1) in Myometrial Activity

Na^+ influx, possibly through NALCN, may contribute to the maintenance of V_m in MSMCs possibly by activating specific K^+ channels. We recently identified a Na^+-activated K^+ channel (SLO2.1) that may contribute to the V_m of MSMCs and counteract Na^+ influx in human MSMCs [155].

In many excitable cells, including neurons and cardiac cells, a high conducting K^+ current that was activated by Na^+, but not activated by Ca^{2+}, Li^+, or choline was identified [156-162]. Additionally, the activation of this channel contributed to maintaining V_m in mouse neurons [163-165]. Further work identified that members of the Slo gene family, *slo2.1* and *slo2.2* encode for K_{Na} channels, SLO2.1 and SLO2.2, respectively [158, 160]. The *slo2* genes are related to the *Slo1* family, which encodes the large-conductance Ca^{2+}-activated K^+ channels and *Slo3* family, which encodes a pH-sensitive K^+ channel [158, 166, 167]. Specifically, SLO2.1 currents are rapidly inactivating and less voltage dependent, while SLO2.2 currents are slow inactivating and voltage sensitive [160, 168]. Finally, initial expression studies observed that *slo2.1* mRNA was widely distributed in the uterus, brain, testis, lung, heart, and liver, while *slo2*.2 was predominantly in the brain [155, 158].

The expression of SLO2.1 in the uterus is a candidate for the Na^+-activated K^+ channel in the MSMCs and may suggest a functional partner for NALCN. To determine if SLO2.1 functioned similarly in MSMCs as described in other excitable cell types, Ferreira *et al.* performed whole-cell and inside-out macro patch clamp experiments in MSMCs and identified a non-inactivating K^+ current that was resistant to TEA and activated by Na^+ [155]. This current had high conductance at both +80 mV and -60 mV (V_m of MSMCs), consistent with a K_{Na} current that could contribute to the V_m. Additionally, mRNA and protein analysis indicated that this current was attributed to SLO2.1 but not SLO2.2 in human MSMCs [155]. When siRNA targeted against SLO2.1 was introduced into human MSMCs, this K_{Na} current disappeared. Thus, the investigators concluded K_{Na} current was conducted by SLO2.1. These studies point to the broad importance of SLO2.1 as a modulator of V_m and excitability. This thesis investigates if Na^+ entry through NALCN activates SLO2.1 and if their association regulates myometrial V_m and excitability.

1.5 The Regulation and Role of NALCN in Human Myometrial Cells

In my thesis work, I investigated the hormonal regulation of NALCN and its contribution to myometrial excitability in human MSMCs. First, it was established that P4, the pro-quiescent hormone, upregulates the expression of NALCN by acting through progesterone response elements in the NALCN promoter. On the other hand, E2, the pro-contractile hormone, downregulates this channel in humans MSMCs, independent of binding to the estrogen response element within the NALCN promoter. These effects on NALCN expression translated functionally as P4 significantly enhanced, and E2 significantly inhibited a NALCN-dependent leak current in human MSMCs. These findings also show that NALCN contributes to the V_m and

excitability of human MSMCs. Specifically, a Gd^{3+}-sensitive Na^{+}-conducting leak channel activates the SLO2.1 current and NALCN and SLO2.1 are in proximity. The NALCN-dependent leak current modulates V_m and responses to intracellular Ca^{2+} by activating SLO2.1 currents. This work has identified novel hormonal regulators of NALCN and recognized NALCN as an important player in contributing to the V_m in order to modulate myometrial excitability.

1.6 References

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Chapter 2: Progesterone and estrogen regulate NALCN expression in human myometrial smooth muscle cells

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2.1 Introduction

The strength and frequency of myometrial contractions are regulated to support the multiple functions of the uterus including expulsion of the endometrial lining during menstruation, sperm transport during fertilization, embryo implantation, fetal growth, and

parturition [1]. During pregnancy, the uterus remains quiescent to support fetal development, but at term, it produces synchronous and forceful contractions to deliver the fetus [2]. Two hormones essential for this transition are progesterone (P4) and estrogen (E2).

P4 represses expression of several genes that increase responsiveness to uterotonic agonists and enhance myometrial contractility during the majority of pregnancy. P4 functions through two major progesterone receptor (PR) isoforms, PRA and PRB. PRB, the full-length canonical form, predominates throughout pregnancy and mediates myometrial quiescence [3]. For example, after binding to P4, nuclear PRB represses transcription of genes encoding interleukin-8, cyclooxygenase 2, oxytocin receptor, connexin-43, and nuclear factor kappa B (NF-κB) subunit [4-6], all of which are associated with inflammation and contractility. In addition to directly binding to the progesterone response elements (PRE) within a gene, the P4-PR complex can tether to pro-inflammatory transcription factors (e.g., NF-κB or AP-1) and recruit co-repressors, such as p54, to inhibit transcription [7-11]. P4-PR repression of pro-inflammatory genes also occurs by two other mechanisms. First, P4-PR can decrease the recruitment of RNA polymerase II and NF-κB p65 to *COX-2* and *IL*-8 promoters and thus decrease their expression [12]. Second, P4-PR increases the expression of the zinc finger E-box binding homeobox protein, ZEB1, which inhibits expression of contractile genes and the miR-200 family during pregnancy and thus delay the myometrial transitions to a contractile phenotype [8, 13, 14].

As pregnancy progresses to term and into labor, P4 function is decreased in human myometrium, leading to a pro-contractile state [15, 16]. One mechanism by which this P4 functional withdrawal may occur is that expression of PRA, which lacks 164 amino acids including the AF3 activation domain in the N-terminus, increases at the end of pregnancy. This

increased PRA expression leads to an increase in the PRA:PRB ratio, resulting in decreased P4/PRB-mediated gene expression. Other mechanisms underlying this P4 functional withdrawal include changes in the levels of PR-associated co-regulators [17], increased expression of specific endogenous PR antagonists [18], and increased expression of the P4 metabolizing enzyme, 20α HSD [14]. Whereas P4 promotes quiescence, E2 promotes contraction by binding to the estrogen receptor α (ERα) in the myometrium [4, 11, 14]. E2 serum levels also increase as the pregnancy moves towards labor. The E2-ERα complex binds to estrogen response elements (EREs) in genes encoding contractile-associated proteins and inflammatory genes, leading to their upregulation and promoting labor [16, 19, 20]. In addition to the genomics effects, E2 exerts non-genomic effects through ERα activation of the extracellularly regulated kinase/mitogen-activated protein kinase pathway [21].

Other proteins that regulate the transition from uterine quiescence to contractility are the multiple ion channels that are expressed in myometrial smooth muscle cells (MSMCs). Together, these ion channels regulate MSMC membrane potential and excitability by controlling sodium ion (Na^+) influx, potassium ion (K^+) efflux, and calcium ion (Ca^{2+}) influx [22-25]. At a resting state, the myometrial membrane potential is approximately -60 mV. As positive charge 'leaks' into the cell, the membrane becomes less polarized until it reaches a threshold potential at which L-Type Ca^{2+} channels are activated, causing an influx of Ca^{2+}. This Ca^{2+} influx both depolarizes the membrane and leads to activation of myosin and contraction [26, 27]. Membrane potential is restored by efflux of K^+ through K^+ channels [28-31]. The activities of the many ion channels controlling these changes in membrane potential are regulated to promote uterine quiescence throughout pregnancy and contractility at term [29].

Voltage-independent, non-selective cationic leak currents can be important contributors to cell excitability, and RNA sequencing data has detected the expression of a Na^+ leak channel, non-selective (NALCN) in human myometrial tissue [29]. Recently, our lab showed that NALCN contributes to at least 50% of the Na^+ leak current in MSMCs isolated from non-laboring women at term [32], and that mice lacking NALCN in smooth muscle had dysfunctional labor [33]. Collectively, these findings suggest that NALCN contributes to myometrial exciability and reproductive outcomes. Given the importance of ion channels such as NALCN in MSMC excitability, it is not surprising that expression of many of these channels is regulated by E2 and P4 [22, 34-36]. Specifically, Soloff *et al.* reported microarray data indicating that NALCN was upregulated five-fold in human MSMCs after exposure to P4 [34]. Although these data suggest that E2 and P4 regulate NALCN expression, this idea has not been directly tested. Here, we tested the hypothesis that E2 and P4 regulate NALCN expression and current activity in human MSMCs.

2.2 Materials and Methods

2.2.1 Cell Culture

All cell lines were maintained in 95% air, 5% CO_2 at 37 °C and were confirmed mycoplasma-negative with MycoAlert Mycoplasma Detection Kit (Lonza) before experiments.

Generation and characterization of the HM6ERMS2 cells was previously described [34, 37]. HM6ERMS2 cells were maintained in phenol red-free Dulbecco's modified Eagle medium/Ham's F12 (1:1) (DMEM) (Ref. 11039, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Ref. 26140079, Gibco) and 25 μg/mL gentamicin (Sigma, St. Louis, MO). During E2 and P4 treatments, the cells were maintained in DMEM supplemented with 0.5% bovine serum albumin (Sigma) and 25 μg/mL gentamicin.

Generation and characterization of the human telomerase reverse transcriptase-immortalized-human myometrial (hTERT-HM) cells stably expressing FLAG-tag PRA or PRB (hTERT-HM FLAG-tag PR) or lentiviral mock vector was previously described [11, 38]. The hTERT-HM mock, FLAG-tag PRA, and FLAG-tag PRB cells were maintained in phenol red-free DMEM supplemented with 10% FBS and 5 μg/ml blasticidin (Gibco). Twenty-four hours before and during hormone treatments, cells were switched to media with 10% charcoal-stripped FBS (Ref. 12676029, Gibco).

The Michigan Cancer Foundation 7 (MCF-7) cells, a breast cancer cell line, were maintained in ATCC-formulated Eagle's Minimum Essential Medium (Catalog No, 30-2003, ATCC, Manassas, VA) supplemented with 10% FBS. For transfection and hormonal treatments, MCF-7 cells were maintained in their media supplemented with 10% charcoal-stripped FBS.

2.2.2 Hormone Treatment

Progesterone (P4), 17β-estradiol (E2), RU486 (PR antagonist), and ICI 182,780 (ICI, ER antagonist) were of the purest quality available from Sigma. For HM6ERMS2 cells, vehicle treatment was as follows: 0.05% EtOH on all three days. E2 treatment was as follows: 20 nM E2 on day 1, and 2 nM E2 on days 2 and 3. ICI 182,780 was administered every day at 1 μM along with the estradiol concentrations listed above. E2/P4 treatment was as follows: 20 nM E2 on day 1, 2 nM E2 on day 2, and 2 nM E2 and 100 nM P4 on day 3. RU486 was administered at 1 μM on Day 3 along with the E2/P4 treatments. P4 treatment was as follows: 0.05% EtOH for days 1 and 2 and 100 nM P4 on day 3. For hTERT FLAG-tag PR cells, progesterone treatment was as follows: 100 nM P4 on day 1. For both cell types, all assays were performed on the day after the last treatment day.

2.2.3 RNA Isolation, cDNA Synthesis, and Quantitative Real-time PCR

Total RNA was isolated from HM6ERMS2 and hTERT-HM FLAG-tag PR cells by using the RNeasy/QIAshredder Mini Kit (Qiagen, Germantown, MD) and treated with RNase-Free DNase (Qiagen) according to the manufacturer's instructions**.** RNA quality was confirmed by two methods: 1) electropherogram bioanalyzer of the 28S to 18S rRNA ratio, and 2) 260/280 nm and 260/230 nm absorbance ratios above 1.8 and 2.0, respectively. RNA (200 ng) was reverse transcribed to generate cDNA by using the iScript Reverse Transcription Supermix for quantitative real-time PCR (Bio-Rad, Hercules, CA, USA). cDNA was stored at -20°C until quantitative real-time PCR was performed. Gene targets were amplified with iQ SYBR Green Supermix (BioRad) and quantified with the CFX96 BioRad Real-Time PCR Detection System. Temperature cycles were as follows: 95 °C for 3 min, 40 cycles of (95 °C for 10 sec, 60 °C for 30 sec), and a 0.5 °C increment melt curve from 65 °C to 95 °C. Primer efficiencies were ≥80% with human brain cDNA and hTERT-HM lentiviral mock vector. Samples were run in parallel with standard curves to generate accurate copy numbers from threshold cycles. Copy numbers of the measured genes were normalized to the geometric mean of the copy numbers of the reference genes topoisomerase 1 (TOP1), succinate dehydrogenase complex subunit A (SDHA), and smooth muscle alpha actin 2 (ACTA2). These genes are stably expressed in the myometrium [39]. The primer sequences are listed in Table 2.1.

Table 2.1 Primer sets used for quantitative RT-PCR

Primer name

mRNA Primers	**Sequence**
hNALCN_F	ACTGACTTTGGTCCTGATGA
hNALCN_R	ACTGAAGTGGAGGATAGTGC
hTOP1_F	CCAGACGGAAGCTCGGAAAC
hTOP1_R	GTCCAGGAGGCTCTATCTTGAA
hSDHA_F	CAAACAGGAACCCGAGGTTTT
hSDHA_R	CAGCTTGGTAACACATGCTGTAT
hACTA2_F	GCCTTGGTGTGTGACAATGG
hACTA2_R	AAAACAGCCCTGGGAGCAT
hPRB_F	TCCAGTCCTCGGACAGAAGT
hPRB_R	GGAACTGTGGCTGTCGTTTG
hFKBP5_F	ATGCCATTTACTGTGCAAACCAG
hFKBP5_R	AAGAGAGTTGCATTCGAGGAA

Cloning/Restriction Primers	**Cloning Sequence**
hNALCN_F	GCAAATGGGAAGTATATGCC
hNALCN_R	GCTCAGAGCTGTCATGGTG
hNALCN_Nhe1_F	CTAGCTAGCTAGGCAAATGGGAAGTATATGCC
hNALCN_Xho1_R	CCGCTCGAGCGGGCTCAGAGCTGTCATGGT

2.2.4 Whole-cell Lysate Preparations and Western Blotting

Cultured HM6ERMS2 cells were transferred into RIPA Buffer (150 mM NaCl, 50 mM Tris, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], pH 8.0) (Sigma) containing cOmplete Mini EDTA-free Protease Inhibitor Cocktail and PhosStop *EASY*pack (Roche, Indianapolis, IN) and homogenized with the Bullet Blender 24 (Next Advance Inc., Troy, NY) at 4 °C. Lysates were centrifuged at 14,000 x g for 15 min at 4°C. Supernatants were stored at -80 °C. Protein concentrations of whole-cell lysates were measured by using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Lysate samples containing equal amounts of protein were mixed with 6X SDS gel loading buffer (375 mM Tris-HCl, 6% SDS, 48% glycerol, 9% β-mercaptoethanol, and 0.03% bromophenol blue, pH 6.8), and incubated for 5 min at 100°C. Denatured samples were electrophoresed on precast 4% to 15% Tris-Glycine polyacrylamide gels, transferred to nitrocellulose membrane, and blocked in 5% non-fat dried milk (Bio-Rad, Hercules, CA) in 0.075% Tween-Phosphate buffered saline (PBS-T) for 1 h at room temperature. The membranes were incubated overnight at 4 ºC with primary antibodies, then incubated for 1 h at room temperature with appropriate secondary antibodies in 3% milk in 0.075% PBS-T. Antibodies are listed in Table 2.2. Signal was detected with Supersignal West Femto (Thermo Fisher Scientific, Waltham, MA, USA) and viewed with a ChemiDoc MP Imaging System (BioRad). For analysis, a flat line was drawn across the base of the intensity peak for each band to subtract background, and the area under the curve was quantified. NALCN expression was normalized to Histone H3 expression by densitometry with ImageJ software.

Table 2.2 Primary and Secondary Antibodies for Western Blot

Name of Antibody	Manufacturer, Catalog Number, RRID	Species raised in; monoclonal (mAb) or polyclonal (pAb)	Dilution Used
NALCN [S187-7]	StressMarq, SMC-417, AB_2701253	Mouse, mAb	1:1000
PR (H-190)	Santa Cruz Biotechnology, sc-7208, AB_2164331	Rabbit, pAb	1:1000
Erα (HC-20)	Santa Cruz Biotechnology, sc-543, AB_631471	Rabbit, pAb	1:1000
Histone H3	Abcam, ab1791, AB_302613	Rabbit, pAb	1:5000
β-Actin	Millipore Sigma, A1978, AB_476692	Mouse, mAb	1:5000
GAPDH	Millipore, MAB374, AB_2107445	Mouse, mAb	1:2000
Anti-Rabbit IgG (H+L) HRP-linked	Jackson ImmunoResearch, 111-035-144, AB_2307391	Goat, pAb	1:2000
Anti-Mouse IgG (H+L) HRP-linked	Jackson ImmunoResearch, 115-035-003, AB_10015289	Goat, pAb	1:2000

2.2.5 Immunocytochemistry

HM6ERMS2 cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, briefly washed with PBS, and then permeabilized with 0.1% NP40 in PBS for 5 min. Cells were then washed with PBS and 10 mM glycine and incubated in blocking buffer (PBS with 5% Normal Goat Serum [NGS]) for 1 h, incubated with an NALCN-specific antibody (1:500, S187-7, StressMarq, Victoria, British Columbia) diluted in antibody buffer (PBS with 1% NGS and 0.1% Tween-20) overnight, and washed 3 times for ~30 min in wash buffer (250 mM NaCl, 25 mM Tris HCl, 0.1% Tween-20, pH 7.5). Cells were incubated with goat anti-mouse IgG (H+L) Alexa Fluor Plus 488 (1:2000, Invitrogen, Carlsbad, CA) diluted in antibody buffer for 1 h, washed, and incubated with 1 μg/mL 4',6-diamidino-2-phenylindole, and then mounted in Vectashield Antifade Mounting Medium (Vector Laboratories, Burlingame, CA). Cells were imaged with a 63x oil objective on a Leica DMI4000 B microscope. To assess the specificity of NALCN immunostaining, cells were stained with primary antibody only, secondary antibody only, or in the absence of permeabilization. All controls were processed with the same parameters employed for the NALCN antibody.

2.2.6 Whole-cell Electrophysiology

Glass pipettes were pulled and polished to 2-6 MΩ and filled with a pipette solution containing 125 mM Cs-Aspartate, 20 mM tetraethylammonium (TEA)-Cl, 5 mM Mg-ATP, 5 mM EGTA, 100 nM free Ca^{2+} (calculated with Maxchelator software [Stanford University]), and 10 mM HEPES, pH 7.2 [32]. In the intracellular solution, K^+ was replaced with Cs^+. Extracellular bath solution contained 125 mM NaCl or N-methyl-d-glucamine (NMDG), 20 mM TEA-Cl, 0.1 mM $MgCl_2$, 5 mM HEPES, 11 mM glucose, 1 μM $CaCl_2$, and 5 μM nifedipine, pH 7.4. TEA-Cl and nifedipine were added to the bath solution to isolate the leak current by

blocking voltage-gated K^+ and Ca^{2+} channels, respectively. Additionally, 10 μM gadolinium (Gd^{3+}), a concentration known to inhibit NALCN current [40], 500 nM *Grammastola spatula* mechanotoxin #4 (GsMTx4; blocks TRPC1 and TRPC6), and 1 μM pyrazole compound (Pyr3; blocks TRPC3) were added to the bath solution to determine NALCN's contribution to the leak currents [40-42]. Currents in HM6ERMS2 cells were recorded at a sampling rate of 10 kHz and filtered at 1 kHz by using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Cells were held at -60 mV and currents were elicited by stepping from −55 mV (50 ms) to +40 mV for 100 ms (to inactivate voltage-gated Ca^{2+} channels followed by 20 mV steps (500 ms) from −100 mV to +20 mV using the pClamp 10 software (Molecular Devices). Currents were normalized to cell capacitance, and the mean and standard error of the mean of the current density were calculated for each group of samples. In all whole-cell patch-clamp recordings, the investigator was blinded to the cell treatments.

2.2.7 Cloning of NALCN promoter region and mutagenesis of PRE and ERE sites

The human NALCN promoter region, from 2479 bp upstream to 183 bp downstream of the transcription start site, was amplified from genomic DNA in hTERT-HM FLAG-tag PR cells with Phusion High Fidelity DNA Polymerase (NEB, Ipswich, MA). Primers are listed in Table 2.1. The amplified insert was re-amplified with primers that contained Nhe1 and Xho1 sites (Table 2.1), digested with Nhe1 and Xho1, and ligated into the pGL3-Basic firefly luciferase-reporter vector (Promega, Madison, WI) to create the construct pGL3-Basic-NALCN, which was confirmed by restriction digests and Sanger Sequencing (Genewiz, South Plainfield, NJ). Standard procedures were used for plasmid amplification and purification. Genewiz performed site-directed mutagenesis of putative progesterone response elements and estrogen response element.

2.2.8 Transfection and luciferase reporter assay

MCF7 cells were seeded in 96-well plates, and FuGENE HD transfection reagent (Roche Applied Science) was used to co-transfect cells with a *Renilla* luciferase-expressing plasmid (pGL4.74) (Promega) and the pGL3-Basic-NALCN wild-type or mutant constructs. Sixteen hours post-transfection, cells were treated with either EtOH or 20 nM E2. Twelve hours later, cells were treated with EtOH, 2 nM E2, or 100 nM P4. Twenty-four hours later, firefly luciferase and *Renilla* luciferase activities were assayed by using a Dual-Luciferase assay system (Promega). Relative luciferase activities were calculated by normalizing firefly luciferase activity to *Renilla* luciferase activity within the same samples to correct for transfection efficiency.

2.2.9 Statistical Analysis

All data are presented as mean ± SEM. Statistical significance was determined by unpaired t-test (two groups), or one- or two-way ANOVA (three or more groups) correcting for multiple comparisons by using GraphPad Prism 7 (San Diego, CA, USA), and $P < 0.05$ was considered significant. The 'n' refers to the number of experiments analyzed from different days or transfections.

2.3 Results

2.3.1 NALCN Expression is Decreased by Estrogen and Increased by Progesterone

To determine whether the hormones E2, P4, or both regulate NALCN mRNA and protein expression in human myometrial cells, we used HM6ERMS2 cells, a myometrial cell line derived from uterine tissue from a woman late in pregnancy [37]. The cell line stably expresses ERα and expresses PRA and PRB after at least 12 hours of E2 treatment (Figure 2.1A) [34, 37]. We performed hormonal treatments as previously described [15, 16, 43] in HM6ERMS2 cells in

four conditions: 1) vehicle-treated, 2) E2-treated, 3) E2/P4-treated, and 4) P4-treated. To confirm that ERα was functional, we measured mRNA expression of the E2/ERα target PRB [34, 44]. Quantitative RT-PCR showed that PRB mRNA expression was 13-fold higher in cells exposed to E2 alone and 5.2-fold higher in cells exposed to E2/P4 than in cells exposed to vehicle (Figure 2.1B). Cells exposed to E2/P4 expressed 2.4-fold less PRB mRNA than cells exposed to E2 alone, likely due to down-regulation of PRB transcription by P4 signaling [45-48]. To confirm that PR was functional, we measured mRNA expression of the PR target, FK506 binding protein 51 (FKBP5) [5, 49]. Cells exposed to E2/P4 expressed 2.5-fold more FKPB5 mRNA than cells exposed to any of the other treatment conditions (Figure 2.1C). Together, these results confirmed that ERα and PR were functional in the HM6ERMS2 cells.

Next, we investigated the effects of hormones on NALCN expression in myometrial cells. First, qPCR revealed that NALCN mRNA expression was 2.3-fold lower in cells exposed to E2 alone than in cells exposed to vehicle (Figure 2.2A). Conversely, NALCN mRNA expression was 5.6-fold higher in cells exposed to E2/P4 than in cells exposed only to E2. NALCN mRNA was similar in cells exposed to P4 alone and cells exposed to vehicle (Fig 2.2A). The fact that NALCN expression was not increased in cells treated with P4 alone (which do not express PR) supports the idea that P4 requires the presence of PR to regulate NALCN expression.

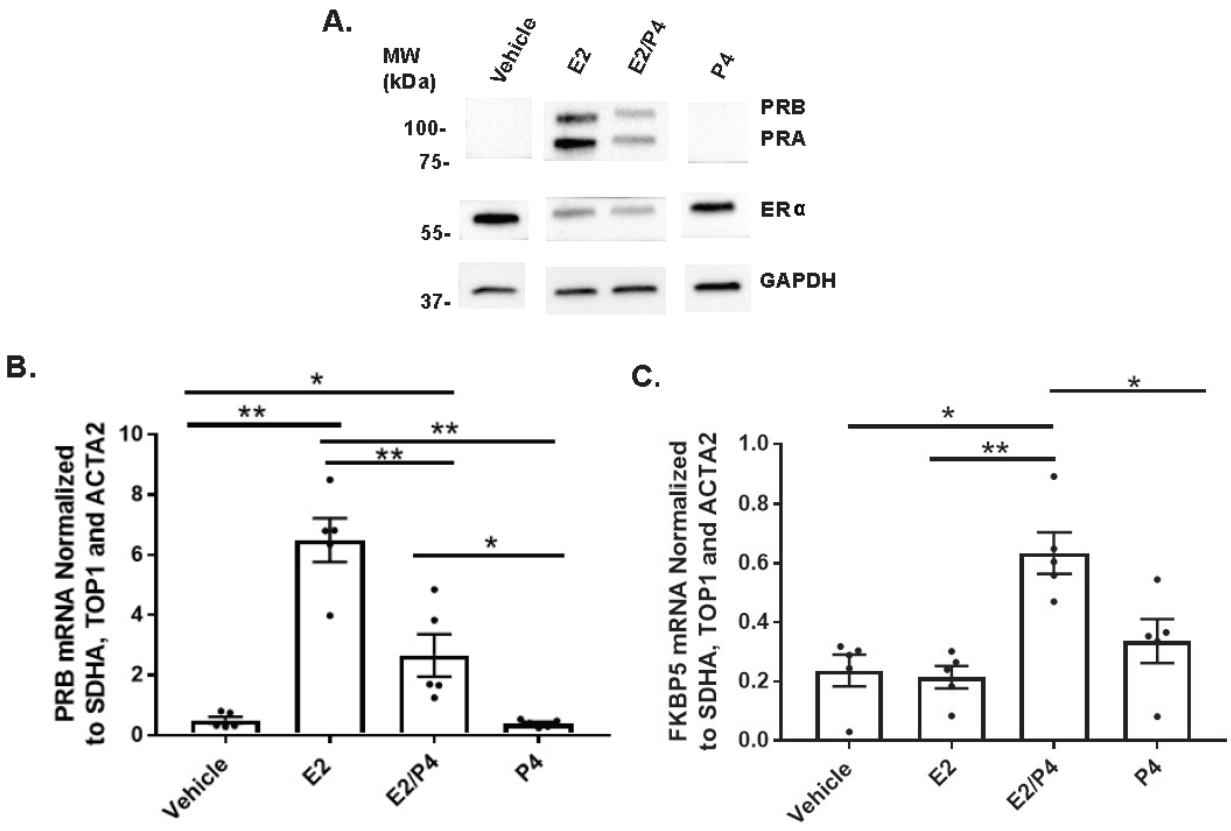


Figure 2.1: ER and PR regulate transcription of PRB and FKBP5
A) Representative immunoblot of ERα, PR-A, and PR-B proteins in HM6ERMS2 whole-cell lysates. GAPDH served as the loading control. All samples were run on the same gel and exposed at the same time. B) Quantification of qRT-PCR of PRB mRNA in HM6ERMS2 treated with vehicle (N=5), E2 (N=5), E2/P4 (N=5), or P4 (N=5). C) Quantification of qRT-PCR of FKBP5 mRNA in HM6ERMS2 treated with vehicle (N=5), E2 (N=5), E2/P4 (N=5), or P4 (N=5). The geometric means of TOP1, SDHA and ACTA2 were used as standards for quantification. Data are presented as mean and standard error of the mean. **P*<0.05, ***P*<0.01 by Dunnett's multiple comparison test one-way ANOVA. MW, molecular mass.

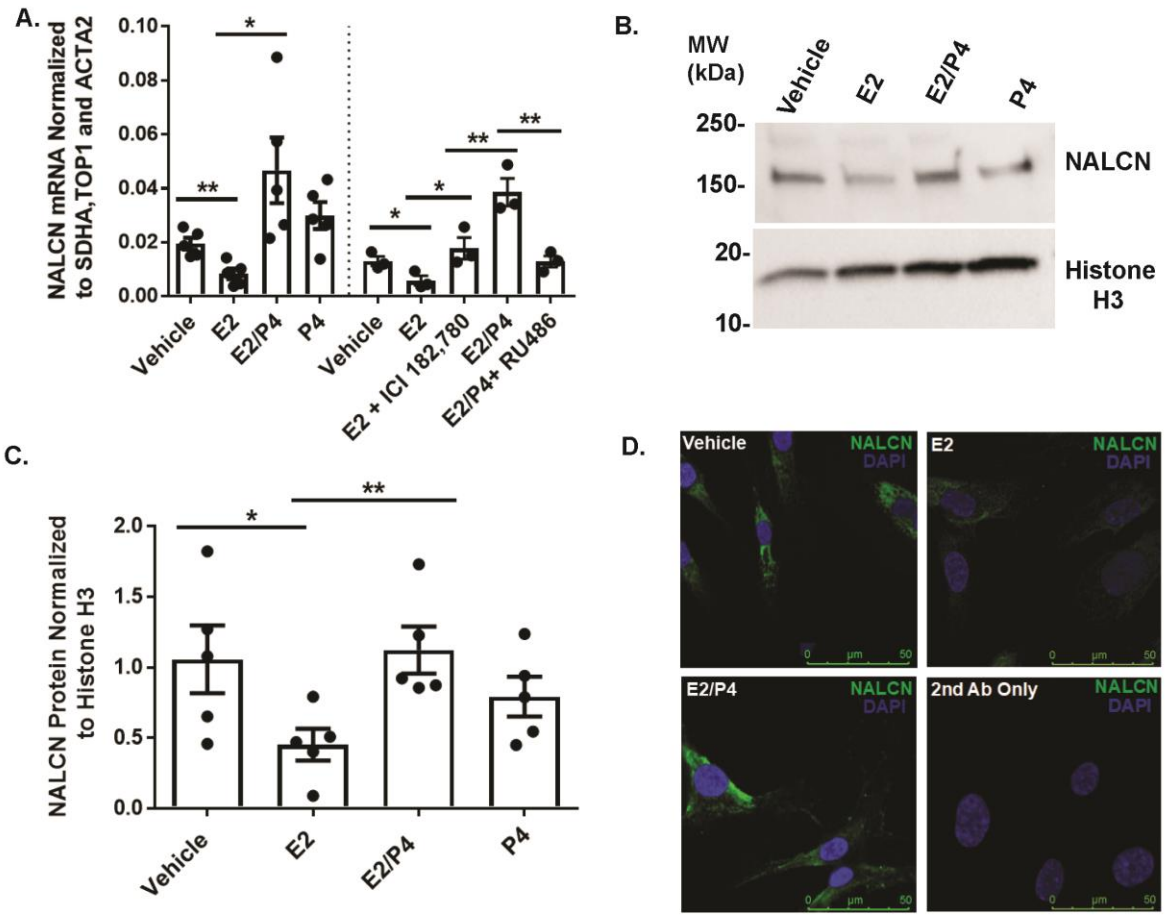


Figure 2.2: E2 and P4 regulate NALCN mRNA and protein expression

A) Quantification of qRT-PCR of NALCN mRNA in HM6ERMS2 treated with vehicle (N=5), E2 (N=5), E2/P4 (N=5), P4 (N=5), E2 and ICI 182,780 (ICI) (N=3), or E2/P4 and RU486 (N=3). The geometric means of TOP1, SDHA, and ACTA2 were used as standards for quantification. B) Representative Western blot of NALCN in HM6ERMS2 whole-cell lysates. Histone H3 served as the loading control. C) Quantification of NALCN protein in the HM6ERMS2 whole-cell lysates; N=5. NALCN signal was normalized to Histone H3. D) Representative immunofluorescence images of NALCN in HM6ERMS2 cells treated as indicated. (Scale Bar, 50 µm). Data are presented as mean and standard error of the mean. **P*<0.05, ***P*<0.01 by Dunnett's multiple comparison test one-way ANOVA. MW, molecular mass.

To confirm that E2 and P4 regulated NALCN expression via their respective receptors, we used the ER antagonist ICI 182,780 and the PR antagonist RU486. Cells treated with both E2 and ICI 182,780 had higher NALCN mRNA expression than cells treated with E2 alone, indicating that E2 represses NALCN expression via acting on ER. Additionally, cells treated with E2/P4 and RU486 had significantly less NALCN mRNA than cells treated with E2/P4, indicating that P4 promoted NALCN expression by acting on PR (Fig 2.2A). These results suggest that E2 decreases, and P4 increases NALCN gene expression.

To determine whether E2 and P4 also regulated NALCN protein expression, we performed immunoblots detecting NALCN in the hormone-treated HM6ERMS2 cells. A representative blot (Figure 2.2B) and quantitation of five replicates (Figure 2.2C) showed that NALCN protein was 2.3-fold lower in cells exposed to E2 than in cells exposed to vehicle. Conversely, we detected no statistically significant difference in NALCN protein expression in cells exposed to vehicle, E2/P4, and P4 alone (Figure 2.2B, C). Immunofluorescence experiments confirmed these findings, as NALCN staining was less evident in cells exposed to E2 than in cells exposed to vehicle or E2/P4 (Figure 2.2D). Together, these experiments indicate that E2 (the pro-contractile hormone) reduces NALCN mRNA and protein expression, whereas P4 (the pro-quiescent hormone) restores NALCN expression in cells in which PR is expressed (cells exposed to E2/P4).

Cellular responses to P4 differ depending on whether they express more PRA, which promotes contractility, or PRB, which promotes quiescence [5, 11]. In HM6ERMS2 cells, PRA and PRB are expressed in similar amounts (Figure 2.1A). Thus, to determine which receptor promoted NALCN expression, we used a human myometrial cell line (hTERT-HM) stably expressing FLAG-tag PRA or FLAG-tag PRB [11] (Figure 2.3A).

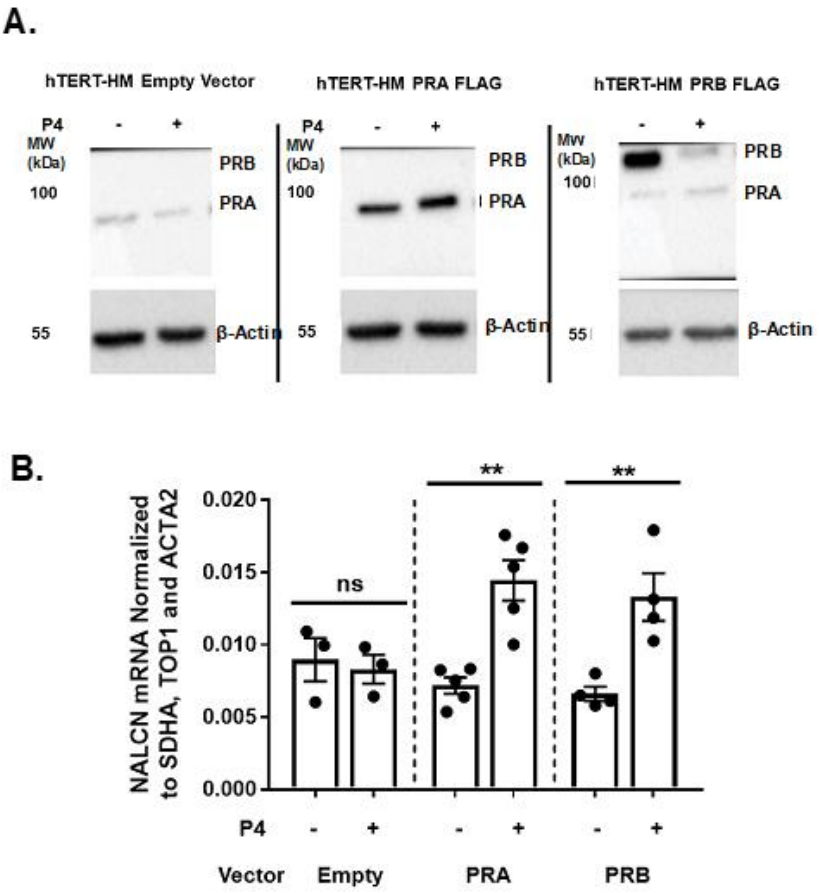
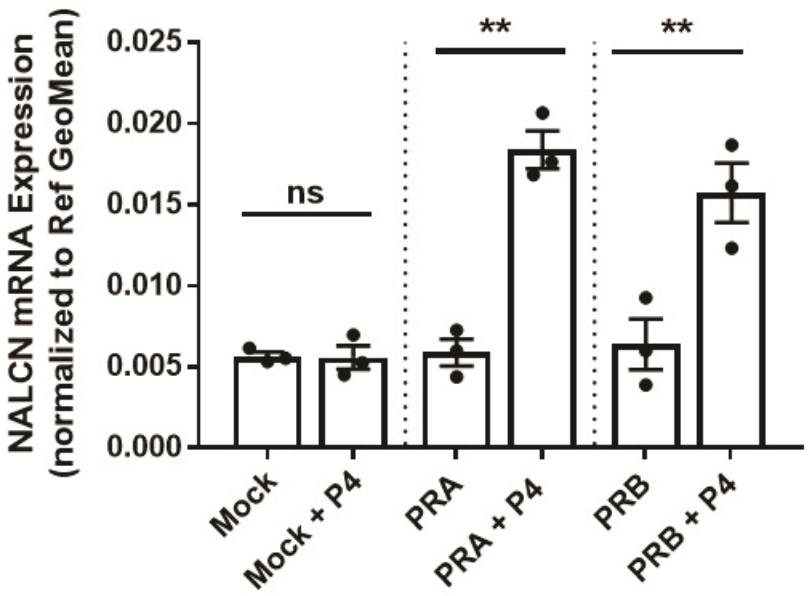


Figure 2.3: NALCN mRNA expression is upregulated by both PRA and PRB
A) Representative Western blot of PRA and PRB in hTERT-HM cells expressing empty vector, FLAG-tag PRA, and FLAG-tag PRB. β-actin served as a loading control. B) Quantification of qRT-PCR of NALCN mRNA in hTERT FLAG-tag PR cells as indicated; N=3 for Empty vector, N=5 for PRA, and N=4 for PRB. The geometric means of TOP1, SDHA, and ACTA2 were used as standards for quantification. Data are presented as mean and standard error of the mean. ***P* <0.01 by unpaired t-test. MW, molecular mass.



Supplementary Figure 2.1: NALCN mRNA expression is upregulated by both PRA and PRB.

Quantification of qRT-PCR of NALCN mRNA in hTERT FLAG-tag PR cells as indicated; N=3 for Empty vector, PRA, and PRB. The geometric means of TOP1, SDHA, and ACTA1 were used as standards for quantification. Data are presented as mean and standard error of the mean. ***P* <0.01 by unpaired t-test.

Whereas P4 had no effect on cells expressing empty vector, P4 treatment increased NALCN mRNA expression ~2.0 fold in both the FLAG-tag PRA and FLAG- tag PRB cells (Figure 2.3B and Supp. Figure 2.1). Thus, P4 signaling through both PRA and PRB can promote NALCN expression.

2.3.2 NALCN Activity is Reduced by E2 and Increased by P4

To determine whether E2- and P4-mediated changes in NALCN expression translated into changes in Na^+ current in MSMCs, we performed whole-cell patch clamping experiments in treated HM6ERMS2 cells. We first measured Na^+ leak current in vehicle-, E2-, and E2/P4-treated cells in the absence and presence of the non-specific NALCN blocker gadolinium (Gd^{3+}) [32, 40]. In cells treated with vehicle, a leak current was identified at all voltages (Figure 2.4A, B). In vehicle-treated cells, currents at -60 mV, near the resting membrane potential, were significantly larger in cells in the absence of Gd^{3+} than in the presence of 10 μM Gd^{3+} (-2.59 ± 0.53 pA/pF vs. -1.57 ± 0.50 pA/pF) (Table 2.3). This indicated the presence of a Gd^{3+}-sensitive current with an average pA/pF of -1.01 ± 0.22 (Figure 2.4C), consistent with our previous observations [32]. At all voltages, the Gd^{3+}-sensitive current was smaller in E2-treated cells than in vehicle-treated cells and higher in E2/P4-treated cells than in E2-treated cells (Fig 2.4B). In particular, the Gd^{3+}-sensitive current, measured at -60 mV, was significantly smaller in cells treated with E2 than in cells treated with vehicle (-0.24 ± 0.08 pA/pF vs. -1.01 ± 0.22 pA/pF) (Figure 2.4C). Finally, the Gd^{3+}-sensitive current at -60 mV was significantly larger in cells treated with E2/P4 than in cells treated with E2 (-0.75 ± 0.18 vs. -0.24 ± 0.08 pA/pF) (Figure 2.4C).

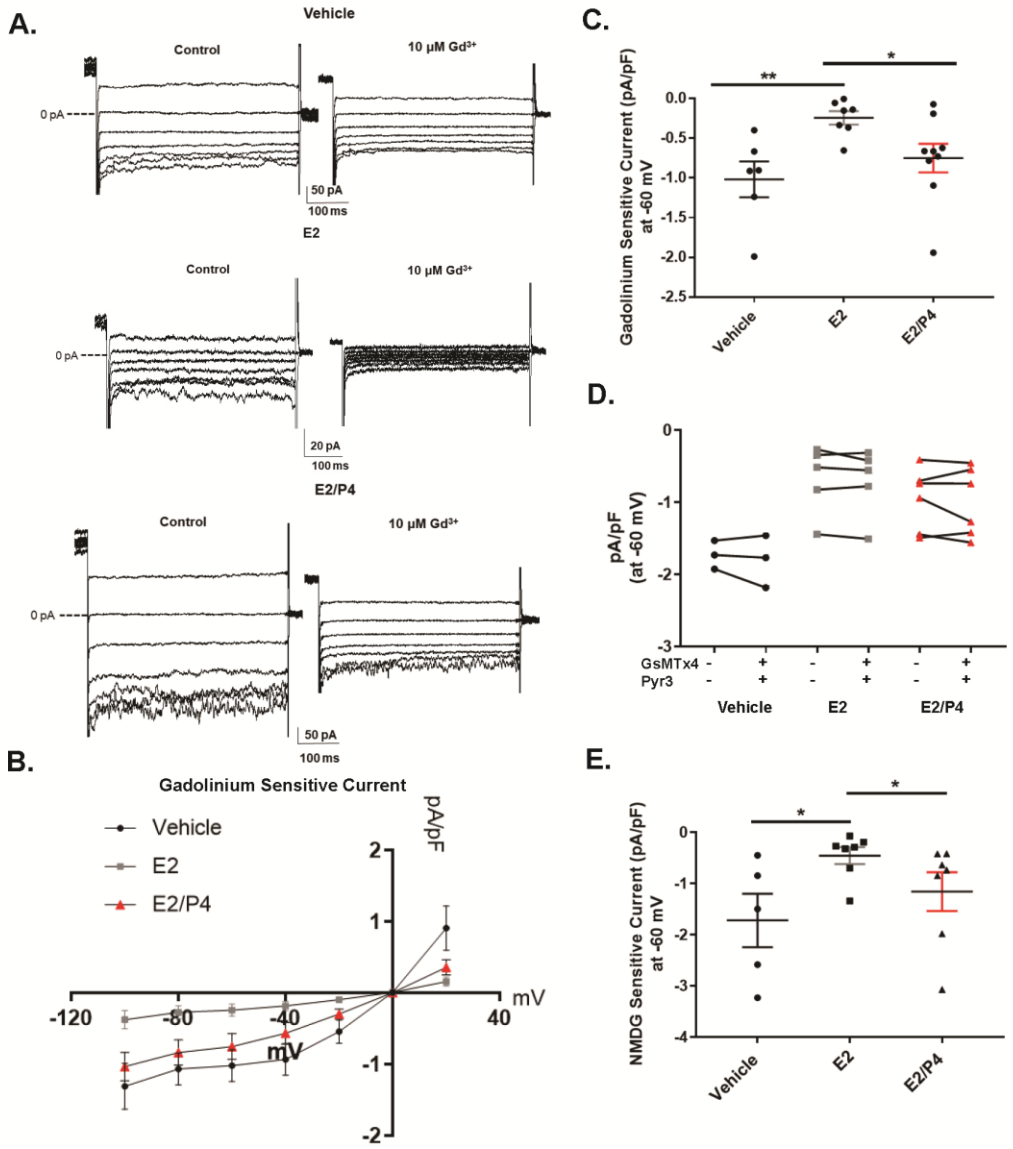


Figure 2.4: NALCN activity is reduced by E2 and increased by P4 in HM6ERMS2 cells
A) Representative traces of leak currents evoked from HM6ERMS2 cells treated with vehicle, E2, and E2/P4 by using a voltage step protocol with 20 mV increments from -100 mV to +20 mV, before and after treatment with 10 μM Gd^{3+}. Capacitive currents were removed from these traces. B) Current-voltage relationships of the Gd^{3+}-sensitive current obtained from HM6ERMS2 cells treated with Vehicle (n=6), E2 (n=7), or E2/P4 (n=9). C) Gd^{3+}-sensitive current density analysis at -60 mV after treatment with Vehicle, E2, or E2/P4. Each symbol represents an individual cell. D) Control and TRPC inhibitor-resistant current density analysis at -60 mV in cells treated with Vehicle (n=3), E2 (n=5), or E2/P4 (n=6) in the absence or presence of 500 nM GsMTx4 and 1 μM Pyr3. Each symbol represents an individual cell. E) NMDG-sensitive current density analysis at -60 mV after treatment with Vehicle (n=5), E2 (n=7) or E2/P4 (n=7). Data are presented as mean and standard error of the mean. *P<0.05, **P<0.01 by Dunnett's multiple comparison test one-way ANOVA.

In addition to blocking NALCN, Gd^{3+} blocks Na^{+} current through TRPC channels, which are expressed in human MSMCs [50-53]. To confirm that the Gd^{3+} sensitive current measured in HM6ERMS2 cells was carried by NALCN, we repeated our experiments in the presence or absence of the TRPC1/TRPC6 blocker GsMTx4 and the TRPC3 blocker Pyr3. At -60 mV, the leak current in all treatment conditions was unaffected by the TRPC blockers (Figure 2.4D, Table 2.3). Therefore, NALCN current is largely responsible for the Gd^{3+} sensitive current in these cells.

Because NALCN is a non-selective channel, we wanted to determine the predominant cations contributing to the current in HM6ERMS2 cells. Thus, we performed patch-clamp experiments in which we replaced Na^{+} in the bath with the channel-impermeable monovalent cation NMDG. This reduced the conductance to a similar extent as the addition of Gd^{3+} (Figure 2.4E, Table 2.3). Taken together, these results suggest that NALCN is the predominant Na^{+}-conducting channel regulated by E2 and P4 in HM6ERMS2 cells.

2.3.3 P4 regulates NALCN through progesterone response elements in the NALCN promoter

To further define the mechanisms underlying E2- and P4- mediated regulation of NALCN expression, we searched the promoter region of the gene that encodes NALCN, *VGCNL1*, for estrogen and progesterone response elements (EREs and PREs). The ERE consensus sequence is GGTCAnnnTGACC and the PRE sequence is GnACAnnnTGTnC (Fig 2.5A).

Table 2.3 Summary of Leak Current Parameters in HM6ERMS2 cells

Treatment (n)	Current Density[#] (pA/pF)		Current Density[#] (pA/pF)		Current Density[#] (pA/pF)	
	Control	**10 μM Gd^{3+}**	**Control**	**500 nM GsMTx4 & 1 μM Pyr3**	**Control**	**NMDG Resistant**
Vehicle	-2.59 ± 0.53 (6)	-1.57 ± 0.50** (6)	-1.73 ± 0.11 (3)	-1.80 ± 0.21 (3)	-3.14 ± 0.69 (5)	-1.42 ± 0.40* (5)
E2	-0.66 ± 0.28 (7)	-0.42 ± 0.20* (7)	-0.68 ± 0.21 (5)	-0.72 ± 0.21 (5)	-1.01 ± 0.27 (7)	-0.55 ± 0.21* (7)
E2/P4	-1.65 ± 0.40 (9)	-0.90 ± 0.28** (9)	-0.96 ± 0.18 (6)	-0.99 ± 0.19 (6)	-2.05 ± 0.52 (7)	-0.89 ± 0.17* (7)

Mean Values ± SEM

Calculated at -60 mV

*P< 0.05 and **P< 0.01 from paired Student's t-test comparing before and after treatment with Gd^{3+}-, GsMTx4/Pyr3-, or NMDG-containing solution.

We used the AliBaba 2.1 TRANSFAC program [54] to search the region between the *VGCNL1* transcription start site and approximately 2.5 kb upstream and found two consensus PREs located 1023 bp (PRE1) and 2447 bp (PRE2) upstream of the *VGCNL1* transcription start site. Additionally, we identified a sequence that matched both ERE and PRE consensus sequences 390 bp (ERE1/PRE3) upstream of the transcription start site (Figure 2.5A). We cloned the ~2.5-kb wild-type (WT) *VGCNL1* promoter sequence upstream of the firefly luciferase gene in the pGL3-Basic Luciferase vector (*VGNCL1*-pGL3BasicLuc) and co-transfected this vector with a vector encoding *Renilla* luciferase into the breast cancer cell line MCF-7. MCF-7 cells constitutively express ERα and express PR after 12 h of E2 treatment [55-57].

In the cells with WT promoter, firefly luciferase activity was 42% lower in MCF-7 cells exposed to E2 than in cells exposed to vehicle and 40% higher in cells exposed to E2/P4 than in cells exposed to vehicle (black bars in Figure 2.5C), indicating that the *VGCNL1* promoter was regulated by E2 and P4. To confirm that any low concentration of E2 in the phenol red-containing media in which MCF-7 cells are normally maintained [58] did not confound our results, we repeated the experiments in phenol red-free media. We observed similar results in both types of media (Supp. Figure 2.2).

To test whether the putative PREs and the ERE were required for PR- and ER-mediated regulation of *VGCNL1* promoter activity, we introduced site-specific mutations that are known to decrease PR and ER binding to their respective response elements (Figure 2.5B) [59-62]. None of the PRE or ERE point mutations had a significant effect on firefly luciferase activity in cells treated with E2 (Figure 2.5C).

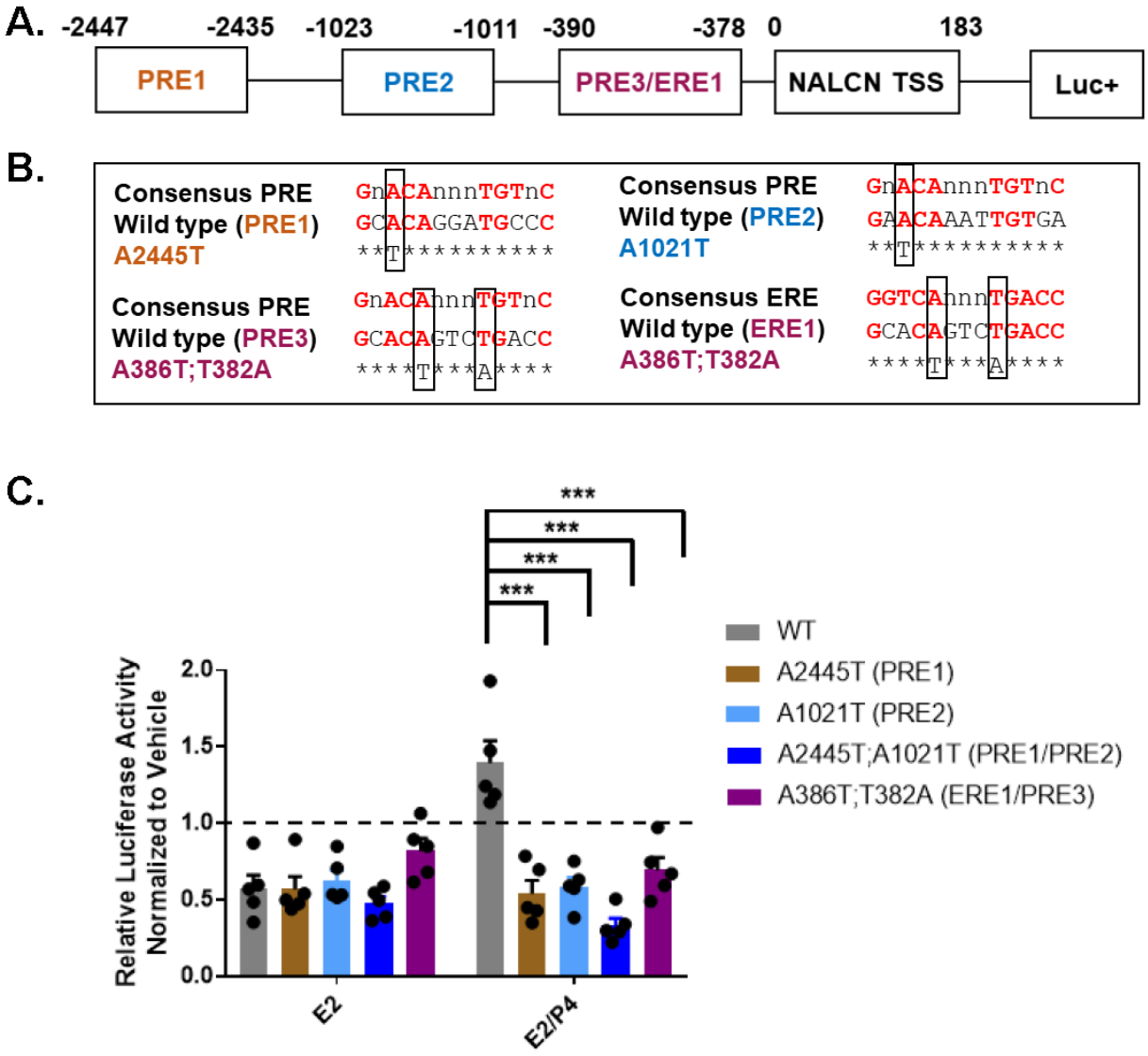
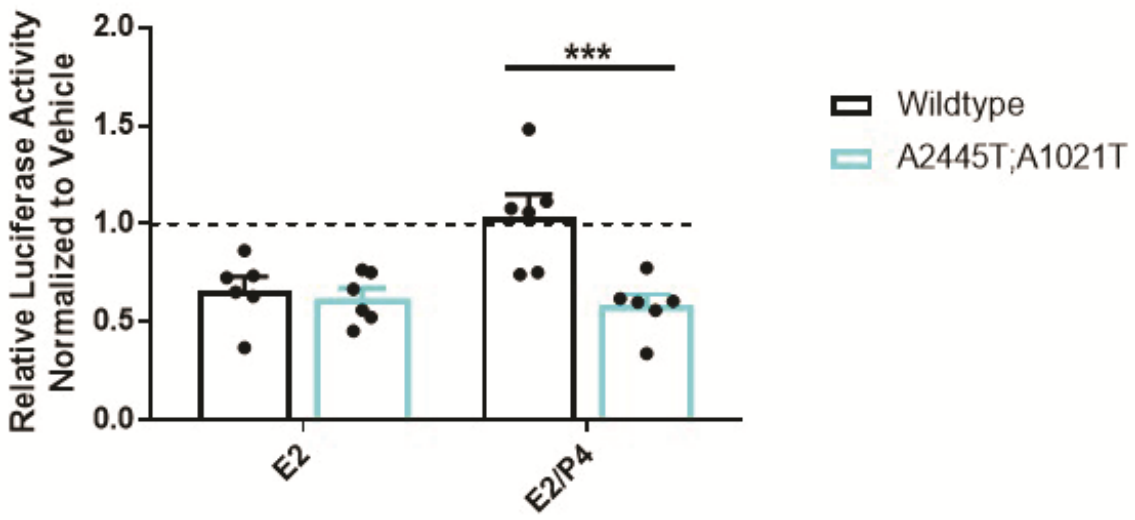


Figure 2.5: P4 regulates progesterone response elements in the NALCN promoter
A) Schematic showing the structure of the NALCN promoter sequence, including the putative PREs and ERE. B) Alignment of the PRE and ERE consensus, wild type, and mutant sequences. C) Relative firefly luciferase activities (normalized to *Renilla* luciferase activity in the same samples) of cells transfected with expression vectors carrying the indicated constructs after treatment with E2 or E2/P4. Data are shown as mean and standard error of the mean. N=5 for each treatment group. ****P*<0.001 by Dunnett's multiple comparison test two-way Anova.



Supplementary Figure 2.2: P4 regulates progesterone response elements in the NALCN promoter.
Relative firefly luciferase activities (normalized to *Renilla* luciferase activity in the same samples) of cells transfected with expression vectors carrying the indicated constructs after treatment with E2 or E2/P4 in phenol red-free EMEM media in 1% charcoal-stripped FBS. Data are shown as mean and standard error of the mean. N=6 for each treatment group. $P<0.001$ by two-way Anova with Dunnett's multiple comparison test.

In contrast, whereas E2/P4-exposed cells produced more activity than vehicle-exposed cells when driven by the WT promoter, E2/P4-exposed cells expressed less activity than vehicle-exposed cells when driven by promoters bearing any of the PRE or ERE mutations (Figure 2.5C and Supp. Figure 2.2). Together, these data suggest that PR regulates NALCN transcriptional activity, in part, by binding to their respective PRE sites within the NALCN promoter.

2.4 Discussion

Two major regulators of uterine contractility are hormonal signaling and ion channel activity [4, 6, 32-35, 63-65]. Previously, we showed that NALCN contributes to the leak current in human myometrial cells and that loss of this channel in mice results in decreased electrical bursting activity in myometrial tissue, dysfunctional reproductive outcomes, and smaller litter sizes [32, 33]. Here, we present evidence that NALCN mRNA and protein expression are downregulated by E2 and upregulated by P4. Additionally, E2 decreased and P4 increased a Na^+ conducting and Gd^{3+}-sensitive current that was predominantly carried by NALCN in MSMCs. We also showed that blocking ER or PR with competitive antagonists blocked the effects of E2 and P4. Finally, mutating the PRE sites in the NALCN promoter region blocked the effects of P4, suggesting that P4 acts through its nuclear receptors and response element sites to regulate NALCN transcription. Mutations of the ERE site did not abolish the effect of E2, suggesting that E2 and ERα work through another mechanism to modulate NALCN transcription.

Our conclusion that NALCN is upregulated by P4, a pro-quiescent hormone, and downregulated by E2, a pro-contractile hormone, is contrary to the traditional concept that Na^+ influx depolarizes the cell to enhance cell excitability. We suggest three potential reasons to

explain NALCN upregulation by a pro-quiescent hormone during pregnancy. First, when serum P4 concentration is high, Na^+ influx through NALCN may counteract K^+ efflux to maintain the resting membrane potential at ~-60 mV. Consistent with this idea, Lu *et al*. showed that, in neurons, NALCN conducted a Na^+ background current that opposed the K^+ conductance to maintain the resting membrane potential [40]. Furthermore, we showed that NALCN contributed to the background leak current in term non-labor human MSMCs [32]. Second, Na^+ influx through NALCN may activate other signaling pathways associated with maintaining resting membrane potential. For example, K^+ efflux through Na^+-activated K^+ channels has been reported in MSMCs, and increased NALCN expression and activity may directly activate more K^+ channels, thus making the resting membrane potential more negative and promoting quiescence [65, 66]. Third, in the presence of increased P4, NALCN and Na^+ influx can activate Na^+/K^+-ATPase pumps, which are necessary for maintaining the resting membrane potential in MSMCs [35, 67]. Overall, P4-mediated upregulation of NALCN expression and activity may contribute to maintaining uterine quiescence by stabilizing the resting membrane potential.

In previous work, we found that in the pregnant mouse uterus, NALCN mRNA and protein expression were high in early pregnancy, decreased during mid-pregnancy, increased at labor, and remained high post-partum [33]. The observed NALCN expression in the pregnant mouse suggests that NALCN protein levels are downregulated by P4 and upregulated by E2, which differs from what we observe in human myometrium. Yet, in our previous work, we did not measure P4 and E2 at the time points during pregnancy when we measured NALCN. Thus, a temporal analysis of NALCN expression and hormone concentrations could help explain the species-specific differences in hormonal regulation of NALCN.

Given the role of E2 as a pro-contractile hormone, one might expect that the depolarizing current through NALCN would increase in the presence of E2, but we found the opposite. Recent neuronal studies showed that overexpression of NALCN dampened excitability and pain sensitivity in sensory neurons of mole rats by depolarizing the membrane potential to a value at which voltage-gated Na^+ channels were inactivated [68]. Similarly, in myometrial cells, increased NALCN expression in the myometrium may indirectly dampen excitability by inactivating other excitatory channels. If this is the case, then E2 may downregulate NALCN to maintain the membrane potential at a certain value to ensure that these excitatory channels can be activated.

Previous evidence has suggested that PRB promotes quiescence by downregulating expression of inflammatory and contractile-associated genes [5, 15]. At the end of pregnancy, PRA:PRB ratio increases, leading to functional suppression of PRB, upregulation of inflammatory and contractile-associated genes, and labor induction [5, 15]. However, we found that both P4-bound PRA and P4-bound PRB increased NALCN expression. Likewise, Tan *et al.* showed that P4-bound PRA and PRB regulate several genes in the same direction, including bromodomain-containing protein 7, apolipoprotein B editing enzyme catalytic subunit 3B, protocadherin beta 8, and plasminogen activator, tissue type. These genes are involved in cell cycle regulation, cellular remodeling, and breakdown of blood clots, which are all necessary for the entirety of pregnancy [5]. Thus, future efforts could be directed at determining whether NALCN similarly functions in the cell cycle, cell remodeling, or vasculature events. Regardless, our data provide another example in which PR-A and PR-B work together to regulate gene expression.

Hormone receptors bind to their specific hormone response elements within promoters to regulate transcription. In our study, mutation of each putative PRE site in the NALCN promoter caused a decrease in reporter expression. In contrast, mutation of the ERE1/PRE3 site did not abrogate E2-mediated transcriptional repression. One possibility is that additional ERE sites exist further upstream than 2.5 kb from the NALCN transcriptional start site. Alternatively, E2-ER could regulate NALCN expression via interaction with transcription factors, such as Sp1 or AP-1, that bind to their own canonical binding sites [69-73]. For example, PR-bound JUN/JUN homodimer (AP-1 type protein) forms a complex with P54/Sin3A/HDAC and binds to the AP-1 consensus site to regulate Connexin-43 expression in MSMCs [11, 74, 75]. Putative AP-1 and Sp1 sites have been identified in the NALCN promoter region [54], but whether these sites are required for ER-dependent NALCN regulation in MSMCs has not been determined.

As pregnancy progresses, concentrations of both E2 and inflammatory mediators increase [76, 77]. Thus, E2 could down-regulate NALCN indirectly through inflammatory mediators. For example, in macrophages, the E2-ERα complex upregulates inflammatory mediators such as IL-1β and IL-6 and thereby affects genes downstream [78]. Additionally, in the myometrium, IL-1β regulates expression of channels and transporters (e.g., sarco/endoplasmic reticulum Ca^{2+}-ATPase) and increases Ca^{2+} transients through store-operated calcium entry [79]. Thus, E2-ERα may upregulate pro-inflammatory mediators in the myometrium, directly affecting NALCN expression and activity. This could provide an indirect mechanism by which NALCN is downregulated by E2.

In addition to regulating NALCN expression, E2 and P4 may regulate expression of accessory subunits that affect NALCN localization and function. For example, in mouse hippocampal and ventral tegmental area neurons, NALCN activity is modulated by the

interacting proteins UNC80, NCA Localization-Factor 1 (NLF-1), UNC79, Ca^{2+} Sensing Regulator, and Src family kinase [80-82]. UNC80 is of particular interest because it contributes to the localization and/or stabilization of NALCN at the cell membrane in neurons [82]. UNC80 also provides a scaffold for UNC79 and Src family kinase binding, which leads to activation of NALCN in various cell types [83]. Additionally, in *Drosophila,* an UNC80 mutation led to decreased NALCN protein concentration [84]. We have shown that UNC79 and NLF-1 mRNAs, but not UNC80, are expressed in uterine samples from term non-laboring women [32]. Thus, future effort should be directed at determining the effects of pregnancy stage, E2, and P4 on expression of these NALCN complex components.

In conclusion, this study provides evidence that NALCN is upregulated by P4 and downregulated by E2 in human myometrial cell lines. Our findings provide a foundation to further explore the function of NALCN during the quiescent period of pregnancy. For example, NALCN may play an important role in maintaining resting membrane potential during this period [32]. In the long term, defining the interplay between hormones and ion channels in the uterus will allow us to design targeted therapeutic approaches to treat uterine pathophysiology of pregnancy and labor.

2.5 Acknowledgments

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Chapter 3: SLO2.1 and NALCN channels form a functional complex that modulates myometrial cell excitability

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3.1 Introduction

Electrical activity of myometrial smooth muscle cells (MSMCs) is a key regulator of uterine contractions. The modulation of uterine electrical and contractile activity are required for several functions of the female reproductive tract, including contractile waves associated with

menstrual cycle, transport of sperm for fertilization, implantation of the embryo at the beginning of pregnancy, and contractility during parturition [1]. Unfortunately, aberrant electrical signaling can cause unproductive uterine contractions with several health consequences during the menstrual cycle and during pregnancy. Some of the consequences during pregnancy result in preterm or post-term deliveries.

As pregnancy progresses from the quiescent state towards term labor, the resting membrane potential (V_m) of MSMCs undergo a gradual depolarization from -75 mV at the end of the 2nd trimester to -50 mV at term or at the time of labor. These changes in the V_m are followed by an increase in the frequency of uterine contractions [2, 3]. The regulation of the V_m involves multiple ion channels, however the precise identity of the channels involved and how their activity changes during pregnancy remains unclear. It is well established that the V_m is determined primarily by an equilibrium between an outward K^+ leak current and an inward Na^+ leak current across the plasma membrane. The relative magnitude of these currents is regulated by the membrane permeability/conductance to K^+ and Na^+ ions. If the membrane permeability to K^+ is increased, the V_m becomes more negative, promoting quiescence. Conversely, when the permeability to Na^+ ions is increased, the V_m becomes more positive, and the uterus is in a more excitable and contractile state. Thus, identifying the ion channels and the molecular mechanisms that control the V_m is key in understanding how MSMC excitability and uterine contractions are regulated.

Our previous studies identified SLO2.1, a member of the SLO2 family of Na^+-activated K^+ channels, in human MSMCs [4-6]**.** This channel has low voltage dependence, high conductance, and can be significantly active at physiological intracellular Na^+ concentration [4, 7]. The characteristics of the channel suggests that SLO2.1 may modulate V_m and cell

excitability by regulating the membrane permeability to K^+. SLO2 channels are also highly expressed in brain and other smooth muscle cells [6, 8-10], where they form complexes with voltage-gated Na^+ channels to modulate the cell membrane potential [4, 11]. These complexes allow the Na^+ conducted through the Na^+ channels to modulate K^+ currents and have a greater effect on the V_m. However, it is unknown if SLO2.1 couples with a Na^+ channel to form a functional complex that can modulate V_m and cell excitability in human MSMCs.

A recent study identified the Na^+ Leak channel, non-selective (NALCN) [12]. This voltage-independent channel conducts about 50% of the sodium leak current at the V_m in human MSMCs [12]. Overall, these characteristics make NALCN an excellent candidate to be the source of Na^+ to modulate the SLO2.1 channel, but no evidence has been reported about the existence of this type of complex in human MSMCs. Here we tested the hypothesis, that NALCN, by forming a complex with the SLO2.1 channel, can be the source of Na^+ that modulates SLO2.1 activity and induce changes in the V_m in human MSMCs. We provide evidence that SLO2.1 and NALCN comprise a functional complex in human MSMCs that can regulate MSMC excitability and uterine contraction.

3.2 Methods and Materials

3.2.1 Ethical Approval and Acquisition of Human Samples

This study conformed to the Declaration of Helsinki and was approved by the Institutional Review Board at Washington University School of Medicine (approval no. 201108143) except for registration in a database. We obtained a signed written consent from each patient with forms approved by the Washington University in St Louis Internal Review Board. Human tissue samples from the lower uterine segment were obtained from non-laboring

women at term (37 weeks of gestation) during elective Cesarean section under spinal anesthesia. Samples were stored in 4°C in PBS and processed for myometrial smooth muscle cell isolation within 60 min of acquisition.

3.2.2 Culture of Myometrial Smooth Muscle Cells

Primary cultures of human myometrial cells from tissue samples obtained during cesarean procedures from women were obtained and cultured as previously described [13]. Briefly, tissue was pre-treated with PBS with 50μg/mL gentamicin and 5μg/ml fungizone. After, tissues were cut into 2- to 3-mm pieces and cultured in DMEM:F12 medium with 5% Fetal Bovine Serum (FBS), 0.2% fibroblast growth factor-β, 0.1% epidermal growth factor, 0.05% insulin, 0.05% gentamicin, and 0.05% fungizone. Colonies were amplified to form primary cell cultures. Primary MSMCs culture and human telomerase reverse transcriptase-immortalized myometrial cells (hTERT-HM) were incubated at 37°C and 5% CO2, in phenol–red free DMEM: F12 medium with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin or 25 μg/mL gentamicin (Sigma, St. Louis, MO). Primary MSMCs were used within two passages and hTERT-HM cells were used in passages lower than P15 to avoid differentiation.

3.2.3 Electrophysiology

Cells were starved in plain DMEM:F12 for at least for 2 hours prior to experiments. For all experiments, pipettes were obtained from borosilicate glass from Warner Instruments. For inside-out macro-patches and whole-cell recording, pipettes with a resistance of 0.8- to 1.8-Mega Ohms (MΩ) were used. For single channel recording, the pipettes used were in the range of 4- to 6- MΩ. For whole-cell recordings with MSMCs and hTERT-HM cells, the external solutions used (asymmetrical K^+, unless specified) were, in mM: 150 NaCl, 5KCl, 5 HEPES,

and 2 $MgCl_2$, pH adjusted to 7.4 with NaOH. For the 0 mM Na^+ solution, Na^+ was replaced by 150 mMvCholineCl and KOH was added instead of KCl to regulate the pH at 7.4, the concentration of external K^+ varied from 4.5 to 5.5 mM. The pipettes were filled with (in mM): 140 KCl, 5 HEPES, 0.5 $MgCl_2$, 5 Mg-ATP, with 0.6 mM free Mg^{2+} and 10 EGTA for the 0 Ca^{2+} solutions. For pipette solutions with 100 nM Ca^{2+}free we used 1mM EGTA. Inside-out macro patches and single-channel patch clamp recordings were performed using symmetrical K^+ solutions. The internal solution contained (in mM): 80, 140 or 160 KCl, 80 NaCl (or 80 CholineCl for the 0 Na^+ condition), and 10 mM HEPES. The pipette solution was in mM: 80, 140 or 160 KCl, 80 NaCl, 2 $MgCl_2$, and 10 HEPES. All with the pH adjusted to 7.2-7.4. Changes in the solutions are indicated in the figures. During electrophysiological experiments, the cells and the intracellular side of the membrane were perfused continuously. Traces were acquired using an Axopatch 200B (Molecular Devices), digitized at 10 kHz for whole cell or macro-patches recording or at 100 kHz for single-channel recording. Records were filtered at 2 kHz or 20 kHz for macro currents (whole cell and macro-patches) or single channel recording, respectively. We analyzed the data with pClamp 10.6 (Molecular Devices) and SigmaPlot 12 (Jandel Scientific).

3.2.4 *In Situ* Proximity Ligation Assay

The hTERT-HM cells were cultured in chambered slides (8-well; LabTek/Sigma, St. Louis, MO), serum-deprived in 0.5% FBS for 24 h, washed in ice-cold 1X PBS, and then fixed in 4% (wt/vol) paraformaldehyde (PFA) in PBS for 20 min at room temperature with gentle rocking. After 4 x 5-min washed in 1X PBS, cells were permeabilized with 0.1% NP-40 for 5 min at room temperature, washed twice with PBS, and washed once in 100 mM Glycine in PBS to quench remaining PFA. The slides were rinsed in milliQ water to remove residual salts.

Duolink *in situ* proximity ligation assay (Sigma, St. Louis, MO) labeling was performed with antibodies: NALCN (mouse monoclonal; StressMarq), SLO2.1 (rabbit polyclonal, Alomone). The manufacture's protocol was followed completely except that cells with stained with NucBlue Fixed Cell Stain ReadyProbes (Invitrogen, Carlsbad, CA) for 5 min at room temperature before final wash in wash buffer B. Slides were dried at room temperature in the dark, mounted in Vectashield (Vector Laboratories, Burlingame, CA) and stored in the dark at -20 °C until analysis. Images were recorded using a AF 6000LX system from Leica (Buffalo Grove, IL, USA) with a Leica DMi8000 inverted microscope and a Andor-Zyla-VCS04494 camera. A halogen lamp was used as source of excitation the wavelength, excitation wavelength used were 488 nm for PLA signals and 340 nm for NucBlue (DAPI). A63X objective (HC PL FluoTar L 63X/0.70 Dry) was used to obtain the images. Data collection and system control were done using LasX software from Leica. Acquisition parameters used were: 2 and 1.5 seconds of exposure time for 488 and 340 nm respectively, no binning, 2048 x 2048 pixels resolution and voxel size of 0.103 μm. Whole images were taken every 10 seconds. Images analysis was done using LAS X, *ImageJ software (*National Institutes of Health, Bethesda, Maryland, USA), and SigmaPlot 12 (Systat Software Inc., Chicago, IL, USA). Results are presented as punctate per cell. Punctate were counted after background subtraction. All the imaging experiments were done at room temperature.

3.2.5 Determination of Membrane Potential by Flow Cytometry

hTERT-HM cells were centrifuged at 1000 rpm for 5 min. Cells were resuspended in modified Ringers solutions: 80 mM Choline Cl, 10 mM HEPES, 5 mM Glucose, 5 mM KCl and 2 mM $CaCl_2$; pH 7.4). The hTERT-HM cell viability and membrane potential were monitored using fluorescent dyes, Hoechst 33342 (Cayman, Ann Arbor, MI) and DiSC3(5) (ThermoFisher,

Waltham, MA), respectively. Hoechst 33342 sorts living cells based on DNA content and DiSC3(5) is a cationic voltage sensitive dye that follows hyperpolarization and has a emits a different wavelength based on extracellular or cytoplasmic localization. Here we record cytoplasmic DiSC3(5) wavelength. Before recording, 0.02 mg/mL Hoechst and 150 nM$DiSC_3(5)$ was added to 500 µL of cell suspension and data was recorded as individual cellular events using and FACS Canto II TM cytometer (BD Biosciences, Franklin Lakes, NJ). Side scatter area (SSC-A) and forward scatter area (FSC-A) fluorescence data were collected from 100,000 events per recording. The voltages used to record these fluorescence signals were as follows: FSC (231) and SSC (386). Threshold levels for FSC and SSC were set to exclude signals from cellular debris (Figure 3.1A). Doublets, aggregates and additional cell debris were excluded from analysis based on a dual parameter dot plot, in which pulse signal (signal high; SSC-H; y-axis) versus signal area (SSC-A; x-axis) was displayed (Figure 3.1B). Living cells that displayed negative staining for Hoechst was selected by using the filter Pacific Blue (214) and positive cells for $DiSC_3(5)$ were detected using the filter for allophycocyanine (APC; 291) (Figure 3.1C). To establish the effect of Na^+ on membrane potential, 80 mM NaCl, Choline chloride or Lithium (Li^+) was added to the 500 uL suspension. Additionally, 10 µM gadolinium (Gd^{3+}), a concentration known to inhibit NALCN current [14], 500 nM GsMTx4 (a peptide isolated from Grammostola spatulata spider venom; blocks TRPC1 and TRPC6), and 1µM Pyr3 (pyrazole; blocks TRPC3) were added to the cell suspension to determine NALCN's contribution to the leak currents [14-16]. Normalization was performed by adding 1 µM valinomycin (Sigma, St. Louis, MO). Data (median values) were analyzed using FlowJo software.

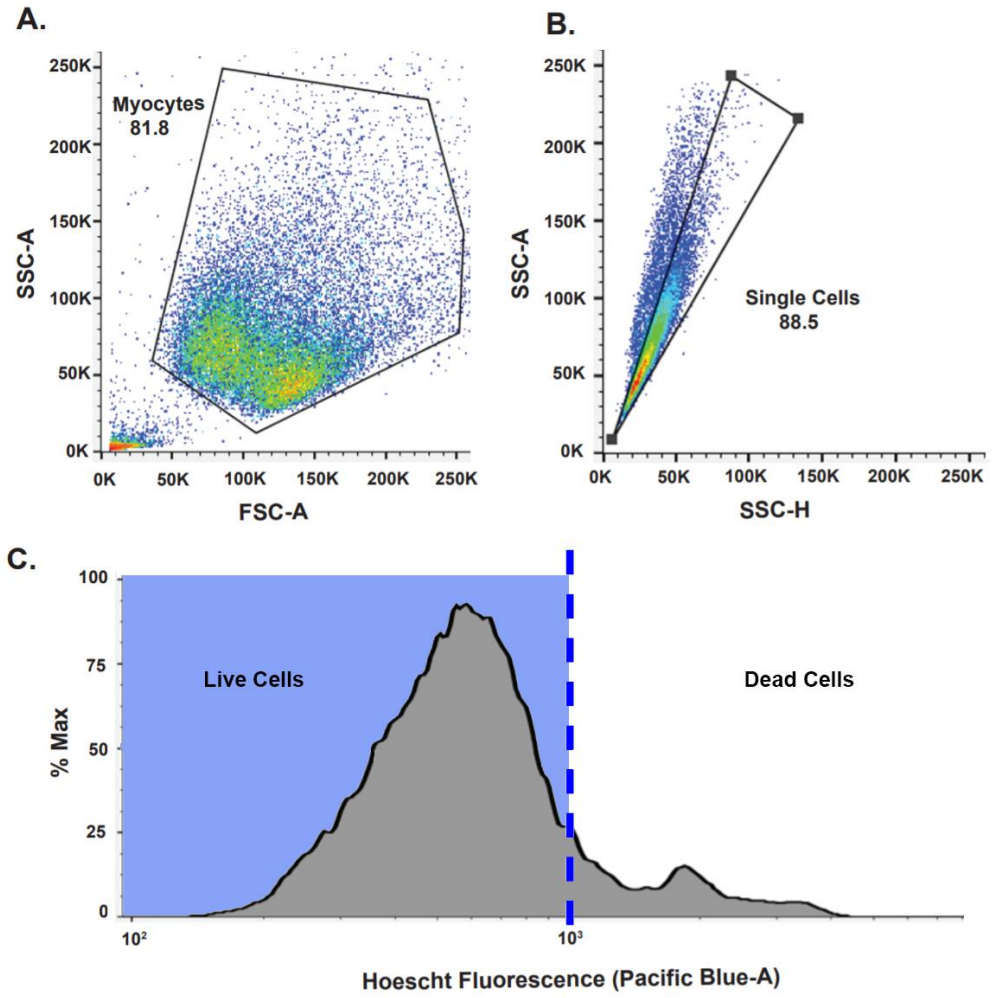


Figure 3.1: Optimized Parameters for Flow Cytometry.
A) SSC-A and FSC-A light detection were used to identify MSMCs based primarily on size. B) Singlets were selected with SSC-A and SSC-H to exclude doublets or debris. C) Hoechst dye was used to differentiate between live and dead MSMCs.

3.2.6 Calcium Imaging

Cells were culture glass coverslips with the media mentioned before for myometrial smooth muscle cells. Cells were pre-incubated with 2μM Fluo-4 AM and 0.05-0.1% Pluronic Acid F-127 in Opti-Mem for 60-90 min. To allow the dye to reach equilibrium in the cells, the cells were removed from the loading solutions and placed in ringer solution for 10 to 20 minutes. A perfusion system with an estimate exchange time of 1.5 s applied the various solutions. Recordings started 2-5 min prior to the addition of the first test solution. Ionomycin (5 μM) was added at the end of the recordings as a control stimulus. Recordings of the calcium signals were done with a AF 6000LX system from Leica (Buffalo Grove, IL, USA) was used with a Leica DMi8000 inverted microscope and an Andor-Zyla-VCS04494 camera. A halogen lamp was used as source of excitation the wavelength filter for excitation was 488 +/- 20 nm. As objectives a 40X (HC PL FluoTar L 40X/0.70 Dry) or a 20X (N-Plan L 20X/0.35 Dry) air objective were used. For the emitted light filters a bandwidth of 530 +/- 20 nm were used. Data collection and system control were done using LasX software from Leica. Acquisition parameters used were: 120 ms of exposure time, 2x2 binning, 512 x 512 pixels resolution and a voxel size of 1.3 μm for the 20X objective. Whole images were taken every 10 seconds. Images analysis was done using LAS X, *ImageJ software (*National Institutes of Health, Bethesda, Maryland, USA)[17], Clampfit 10 (Molecular Devices) and SigmaPlot 12 (Systat Software Inc., Chicago, IL, USA). $[Ca^{2+}]_i$ changes are presented as (F/F_{Iono}) after background subtraction. All the imaging experiments were done at room temperature. Cells with $[Ca^{2+}]_i$ changes of 5-10% relative to ionomycin responses changes were counted as responsive.

3.2.7 Isometric Tension Recording

Human myometrial tissue from four non-laboring women at term were isolated and cut into strips and immediately placed in 4° Krebs solution containing (in mM): 133 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 10 TES, 1.2 $CaCl_2$, and 11.1 glucose, pH 7.4. Strips were mounted to a force transducer in organ baths filled with oxygenated (95% O_2, 5% CO_2) Krebs solution at 35.7°C, and tension was recorded by a muscle strip myograph system - 820MS (DMT, Ann Arbor, MI) data acquisition system. Basal tension (2 g) was applied to the tissue strips and equilibrated for 1 hr prior until spontaneous myometrial contractility appeared. When four stable regular contractile waveforms were observed, we changed the Krebs solution to modified Krebs (lower [Na^+] solution containing (in mM): 33.25 NaCl, 99.75 CholineCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 10 TES, 1.2 $CaCl_2$, and 11.1 glucose, pH 7.4. Tension was recorded for 30 min. Traces obtained in the last 10 minutes before and after modified Krebs solution addition were compared using LabChart 8 (ADInstruments, Colorado Springs, CO). Basal tension and area under the curve (AUC) of phasic contractions of the e myometrial strips was calculated in both solutions.

3.2.8 Statistical analysis

Statistical analysis was performed using Sigmaplot, version 12.0 (Systat Software Inc.). An unpaired Student's t test was used to determine significant differences between independent samples tested. A paired t test was used to determine significant differences in case–control studies performed in the same individuals. Results are expressed as the mean ± SD. $P < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 The Gd^{3+} sensitive, Na^+ leak current is the source of Na^+ for the Na^+-activated K^+ current in human MSMCs

Here we investigate the source of the Na^+ that regulates SLO2.1, the previously described Na^+-activated K^+ channel that can regulate the excitability of the cell by changing the V_m [18]. In order to isolate the responses related to SLO2.1 channels, we used the SLO1 K^+ channel blocker tetraethylammonium (TEA) (5–10 mM) [19]. After adding 80 mM extracellular Na^+ to evaluate the activation of SLO2.1 current, we measured an 80-100% increase in the currents at +80 mV and -60 mV (V_m) (Figure 3.2A, D and E). An influx of extracellular Na^+ can activate K_{Na} channels at V_m as shown in Figure 1A and previously in human MSMCs by us [18] and also in neurons [4, 11]. To investigate if the recently described Na^+ leak channel, NALCN [12] conducts the Na^+ responsible for the activation of SLO2.1 in MSMCs, we performed whole-cell patch clamp experiments with controlled intracellular solution and different Na^+ channel blockers.

When experiments are performed in the presence of gadolinium (Gd^{3+}), a known blocker of sodium leak channels, the Na^+-dependent K^+ current is not activated by the addition of extracellular Na^+ (Figure 3.2B, D and E). This indicates that the source of Na^+ that activates K_{Na} in human MSMCs is conducted by a Na^+ leak current. We confirmed that Gd^{3+}does not have a direct effect on SLO2.1 channels (Figure 3.3). Thus, these results confirm that the Na^+ source to activate K_{Na} in human MSMCs is driven by a Na^+ leak current, which could be conducted by NALCN.

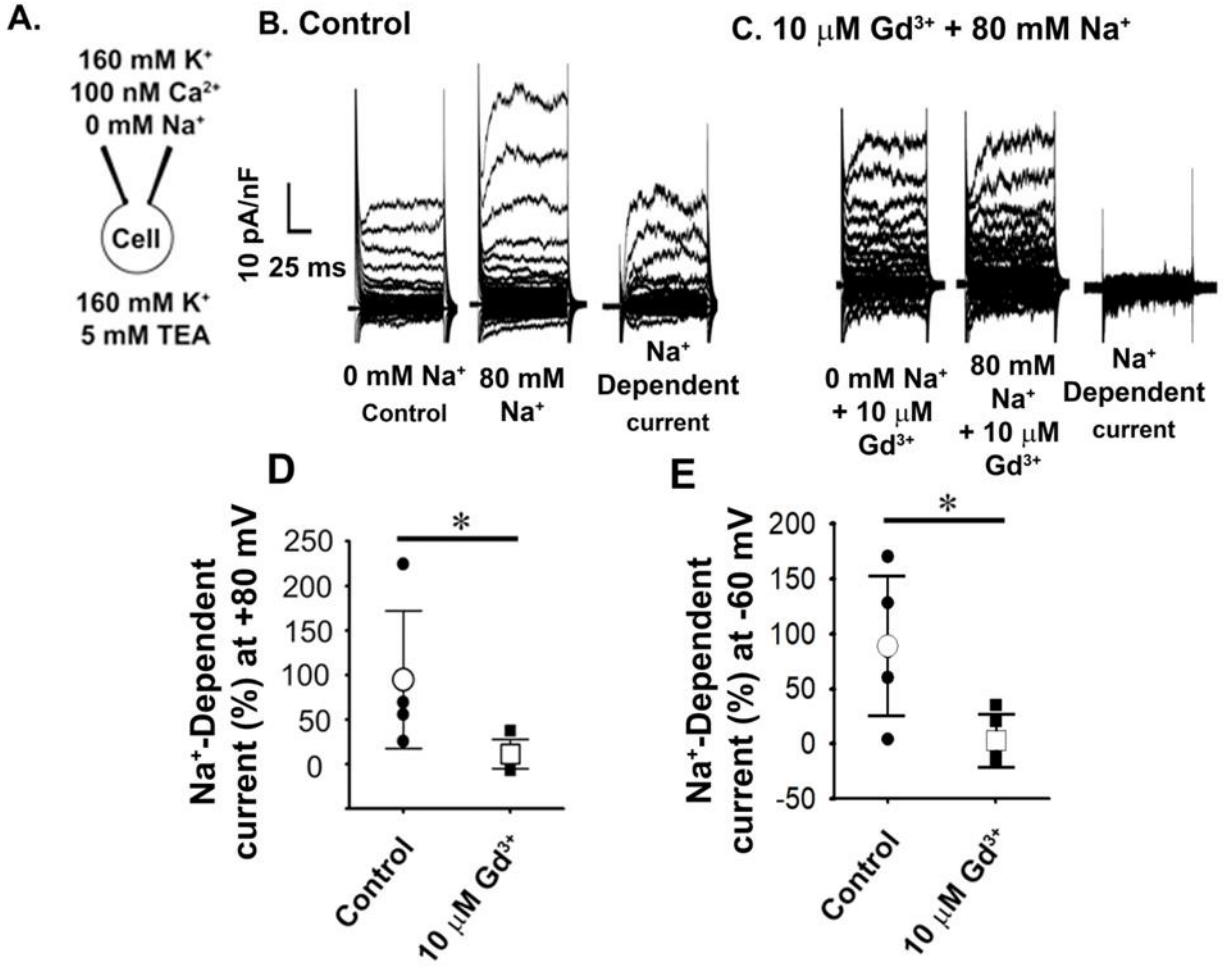


Figure 3.2: SLO2.1 channels are activated by a NALCN-Dependent Gd^{3+} sensitive Na^+ leak current in human MSMCs.

A) Schematic of whole-cell recording set-up. B) Representative whole-cell currents (V_h = −70 mV, with step pulses from −80 to +150 mV) from human MSMC recorded in the absence (control) and presence of 80mM Na^+.The Na^+-dependent currents were calculated by subtracting traces (80 mM –0 mM Na^+). C) Same as B, in the presence of 10 μM Gd^{3+}. Graph D and E: The percentage of Na^+-dependent currents activated with the addition of 80 mM Na^+ at +80 mV and -60 mV, respectively (n = 5 cells, data plotted as the mean ± SD). Values are for control at -60 mV: mean= 89.24, SD= 63.56 and at +80 mV: mean= 94.75, SD= 77.14. Values with Gd^{3+} at -60 mV: mean= 3.51, SD= 23.50 and at +80 mV: mean= 11.20, SD= 16.36. Unpaired t-test was performed.* *P*-Value<0.05.

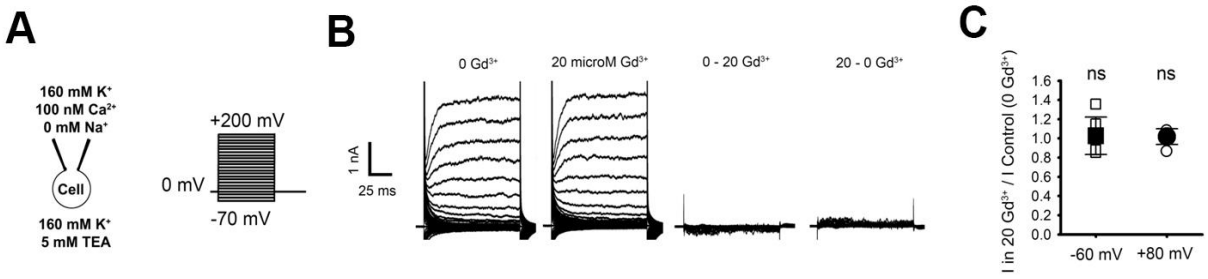


Figure 3.3: Effects of Gd^{3+} over SLO2.1 Currents.
A, schematic of whole-cell recording set-up and voltage steps applied. B) Representative whole-cell currents (V_h = −70 mV, with step pulses from −80 to +150 mV) from human MSMC recorded in the absence (control) and presence of 20 μM Gd^{3+}. The subtraction of traces represent: 0 – 20 μM $Gd^{3+}$and 20 μM Gd^{3+}-0. C.: Graph currents in the presence of 20 μM Gd^{3+}at -60 and +80 mV voltage steps, normalized to currents in control, error bars are SD. Values: 1) at -60 mV, Mean=1.03, SD= 0.195 and n= 6. 2) at 80mV, Mean= 1.018, SD= 0.0814 and n= 6. Paired t-test were performed and *P*-Value are 0.739 and 0.589 for 1 and 2, respectively.

3.3.2 NALCN and SLO2.1 are in close proximity in human MSMCs

Studies have confirmed the expression of functional complexes between SLO2.1 and sodium channels that activate SLO2.1 currents at V_m in neurons [4, 11]. To determine if NALCN and SLO2.1 channels are in proximity, we performed *in* situ proximity ligation assay in both human primary MSMCs and hTERT-HM. Punctate green fluorescent signals were detected and quantified in cells exposed to antibodies specific to NALCN and SLO2.1 (Figure 3.4A, B, and C). No significant signals were detected in our negative controls: 1) NALCN antibody alone, 2) SLO2.1 antibody alone or 3) secondary antibody alone (Figure 3.4A, B, and C). This data confirms that NALCN and SLO2.1 are expressed in close proximity in human MSMCs.

3.3.3 Hyperpolarization of the V_m induced by SLO2.1 activity is modulated by a NALCN-dependent Na^+ leak current

We investigated if an increase in the NALCN-dependent Na^+ leak current induced by the addition of extracellular Na^+ could modulate the V_m due to changes induced in SLO2.1 activity. To measure the changes in V_m, we used DiSC3(5) fluorescence in flow cytometry. DiSC3(5) is a cationic voltage sensitive dye that accumulates on hyperpolarized membranes and increases intracellular fluorescence. To evaluate the effects of extracellular Na^+, we treated the cells with: 1) sodium, 2) choline, an impermeable cation to maintain osmolarity or 3) lithium (Li^+), a permeable cation that does not activate SLO2.1 channels.

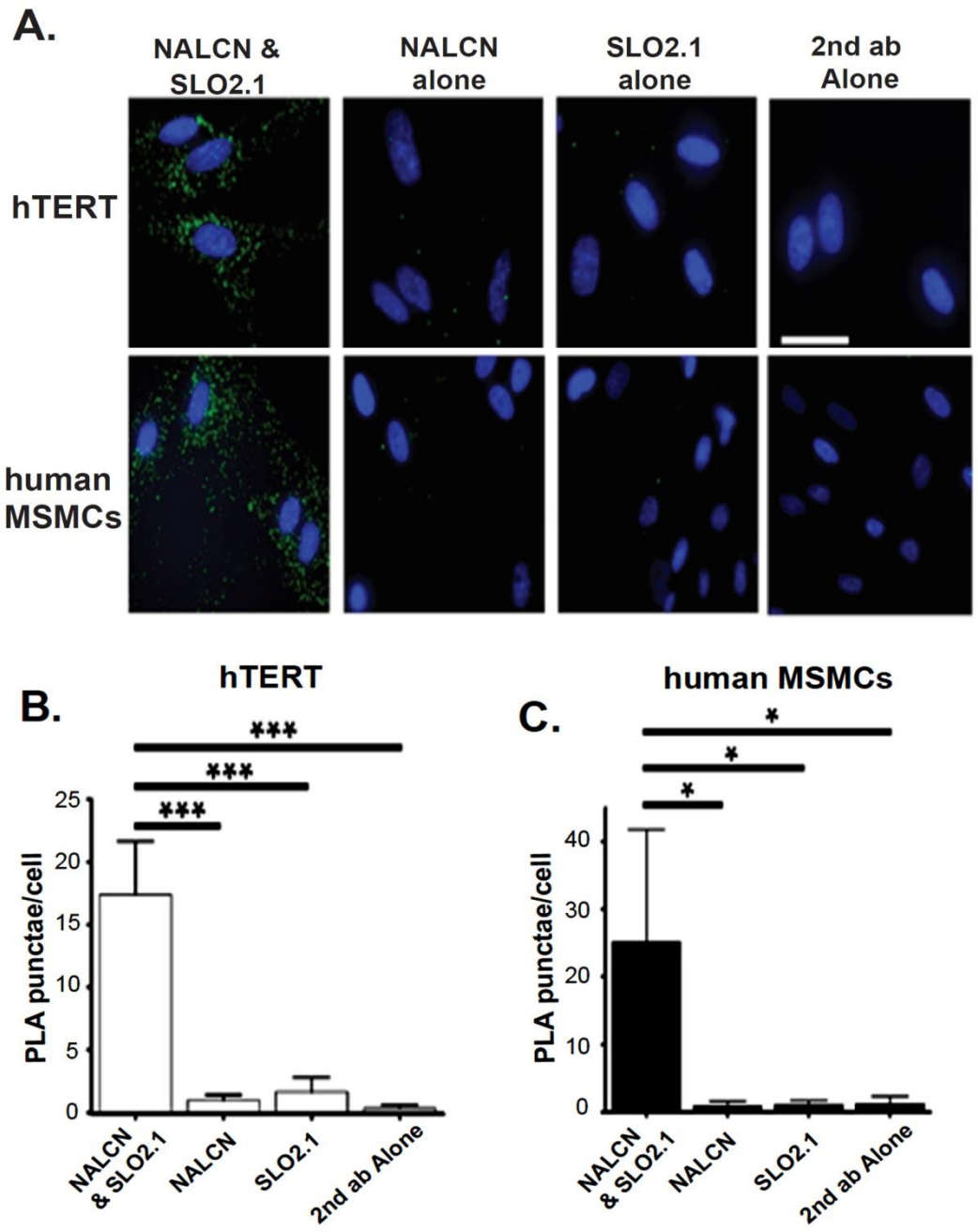


Figure 3.4: NALCN and SLO2.1 are in proximity in human MSMCs cells.
A) Representative PLA labeling of hTERT-HM and human MSMCs with the indicated single Abs and Ab combinations. (Scale bar, 10 μm.) (B,C) Average number of PLA signals in hTERT-HM (N=4) and human MSMCs cells, respectively. Human MSMC experiments were replicated in primary cultures from term non-laboring patients (N=4). Over 300 cells per condition were processed. Data are presented as mean and standard deviation. **P*<0.05, and ****P*<0.001 by unpaired t-test.

There was a significantly higher hyperpolarization when cells were treated with Na^+ than when treated with either choline or Li^+ (30.48% ± 20.62, 9.91% ± 9.76, and 2.01% ± 6.54, respectively) (Figure 3.5A and B). Therefore, Na^+ influx causes a hyperpolarization in the membrane potential of MSMCs presumably by activating SLO2.1.

To determine if Na^+ flux through NALCN was the main source of Na^+, we measured the hyperpolarization induced in the presence of the Gd^{3+}, a blocker of NALCN. Hyperpolarization induced by the addition of Na^+ in cells treated with Gd^{3+} was decreased to 6.01% ± 7.16 (Figure 3.5A and B). This response was similar to the hyperpolarization measured in cells treated with choline or Li^+ (*P*-value of 0.163 and 0.662, respectively) (Figure 3.5A and B). Similarly, in the presence of Gd^{3+}, there was no significant change in the hyperpolarization in cells treated with choline or sodium (mean(s) of 5.63% ± 4.57 vs 6.01%, respectively and *P*-value of 0.833) (Figure 3.6A).

Although, Gd^{3+}blocks NALCN currents, it also inhibits transient receptor potential canonical channels (TRPC). TRPC1, 3 and 6 channels were previously identified in human MSMCs [20-24]. TRPCs are primarily Ca^{2+} conducting channels, but they can conduct Na^+ [20-24]. We evaluated if the influx of Na^+ through TRPC channels could modulate SLO2.1 activity and V_m by using GsMTx-4 and Pyr3, two inhibitors that block TRPC 1 and 6 and TRPC 3, respectively. The hyperpolarization induced in cells by extracellular Na^+ and TRPC blockers was lower than in cells treated with Na^+ alone (17.77% ± 12.10 vs 30.48%, *P*-value of 0.040) (Figure 3.5A and B). Yet, in the presence of TRPC blockers, Na^+ induced a higher hyperpolarization than choline (mean(s) of 17.77% vs 5.21% ± 6.426, respectively, *P*-value of 0.008) (Figure 3.6B).

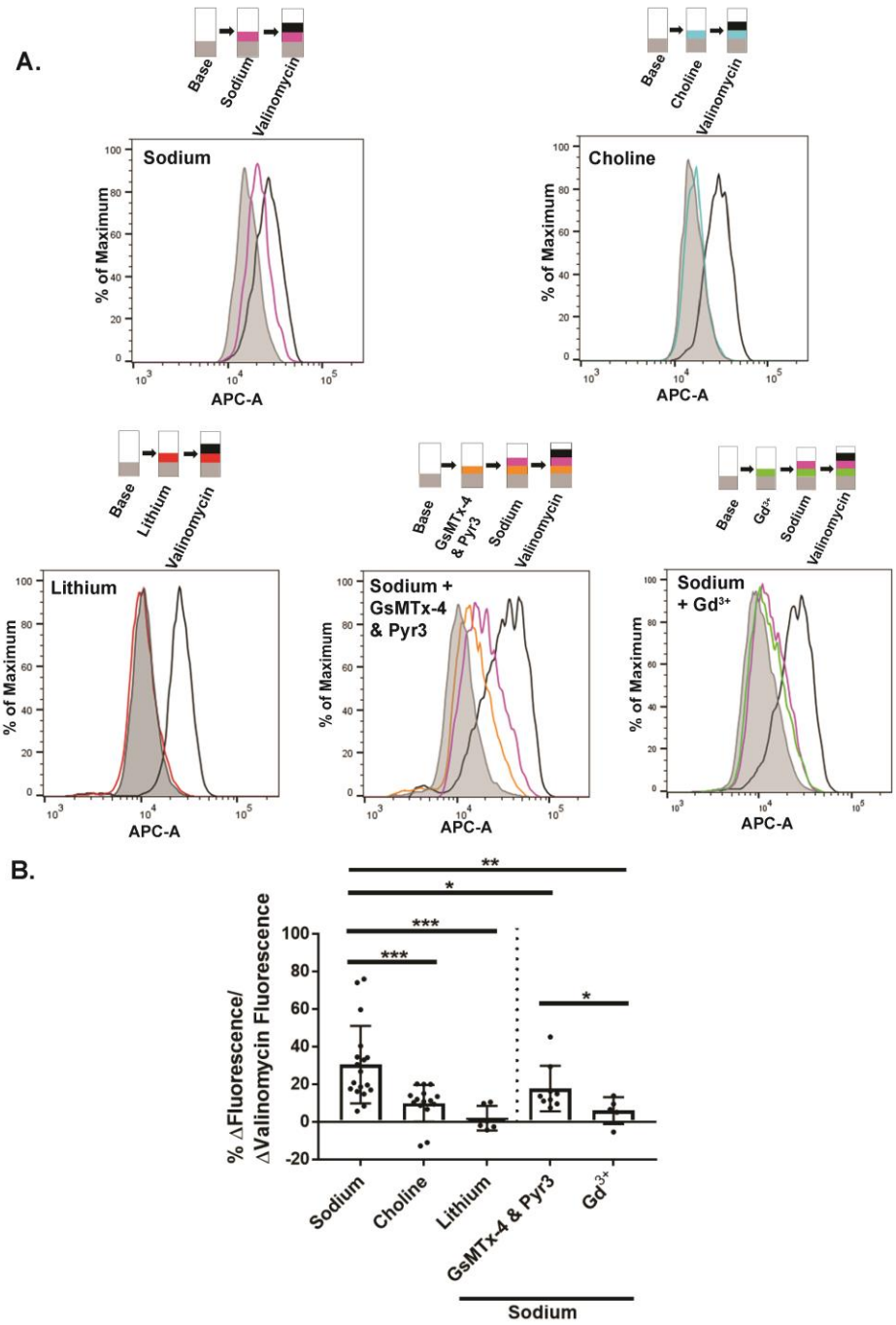


Figure 3.5: Regulation of SLO2.1 activity by the NALCN-dependent Na^+ leak induces hyperpolarization of the V_m.

A. Representative images of experimental schemes and relative shifts of DiSC3(5) fluorescence induced by Sodium, Choline, Lithium, GsMTx-4 and Pyr3 and Gd^{3+} in hTERT-HM cells. B) Quantification of shifts in cells by Sodium (n= 18), Choline (n=15), Lithium (n=6), Sodium (GsMTx-4 and Pyr3; n=9) and Sodium (Gd^{3+}; n=5) normalized to changes in Valinomycin fluorescence. Data are presented as mean and Standard deviation. **P*<0.05, ***P*<0.01, ****P*<0.001 by unpaired t-test with Mann-Whitney corrections.

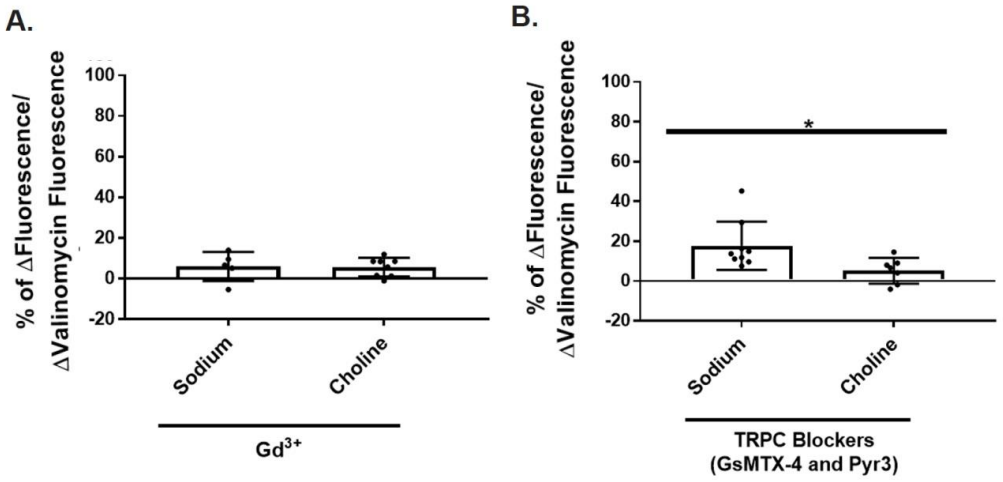


Figure 3.6: Gadolinium blocks hyperpolarization caused by both extracellular Na^+ and Choline while TRPC blockers partially decrease the hyperpolarization caused by extracellular Na^+.

A) Quantification of shifts in cells by Sodium (Gd^{3+}; n= 5) and Choline (Gd^{3+}, n=6) normalized to changes in Valinomycin fluorescence. B) Quantification of shifts in cells by Sodium (GsMTx-4 and Pyr3; n=9) and Choline (GsMTx-4 and Pyr3; n=7) normalized to changes in Valinomycin fluorescence. Data are presented as mean and standard error of the mean. $^{**}P<0.01$byunpaired t-test with Mann-Whitney corrections.

Additionally, the decrease in hyperpolarization induced by extracellular Na^+ was greater in the presence of Gd^{3+} than the TRPC blockers (6.01% vs 17.77%, *P*-value of 0.029) (Figure 3.5A and B). Our data indicate that the NALCN-dependent Na^+ leak current is responsible for ~60% of the Na^+-dependent hyperpolarization in human MSMCs, while the combination of TRPC 1, 3, and 6 contributes to ~40% of this hyperpolarization.

3.3.4 Functional coupling of the NALCN/SLO2.1 modulates intracellular Ca^{2+} responses in human MSMCs

Membrane depolarization triggers intracellular calcium increases through VDCCs in human MSMCs. Ferreira et al suggested that the inhibition of SLO2.1 channels by oxytocin or PMA triggers Ca^{2+} entry through VDCCs [18], possibly by modifying the membrane potential. We confirmed that the regulation of NALCN/SLO2.1 complex by extracellular Na^+ changes the V_m in human MSMCs. We hypothesized that NALCN-dependent Na^+ leak currents, by modulating SLO2.1 channels, can regulate VDCC and the intracellular calcium levels in MSMCs. We performed experiments measuring the intracellular calcium concentration by using the Ca^{2+} indicator Fluo4-AM. First, we used 5, 10, 20, and 50 mM KCl to induce increasing levels of membrane depolarization and observed that 20 and 50 mM KCl induced intracellular Ca^{2+} increases (Figure 3.7A).

Next, to evaluate the effects of extracellular Na^+, we substituted with Choline or with Li^+ [7, 25, 26]. In Figure 3.7B, cells were incubated in 80 mM Na^+ and 80 mM Choline, and then Na^+ component of the solution was replaced with 80 mM choline (160 mM choline). This change in the extracellular solution caused Ca^{2+} oscillations (Figure 3.7B and C) is comparable to those

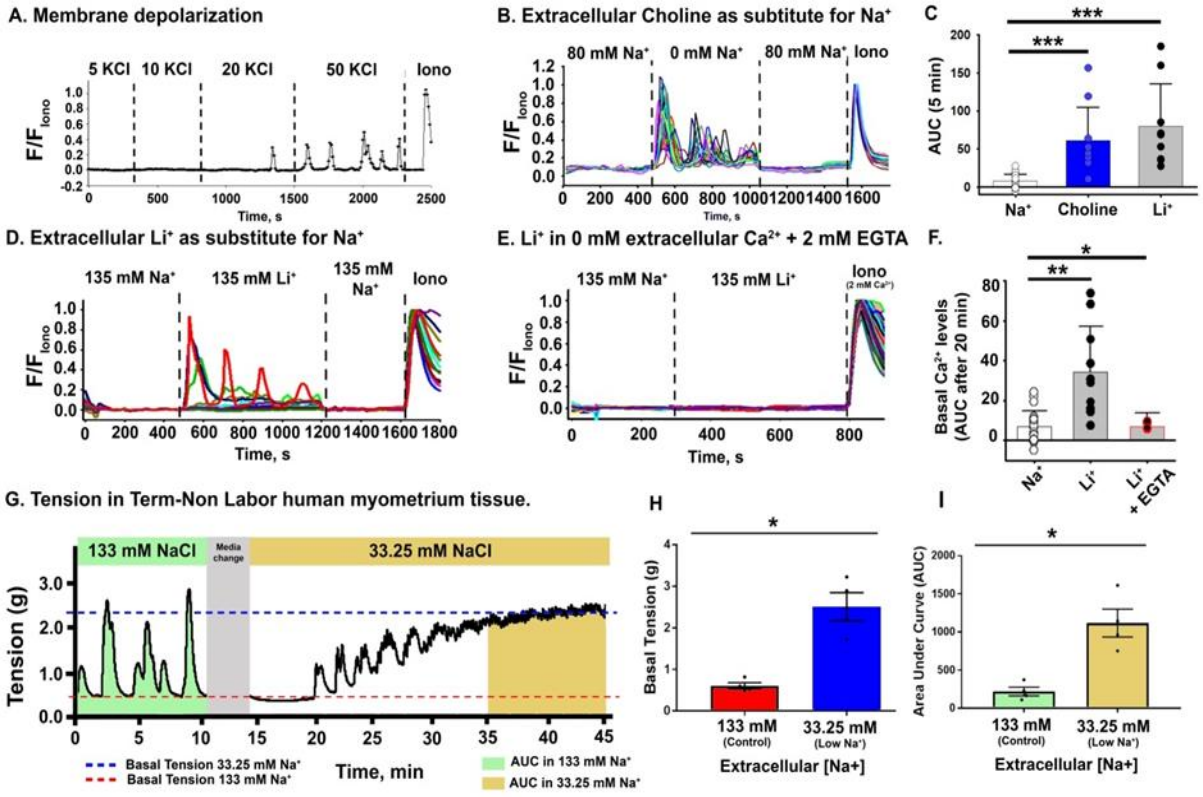
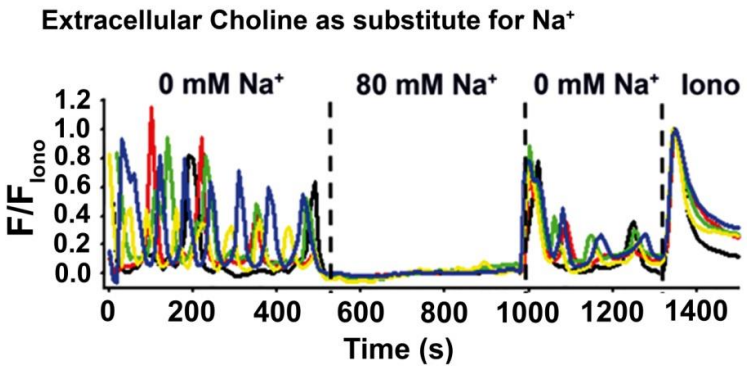


Figure 3.7: Na^+ leak regulates intracellular calcium homeostasis and basal tension in human MSMCs and myometrial tissue.

A, B, D and E**.** Representative fluorescence traces from human MSMCs loaded with 10 μM Fluo-4 AM. A) Membrane depolarization responses to 5, 10, 20, and 50 mM external KCl. B and C) Calcium responses induced by substituting extracellular Na^+ with Choline or Li^+ respectively. Graph I shows the quantification of the area under the curve (AUC) of the first 5 min after changing the solutions. Values are: 8.19 +/- 8.9 (SD), n=21; 61.5 +/- 43.6 (SD), n=10; and 80.3 +/- 55.8 (SD), n=9; for Na^+, Choline, and Li^+ respectively. D) Calcium responses induced by substituting extracellular Na^+ with Li^+ in 0 mM external Ca^{2+} and 2 mM EGTA. Graph II shows basal intracellular calcium levels (5 min AUC) of cells bathed for at least 20 min with 135 mM Na^+, 135 mM Li^+ or Li^+ with EGTA. Values are: 7.0 +/- 7.7 (SD), n=21; 34.2 +/- 22.9 (SD), n=10; and 6.94 +/- 6.7 (SD), n=4; for Na^+, Li^+, and Li^++EGTA respectively. All recordings were normalized to the fluorescence in 5 μM ionomycin and 2 mM extracellular Ca^{2+} (Iono). E) Representative tension-recording trace of myometrial tissue obtained from TNL patient in normal and low extracellular Na^+. Figure H, quantification of basal tension when tissue was bathed with control (red) or low Na^+ (blue) solutions (Error bars = SEM). Figure I, quantification of area under the curve (AUC) when tissue was bathed with control (green) or low Na^+ (light brown) solutions (Error bars = SEM). For Figures C and F unpaired t-test was performed and in Figure H and I, paired t-test were performed, n=4 for both. * *P*-Value<0.05, ** *P*-Value<0.01, and *** *P*-Value<0.001



Supplementary Figure 3.8: Na^+ leak regulates intracellular calcium homeostasis and basal tension in human MSMCs and myometrial tissue.
Representative fluorescence traces from human MSMCs loaded with 10 μM Fluo-4 AM. Calcium responses in MSMCs by substituting extracellular Choline with Na^+. Reverse experiments of Figure 3.4B.

induced by 50 mM KCl (Figure 3.7A). To avoid possible effects of using a non-permeable cation (choline), we performed experiments using Li^+ as a substitute for Na^+, which does not activate SLO2.1. In Figure 3.7D, cells were incubated in 135 mM Na^+, and then replaced with 135 mM Li^+. This change in the extracellular solution induced statistically significant Ca^{2+} increases (Figure 3.7C and D) similar to the responses triggered by 160mM choline (Figure 3.7B) and 50 mM KCl (Figure 4A). We observed similar results when cells were incubated with 160 mM choline and then replaced 80 mM of the choline solution with 80 mM Na^+ (Figure 3.8). To confirm that the Ca^{2+} increases induced by SLO2.1 inhibition are dependent on VDCCs and extracellular Ca^{2+}, we repeated the Li^+ experiment in the absence of 0 mM Ca^{2+} and 2 mM EGTA. No intracellular Ca^{2+} increases were observed under these conditions (Figure 3.7E).

Increases in the basal levels of intracellular Ca^{2+} were measured when Na^+ was replaced with either Choline or Li^+ (Figure 3.7F). These results are in line with our hypothesis where the absence of Na^+ would inhibit SLO2.1, depolarize the MSMCs, activate VDCCs and increase basal Ca^{2+} levels. To investigate if these findings translated to changes in basal tension and contractility, we performed tension recording on myometrial strips obtained from term non-labor patients. In Figure 3.7G, we show a representative trace of a myometrial strip bathed in normal Krebs solution that contains 133 mM Na^+ (control). When the extracellular Na^+ was reduced from 133 mM to 33.25mM, there was a significant increase in both the basal (0.61 ± 0.15 g vs 2.51 ± 0.68 g) and total (AUC) (219 ± 113 g vs 1118 ± 366.4 g) tension produced by the myometrial strips (Figures 3.7H and I). NALCN/SLO2.1 complex by extracellular Na^+ can modulate intracellular Ca^{2+} responses in human MSMCs and these effects can be observed in the tension generated by myometrial tissue. Together these results confirm that the regulation of the

NALCN/SLO2.1 complex by extracellular Na^+ can modulate intracellular Ca^{2+} responses in human MSMCs and these effects can be observed in the contractile activity of the uterus.

3.4 Discussion

Our results support the idea of a novel complex between a NALCN-dependent Na^+ leak current and the SLO2.1 channel modulating the V_m and the myometrial smooth muscle excitability. In patch clamp, we showed that the inhibition of the NALCN-dependent Na^+ leak current by Gd^{3+} reduced the activation of the Na^+-activated current carried by the SLO2.1 channel (Figures 3.2 and 3.3). With proximity ligation assays, we confirmed the close proximity of NALCN and SLO2.1 (Figure 3.4). We found that the increase in the inward Na^+ leak current induces a hyperpolarization of the V_m in human MSMCs (Figures 3.5 and 3.6). Finally, we provide evidence that the modulation of the V_m by NALCN-dependent Na^+ leak current and SLO2.1 channels can increase intracellular calcium by opening VDCC (Figures 3.7 and 3.8).

In MSMCs, action potentials are driven by an influx of Ca^{2+} through VDCC that open in response to a slow recurrent depolarization between action potentials (pacemaker current) [27-30]. Previous works in dopaminergic neurons and gastrointestinal cells showed that the depolarizing Na^+ leak currents could contribute to the pacemaker activity [31-33]. Although NALCN, a Na^+ leak channel, has been identified in MSMCs, the role of this channel [12, 34, 35], in regulating the pacemaker activity is still not well understood. If the channel is involved in setting the pacemaker activity, the absence of NALCN should present with a disrupted inter-burst frequency of action potentials. Yet, Reinl *et al.* only observed a significant reduction in the burst duration and no significant differences in myometrial inter-burst frequency at day 19 of pregnancy in NALCN KO mice [35]. These results and other recent studies have suggested that

NALCN could have a role in regulating myometrial excitability presumably by modulating the permeability of other ions (particularly K^+) and the V_m in MSMCs [12, 18, 35].

Different K^+ channels including the inward rectifier Kir7.1, Ca^{2+}-activated Maxi-K, and Na^+-activated SLO2.1 channels have been identified in human MSMCs [18, 19, 39, 40]. The expression of the Kir7.1 channel peaks in mid-pregnancy in mice and Kir7.1 currents contribute to maintaining a hyperpolarized V_m and low contractility during the quiescence state of pregnancy in mice and humans [39]. This suggests that Kir7.1 could contribute to regulating the transition from the quiescent to the contractile state during pregnancy. Yet, Ferreira *et al.* showed that Maxi-K and SLO2.1 contribute to ~87% of the K^+ current in human MSMCs (52% and 35%, respectively) [18]. Additionally, the authors showed that SLO2.1 is regulated by Na^+ and conducts current at the V_m of these cells [18], thus this channel introduces a novel mechanism to regulate the V_m, excitability and contractility of human MSMCs. Here we provide data showing the possible modulation of SLO2.1 channels by NALCN by demonstrating that the potassium channel SLO2.1 can be activated by an inward Gd^{3+}-sensitive Na^+ leak current (Figures 3.2 and 3.3). These results confirmed that a NALCN-dependent leak current can be the source of Na^+ to activate the SLO2.1 channel. This novel mechanism could regulate the V_m by increasing both the permeability to K^+ and the hyperpolarizing conductance of the cell.

In order for NALCN and SLO2.1 to be physiologically important as a functional complex, these two channels need to be in close proximity. By being in close proximity, the Na^+ leak current (NALCN) can modify the Na^+ concentration several fold in the intracellular microdomain without changing the bulk intracellular Na^+ concentration [4, 10]. We showed using *in situ* proximity ligation assay that NALCN and SLO2.1 are within 40 nM of each other in human MSMCs, confirming the close proximity of the two channels (Figure 3.4). These results

support the possibility of NALCN and SLO2.1 functioning as a complex and provide another example of functional complexes, which has already been found between many channels, including SLO2 channels and Nav1.6 in neurons [4, 11].

We investigated if the inward NALCN-dependent Na^+ leak current induces changes in the V_m by increasing the outward K^+ current carried by SLO2.1. Our hypothesis was that due to the proximity of the channels and the different conductance of NALCN (14.6 pS/pF) and SLO2.1 (78 pS), an increase in the inward Na^+ leak current that activates the SLO2.1 channel would cause a global cell hyperpolarization [12, 18]. We measured the membrane potential of myometrial cells and the results demonstrated that the addition of extracellular Na^+ causes a hyperpolarization of the V_m, supporting our hypothesis (Figure 3.5). Due to the limitations of the experimental conditions, we corroborate that membrane potential results are not a consequence of: 1) changes of the osmolarity when 160 mosm are added or 2) an increase in the cationic inward current. When 80 mM LiCl, a permeable cation that does not activate the SLO2.1 channel was added the V_m remained unchanged (Figure 3.3B). The hyperpolarization induced by extracellular Na^+ was abrogated in the presence of Gadolinium (Gd^{3+}), an inhibitor of Na^+ leak currents (Figures 3.5 and 3.6) [12, 14]. In the presence of Gd^{3+}, the cell becomes almost impermeable to Na^+ and the addition of 80 mM NaCl under this condition induced a small hyperpolarization that we attributed to osmolarity changes. We observed a similar percentage of hyperpolarization when Na^+ was substituted with Choline, an impermeable cation.

Gadolinium blocks both NALCN and transient receptor potential canonical channels (TRPC) channels [36, 37]. Several TRPCs, including TRPC1, TRPC3, and TRPC6, can conduct both Ca^{2+} and Na^+ and have different levels of expression in uterine tissue from rats and humans [20-24]. To determine if these three channels can contribute to the regulation of the Na^+-

activated K^+ current carried by SLO2.1, we used GsMTx-4 and Pyr3, specific blockers for TRPC 1/6 and TRPC 3, respectively. The results led us to the conclusion that TRPC1, 3 and 6 contribute ~40% of the hyperpolarization induced by the addition of 80 mM extracellular Na^+ (Figures 3.5 and 3.6). This highlights that the NALCN-dependent Na^+ leak current contributes to ~60% of the hyperpolarization induced under these conditions. The exact regulation and role of TRPCs channels during pregnancy is still under investigation. On one hand, the expression of TRPC6 (mRNA and Protein) is downregulated, while TRPC1 expression has no major difference in expression during pregnancy in rats [20]. On the other hand, TRPCs channels are sensitive to multiple signals *in vitro,* including mechanical stretch and IL-1β, signals notable important during labor [21, 38, 39]. Our results suggest that TRPC 1, 3 and 6 channels could have a role in regulating the $V_{m,}$ but based on previous evidence these channels may have a higher impact during labor when tension in the myometrium is increased. Further investigation is needed to understand the exact role of these channels during pregnancy.

We recently published evidence showing that oxytocin acting through a non-canonical pathway inhibits the SLO2.1 channel and induces an increase in intracellular Ca^{2+} by opening VDCC in human MSMCs [18]. These results are in line with data published by Arnaudeau *et al.* that suggested oxytocin has a sustained effect over the intracellular Ca^{2+} concentration that is also dependent on VDCC activity in MSMCs from pregnant rats [40]. We attributed this activation of VDCC to the depolarization induced by the inhibition of the SLO2.1 channel [18]. Here we show that the modulation of the NALCN-dependent Na^+ leak current has similar effects over the activity of VDCC and intracellular Ca^{2+} concentration (Figure 3.7 and 3.8). In our results, the absence of extracellular Na^+ increases both the transients and sustained levels of intracellular Ca^{2+} (Figures 3.7 and 3.8). By using Ca^{2+} free extracellular solution, we confirmed

that the responses induced by the absence of extracellular Na^+ are dependent on extracellular Ca^{2+} conducted through VDCC (Figures 3.7 and 3.8). Until here, we showed that NALCN and SLO2.1 by their role as a functional complex can regulate intracellular Ca^{2+} levels by modifying the V_m. We also observed that the reduction of extracellular Na^+ increased the contractility levels produced by human myometrium muscle (Figures 3.7 and 3.8). In MSMCs, contraction is initiated when intracellular Ca^{2+} ions bind to calmodulin, a Ca^{2+}-dependent cytosolic protein [41]. Thus, these results corroborate the novel importance of NALCN and SLO2.1 functional complex in the regulation of MSMC excitability and contractility.

It is known that ion channel activity and hormonal signaling are two major regulators of uterine contractility [12, 18, 35, 42-47]. Our novel model in Figure 3.9 shows how progesterone (P4), estrogen (E2) and oxytocin (OXT) could modulate MSMCs excitability, by regulating the expression and activity of NALCN and SLO2.1 complex. First during the quiescent state, P4 upregulates the expression of NALCN channels [47]. We hypothesize that this would increase the NALCN-dependent Na^+ leak current, which activates SLO2.1 channels, hyperpolarizes the cell and closes VDCCs in human MSMCs. Second, during the contractile state, OXT can induce calcium release from intracellular stores, and inhibit SLO2.1 channels acting through PKC [18] (Figure 3.9). The inhibition of SLO2.1 channels during the contractile state will be potentiated by the downregulation of NALCN by E2, a pro-contractile hormone [47]. Thus, we propose that OXT and E2, by inhibiting SLO2.1 channels in the last stages of pregnancy, will cause a depolarization of human MSMCs, promote intracellular Ca^{2+} increase through VDCC and increase uterine contraction (Figure 3.9). In summary, our model proposes novel insights into the mechanisms of how pregnancy hormones can modulate the activity of NALCN and the SLO2.1 channel and MSMC excitability levels during different stages of pregnancy.

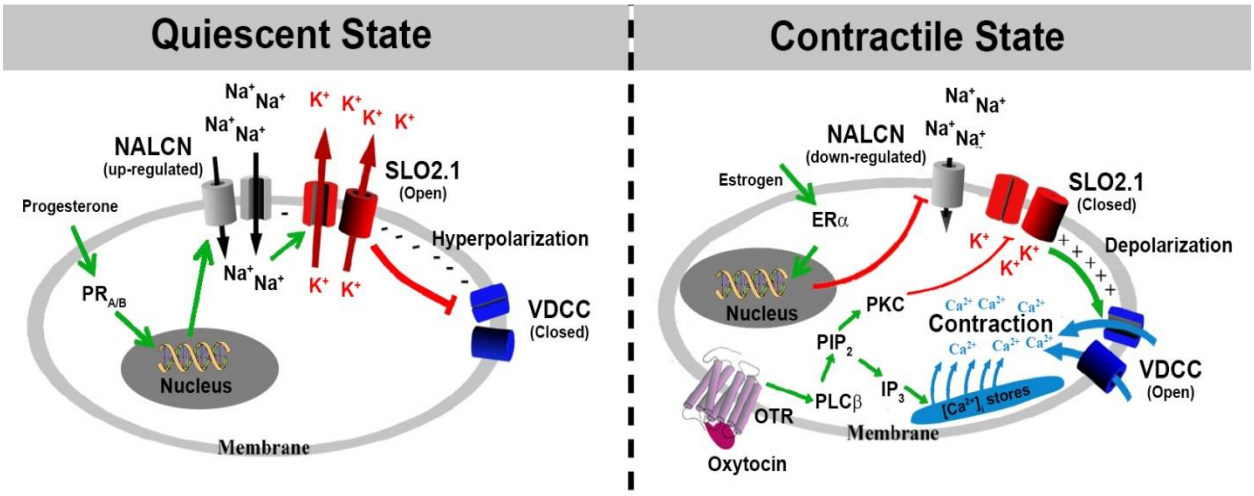


Figure 3.9: Proposed model for the hormonal regulation of NALCN/SLO2.1 complex in regulating myometrial excitability.

During the quiescent state progesterone binding to $PR_{A/B}$ increases the expression of NALCN channels [47]. Sodium current through NALCN activates SLO2.1 channels, increasing the K^+ current to maintain the cell negativity so the voltage-dependent Ca^{2+} channels (VDCC) are closed and uterine contractions do not occur. Our data together with previous publications from our laboratories [18, 47] suggest that the Na^+-activated K^+ current (SLO2.1 channels) that maintain the MSMC resting membrane potential in the presence of Na^+ flux. In the contractile state, estrogen acting on ERα inhibits the expression of NALCN channels [47]. A decrease in NALCN activity prevents SLO2.1 activity resulting in depolarization. VDCC are more active when the membrane depolarization increases, increasing intracellular Ca^{2+}, which can promote uterine contractions. Also, close to labor OXT binds OXTR, increasing diacylglycerol (DAG) that activates protein kinase C (PKC), which inhibits SLO2.1 [18]. Further inhibition of SLO2.1 channels could greatly depolarize the membrane, thus opening more VDCCs, which will greatly increase intracellular Ca^{2+} and activate myosin to cause muscle contraction.

3.5 References

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Chapter 4: Discussion and Proposed Future Directions

4.1 The Field of Myometrial Physiology

The electrical activity of myometrial cells, including resting membrane potential (V_m) and action potential, are caused by controlled changes in membrane permeability to Ca^{2+}, K^+, Na^+ and Cl^- ions [1, 2]. The changes in the permeability primarily result from changes in both expression and activity of the myriad of ion channels that conduct these ions [1, 2]. The myometrial ion channels are classified under five major types: Ca^{2+}, K^+, Cl^-, Na^+ and non-selective channels [1-3]. Historically, studies have primarily focused on Ca^{2+} (L-Type and T-Type) and K^+ (Ca^{2+}-activated) channels because of their significant contributions to the depolarizing and repolarizing phases of action potentials, respectively, that modulate the concentration of intracellular Ca^{2+} [4, 5]. The major Ca^{2+} channels are activated by voltage, while the Ca^{2+}-activated K^+ channels are activated by an increase in intracellular Ca^{2+}, depolarization of the plasma membrane, and uterine relaxants, including β-adrenergic agents, which elevate cAMP in myometrial cells [1, 6, 7]. Inwardly rectifying and voltage-gated K^+ channels contribute to the hyperpolarization of V_m in MSMCs [8, 9]. Functionally, Na^+ channels produce fast inward currents that result in depolarization but their identification and role in uterine excitability is still being investigated [4, 5]. Finally, Cl^- channels and non-selective channels have been identified and are proposed to regulate the pacemaker activity of the myometrial cells [4, 9, 10].

However, fewer studies have delved into the role(s) and regulation of non-selective Na^+ channels in MSMC excitability. In my thesis work, I investigated the hormonal regulation of NALCN, a Na^+ leak channel and discovered a novel role for this channel in regulating V_m and excitability in myometrial smooth muscle cells. My research has answered necessary questions in the field of myometrial physiology and revealed new questions for further investigation.

4.2 Hormonal Regulation of the NALCN Complex in MSMCs

Myometrial excitability changes across pregnancy in response to the changing hormonal expression and activity. Two hormones necessary for these important changes are progesterone (P4) and estrogen (E2). Progesterone works primarily through the nuclear progesterone receptor B (PR-B) to maintain uterine quiescence. The P4-PR-B complex downregulates contractile-associated proteins (CAPs) and inflammatory molecules, while upregulating pro-quiescent proteins and anti-inflammatory molecules [11-20] (Chapter 1). The transition from quiescence to contractility requires an increase of P4 signaling through PR-A and membrane bound PRs and E2 signaling through ERα [12, 18, 19, 21-27]. Both of these hormonal changes upregulate CAPs and inflammatory molecules, leading to increased contractility [20, 27-32] (Chapter 1).

As human pregnancy transitions from quiescence to contractility, the ratio of the pro-quiescent PR isoform PR-B to the pro-contractile isoform PR-A decreases [18, 32, 33]. Before term, the PR-B: PR-A ratio is 3:1; this changes to 1:1 during term non-labor (TNL) and to 1:3 during term labor (TL) [32, 33]. These changes remove the inhibitory actions of P4 and PR-B on CAPs and inflammatory molecules. In my studies, treating HM6ERMS2 cells with E2 induced a 1:1 PR-B:PR-A ratio which resembled the hormone receptor expression observed in TNL samples (Chapter 2). In this environment, I hypothesized that NALCN would be downregulated

by P4 and upregulated by E2 since Na^+ influx depolarizes the cell and enhances cell excitability. However, I found that the expression and activity of NALCN in human MSMCs was upregulated by P4 and downregulated by E2 (Chapter 2). Since P4 is a pro-quiescent hormone, these results suggest that Na^+ influx could promote quiescence. Although these results were unexpected, NALCN, unlike other major Na^+ channels, is constitutively active, voltage insensitive, and conducts Na^+ current at the negative V_m in human MSMCs [34]. Thus, our results are the first to establish the hormonal regulation of NALCN and suggest a novel role of NALCN in promoting quiescence during the TNL period of pregnancy.

One limitation in the HM6ERMS2 cell line is that we could not create a PR-B:PR-A ratio of 3:1 to effectively study the effects of NALCN in a TL-like environment. To address this limitation in future studies, we can use human myometrial tissue and isolated primary MSMCs from both TNL and TL samples. Our preliminary experiments show a decrease in baseline NALCN protein expression in membranes from TL uterine samples (n=3) compared to TNL uterine samples (n=2) (Figure 4.1). The differences observed in the data could be due to: 1) variabilities in the patient demographics within each group (TNL and TL), 2) any potential medications taken by the patient that may affect channel expression and/or 3) differences in cytokine/inflammatory profile at time of acquisition since cytokines can directly affect ion channel expression [35-37]. To address these problems, future experiments including: 1) increasing the sample size of tissue to determine baseline levels of NALCN expression, 2) evaluating the PR-B:PR-A ratios in each sample, and 3) isolating and treating primary MSMCs from these tissue samples with exogenous E2 and P4 to study the changes in NALCN expression from baseline are needed.

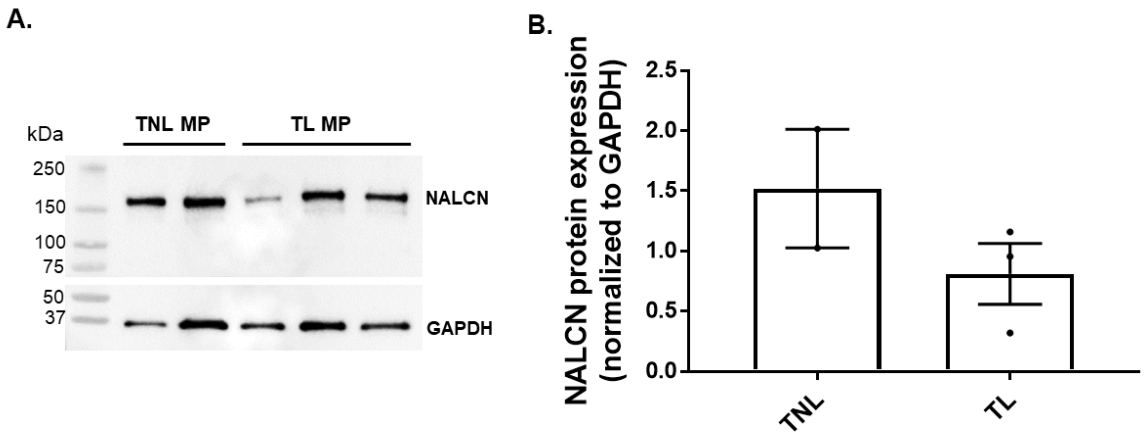

Figure 4.1 NALCN protein expression in primary human myometrial tissue from TNL and TL patients.

A) Representative Western blot of NALCN in myometrial membrane preparations from term, non-laboring (TNL) and term laboring (TL) samples. GAPDH served as loading control. B) Quantification of NALCN protein in the in the TNL (n=2) and TL (n=3) myometrial samples. NALCN was normalized to GAPDH.

NALCN has been shown to interact and form complexes with other proteins to regulate channel activity in various cell types [38-40]. Thus, E2 and P4 may modulate the expression of these accessory subunits (Chapter 1). This regulation can then affect the expression, stability and/or activity of NALCN. As described in detail in chapter 1, the modulation of NALCN in neurons depends on two proposed scaffolding subunits, UNC79 and UNC80 [38, 40, 41]. Our lab previously showed that UNC79 but not UNC80 was expressed in uterine samples from TNL women [34], while unpublished data showed that UNC80 was expressed in myometrial tissue from non-pregnant and TL samples. I investigated whether E2 and/or P4 modulated UNC79 or UNC80 expression in the HM6ERMS2 cell line, a line derived from TNL tissue. Preliminary quantitative RT-PCR revealed that UNC79 mRNA expression increased 2.0-fold in the presence of E2 and no additional increase was observed in the presence of P4 (Figure 4.2A). I further showed that P4 had no effect on UNC79 expression in hTERT-HM PR-Flag cells expressing either PR-A or PR-B (Figure 4.2B). The preliminary data shows that E2 upregulates while P4 does not affect UNC79 expression, which is different from NALCN regulation. In addition, UNC79 requires UNC80 to modulate NALCN activity in neurons [41] but UNC80 expression was negligible in the HM6ERMS2 myometrial cell line, a TNL-like environment (data not shown). In neurons, NALCN conducts a basal Na^+ leak current but its sensitivity to changes in extracellular Ca^{2+} requires UNC79 and UNC80 current [39]. Thus, UNC79 and UNC80 may be necessary during labor were the role and effects of Ca^{2+} are important however, this hypothesis needs further investigation.

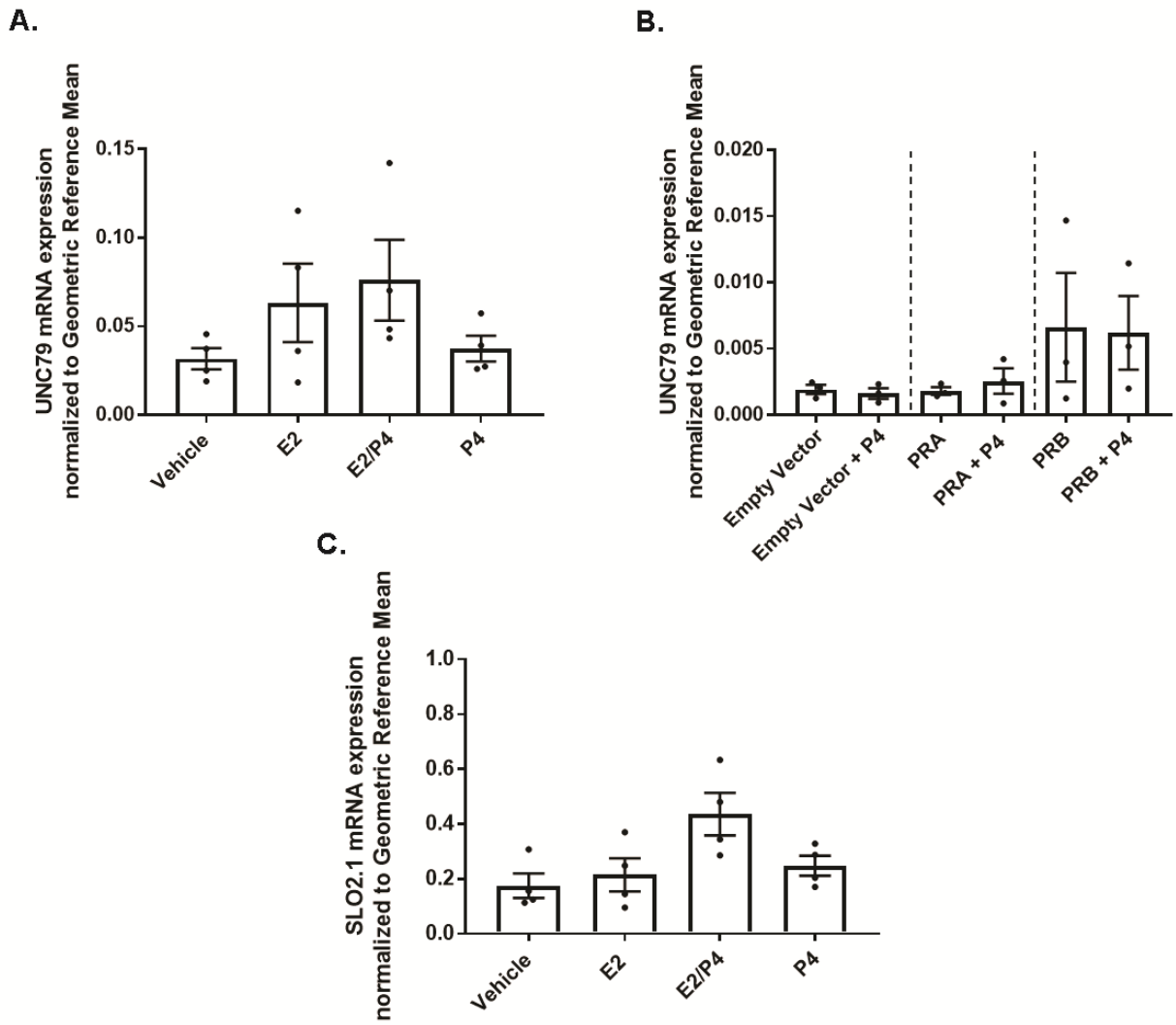

Figure 4.2 UNC79 and SLO2.1 are regulated by estrogen and progesterone in HM6ERMS2 myometrial cells, respectively.

A) Quantification of quantitative RT-PCR of UNC79 mRNA in HM6ERMS2 treated with vehicle (N=4), E2 (N=4), E2/P4 (N=4), and P4 (N=4). B) Quantification of quantitative RT-PCR of UNC79 mRNA in hTERT PR-Flag cells as indicated; N=3 for Empty vector, PRA and PRB conditions. C) Quantification of quantitative RT-PCR of SLO2.1 mRNA in HM6ERMS2 treated with vehicle (N=4), E2 (N=4), E2/P4 (N=4), and P4 (N=4). The geometric means of TOP1, SDHA, and ACTA2 were used as standards for quantification.

In Chapter 3, we proposed that SLO2.1 functionally couples and interacts with NALCN in human MSMCs to promote uterine quiescence by contributing to the V_m of the cell and decreasing intracellular Ca^{2+}. Thus, we investigated if E2 and P4 modulate this novel component of the NALCN complex in human myometrial cells. Preliminary evidence showed that E2/P4 treatment increased SLO2.1 expression 2.0-fold, similar to NALCN regulation, while E2 alone had no effect (Figure 4.2C). The upregulation of SLO2.1 and NALCN by P4 suggests an increased expression of NALCN, which could lead to increased activity of the functional complex, possibly during the quiescent period. Although, E2 inhibits NALCN, it does not affect SLO2.1 expression at the transcriptional level. Estrogen may still modulate SLO2.1 channel activity and/or stability at the membrane, which could affect the functional coupling during the pro-laboring period. For example, in cardiac cells, the hERG channel (the human Ether-à-go-go-Related Gene) contributes to the rapid delayed rectifier K^+ current (I_{kr}) in the action potential, which helps to coordinate the beating of the heart [42, 43]. Estrogen inhibits the trafficking of hERG to the membrane and the I_{kr} resulting in a decrease in hyperpolarization and a subsequent increase in excitability in cardiac cells [44, 45]. Oxytocin another pro-labor hormone regulates SLO2.1 channels in a similar way [46]. Ferreira *et al.* showed that oxytocin inhibits SLO2.1 activity through the protein kinase C (PKC) pathway, increasing intracellular Ca^{2+} and promoting contractions in human MSMCs [46]. Thus, E2 and oxytocin may work in similar ways leading to a depolarized membrane and increased myometrial activity during the laboring period, but this needs further investigation. Overall, we have shown that the major pregnancy hormones dynamically regulate the NALCN complex, which can affect the activity of NALCN channels in the myometrial cells.

4.3 The Novel Roles of Sodium Channels in Myometrial Physiology

Myometrial quiescence depends on the maintenance of a negative V_m in order to prevent increases in intracellular Ca^{2+} and subsequent myometrial contractions. Maintaining this negative V_m in MSMCs requires efflux of K^+ ions and the influx of Na^+ ions, primarily through K^+ and Na^+ channels, respectively. Recently, Ferreira *et al.* identified SLO2.1, Na^+-activated K^+ channel, as a potential significant contributor to the maintenance of V_m in human MSMCs [46]. SLO2.1 activation depends on the influx of Na^+, which we propose may occur through NALCN. Several lines of evidence support this model. First, NALCN is a voltage insensitive and constitutively active channel, meaning it can work at the V_m and possibly help maintain it [34, 47]. Second, our lab recently established that NALCN contributed ~50% of the background sodium leak current in human MSMCs [34]. Third, the upregulation of NALCN expression and activity by P4, a pro-quiescent hormone, provides a novel role of NALCN during the quiescent period [48]. As described extensively in Chapter 3, our results revealed a NALCN-dependent leak current that contributed to the V_m and modulated intracellular Ca^{2+}, through SLO2.1 activation.

This thesis work identified NALCN and SLO2.1 as channels that could contribute to the V_m in human MSMCs in similar mechanisms as the inwardly rectifying K^+ channel (Kir7.1), another regulator of V_m [8, 9]. At an expression level, Kir7.1 expression is high in mid-pregnancy but decreases towards labor in mouse model, similarly to the serum P4 levels, suggesting that P4 may promote the expression and activity of Kir7.1 [8]. Functionally, both Kir7.1 and NALCN remain persistently open, which can contribute to a hyperpolarized V_m [8]. Thus, the inhibition of both Kir7.1 and indirectly SLO2.1, by decreasing NALCN activity, can cause a depolarization, calcium entry and increased contractility in human myometrium [8].

Thus, we suggest that NALCN and SLO2.1 complex, in addition to Kir7.1, may be important in regulating the transition from uterine quiescence to contractility and could be targets for therapeutics to modulate uterine contractility, but further investigation is needed.

Our work also provides insight into the role and regulation of TRPC channels, a Gd^{3+}-sensitive, Na^+-conducting channel [47, 49, 50]. In our study, we found that a Gd^{3+}-sensitive Na^+ current, possibly NALCN and/or TRPCs, contributed to a hyperpolarization of the membrane potential in human MSMCs. To investigate the contribution of TRPCs to this current, we treated the human MSMCs with two TRPC inhibitors that blocked TRPC 1, 3 and 6. We saw a ~40% decrease in hyperpolarization induced by extracellular Na^+ suggesting that TRPCs can conduct Na^+ and modulate SLO2.1 channels in human MSMCs. During pregnancy, TRPC channels increase towards labor, and are activated by mechanical stretch and contribute to the electromechanical coupling in MSMCs, two events that underlie labor [51-55]. On the other hand, NALCN is inhibited by E2, a pro-labor hormone, suggesting that NALCN expression and activity may be decreased during labor. Thus, I hypothesize that NALCN is the primary activator of SLO2.1 during the quiescent period, while, TRPC is the major activator during the laboring period of pregnancy, yet further studies are recommended.

Although, this study focused on the channels that could regulate $V_{m,}$ Na^+ conductance through NALCN and TRPC channels may also regulate myometrial activity in labor through upstream signaling by uterine contractile agonists, such as substance P [56-60]. Substance P works through the GPCR neurokinin 1 receptor (NK1R) to activate a Na^+ current through NALCN, which results in rhythmic stability and pacemaking activity, in neurons and interstitial cells of Cajal (ICCs), respectively [41, 61, 62]. In neurons, this modulation is dependent on the presence of NALCN and UNC-80 [41, 61]. Since UNC80 and NALCN are present in human TL

samples, SP may activate NALCN to promote depolarization during the laboring period of pregnancy and regulate pacemaking activity of action potentials (phase three). Similarly, SP through NK1R induces an inward Na^+ current through TRPC channels to modulate pain transmission and respiratory rhythm regularity in neurons and this current is inhibited by PKC [63, 64]. PKC is downstream of NK1R and oxytocin receptor activation. Thus, better understanding if the PKC-mediated inhibition of TRPC channels may be a negative feedback mechanism to prevent over-excitation of myometrial excitability by SP or oxytocin during labor is necessary.

Overall, this thesis work proposes novels roles for Na^+ entry through NALCN and TRPC channels, which can lead to several impacts on myometrial reproductive physiology and provide a basis to explore similar functional complexes in other excitable organs.

4.4 The Role of NALCN in Translational Studies

We extensively discussed the genetic mutations of NALCN that were found in children and resulted in neurological and/or respiratory deficits (Chapter 1). These human mutations demonstrate the crucial role of NALCN in normal neurologic function and development. Yet, this thesis focused on the role of NALCN in myometrial and reproductive physiology. Thus, it would be important to discover and study if mutations and/or variants in the human *NALCN* gene could significantly affect myometrial or reproductive outcomes. We were recently contacted by a Norwegian research group, led by Dr. Mbarek, that performed a genome wide association study (GWAS) to identify SNPs associated with gestational duration in a sample of 7353 twin and non-twin births from the Netherlands Twin Register (unpublished data). They found one noncoding SNP upstream of the *NALCN* gene that was associated with gestational duration with genome wide significance (unpublished data). However, the mechanism of action of this SNP is unclear.

Further candidate gene studies may be helpful to determine whether variants in NALCN are important in reproductive outcomes. Once further genes are identified, we can perform functional studies to assess NALCN expression, stability and activity using mouse models, CRISPR genome editing and/or myometrial cell lines. The genetics studies coupled with functional experiments can help us determine whether variants in NALCN are associated with reproductive pathophysiological outcomes of patients, such as preterm birth and dystocia.

4.5 Concluding Remarks

Pregnancy is a complex physiological event that requires intricate regulation of quiescence until term and timely onset of contractility at term. The mechanisms and timing rely on many different regulators, which include hormonal regulation and ion channel activity. Thus, it is important that when addressing pathological events in pregnancy, we have a better understanding of the physiology that underlies a successful pregnancy.

In our studies, we were the first establish that NALCN expression and activity are upregulated by P4 and downregulated by E2. We also discovered a novel functional complex between NALCN and SLO2.1, a Na^+-activated K^+ channel, which could be important during the quiescent period of pregnancy to maintain a negative V_m and reduce intracellular Ca^{2+}. In the future, it will be important to characterize the hormonal regulation and role of ancillary subunits of NALCN in myometrial physiology. Additionally, future investigators should determine whether other Na^+ channels in the myometrium can also work through SLO2.1 to maintain the myometrial V_m. Additionally, determining the effect of genetic variants on NALCN functional complex activity could help predict reproductive outcomes.

Our findings of a novel regulation and role of NALCN in contributing to uterine quiescence can provide an opportunity to develop therapeutics that can modulate uterine

excitability and contractility. Most importantly, these findings can develop many new questions to address and further the field of myometrial physiology.

4.6 References

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