A Dominant Loss-of-Function GJA1 (Cx43) Mutant Impairs Parturition in the Mouse

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ABSTRACT

Expression of GJA1 (commonly known as connexin43 or Cx43), a major myometrial gap junction protein, is upregulated before the onset of delivery, suggesting an essential role for Cx43-mediated gap junctional intercellular communication (GJIC) in normal uterine contraction during parturition. To determine how a disease-linked Cx43 mutation affects myometrial function, we studied a mutant mouse model carrying an autosomal dominant mutation (Gja1+/m) in the gene encoding Cx43 that displays features of the human genetic disease oculodentodigital dysplasia. We found that Cx43 level, specifically the phosphorylated species of the protein, is significantly reduced in the myometrium of the mutant mice (Gja1+/m+), as revealed by Western blotting and immunostaining. Patch-clamp electrophysiological measurements demonstrated that coupling between myometrial smooth muscle cells is reduced to <15% of wild-type, indicating that the mutant protein acts dominantly on its wild-type counterpart. The phosphorylated species of Cx43 in the mutant myometrium failed to increase prior to parturition as well as in response to exogenous estrogen. Correspondingly, in vitro experiments with uterine strips revealed weaker contractions of the mutant myometrium and reduced responsiveness to oxytocin, providing an explanation for the prolonged gestation and presence of suffocated fetuses in the uteri that were observed in some of the mutant mice. We conclude that the Gja1+/m mutation has a dominant-negative effect on Cx43 function in the myometrium, severely reducing GJIC, leading to impaired parturition.

connexin43, gap junction, intercellular communication, myometrium, oculodentodigital dysplasia, ODDD, oxytocin, pregnancy, uterus

INTRODUCTION

Gap junctions are specialized plasma membrane domains containing arrays of channels that exchange ions and small molecules between neighboring cells, enabling cells to directly cooperate with each other both electrically and metabolically [1]. An intercellular gap junction channel consists of two hemichannels (connexons) docked end to end, each of which is composed of six connexin proteins. To date, 20 and 21 connexin genes have been identified in the mouse and human genomes, respectively, which have distinct but overlapping patterns of expression [1].

In humans and other mammals, the sudden appearance of numerous gap junctions within the myometrium is one of the many steps that occur during the conversion of the uterus from the quiescent state of pregnancy to the active state of labor [2]. Four different connexins, Cx26 (GJB2), Cx40 (GJA5), Cx43, and Cx45 (GJC1), have been identified in the myometrium, but they are differentially regulated during pregnancy. Expression of Cx26 is highest during late pregnancy but decreases before the onset of labor [3]. Cx45 is present in the nonpregnant uterus and during early pregnancy but declines thereafter [4]. Cx40 was found in human myometrial cells [5], but there is no evidence that its expression is regulated during pregnancy. In contrast, Cx43 gap junctions are scarce in the myometrium of the nonpregnant uterus but increase significantly just before the onset of labor and then disappear shortly after delivery [3, 6], indicating that Cx43 expression is temporally associated with parturition. Functional studies have shown that the ability to propagate action potentials along the myometrium is enhanced during parturition [7, 8]. Direct measurements by dual voltage clamp and dye microinjection also confirmed that gap junctional intercellular communication (GJIC) between myometrial myocytes is significantly elevated during parturition, consistent with the increased Cx43 protein level [9]. It is believed that this extensive connectivity within the myometrium is essential for the synchronized contractions required to expel the fetus and placenta.

Well-defined hormonal regulation of Cx43 expression has been observed in the myometrium. Cx43 expression is suppressed by progesterone and elevated by estrogen [10–12]. It is believed that the downregulation of Cx43 by progesterone maintains the myometrium in a quiescent state during pregnancy, avoiding muscle contraction that could lead to premature labor. Prior to the onset of labor, Cx43 mRNA and protein levels in the myometrial myocytes increase in association with an increase of plasma estrogen:progesterone ratio. In addition to hormonal signals, Cx43 expression is also upregulated by uterine stretch as a result of the increasing intrauterine volume caused by pregnancy [6]. The importance of Cx43-mediated GJIC for parturition was demonstrated by myometrial-speciﬁc ablation of the Gja1 gene in the mouse using Cre-LoxP system. The myometrial Cx43 level was reduced by ~70%, which resulted in a prolongation of pregnancy and intrauterine death of some pups [13].

Oculodentodigital dysplasia (ODDD) is a rare, human, autosomal-dominant disorder caused by mutations in the GJA1 gene encoding Cx43. Common symptoms include syndactyly of hands and feet, enamel hypoplasia, craniofacial abnormalities, ophthalmic defects, and occasionally heart and neurolog-
ical dysfunction [14, 15]. More than 39 different mutations in GJA1 have been identified so far [1]. In vitro studies of the ODDD-linked Cx43 mutants have shown that most will assemble into gap junction plaque-like structures at the cell surface; however, all mutants investigated to date exhibit severely reduced GJIC compared with wild-type Cx43. Furthermore, when coexpressed, the mutants typically act to inhibit the function of wild-type Cx43 [16–18].

In 2005, a line of mutant mice (Gja1Jrt+/+) with several of the classic symptoms of ODDD, including syndactyly, enamel hypoplasia, and craniofacial anomalies, was generated by N-ethyl-N-nitrosourea mutagenesis [19]. These mice carry a G60S amino acid substitution in the first extracellular loop of Cx43, one residue removed from the P59H mutation identified in some human ODDD patients [20]. Consistent with in vitro studies, Cx43G60S also severely impaired Cx43-mediated GJIC in various tissues of the mutant mice [19, 21]. In the present study, we sought to determine the effect of this disease-linked Cx43 mutation on myometrial function. Given the important role of Cx43 in synchronizing myometrial contraction, we hypothesized that the Cx43G60S mutant affects parturition by inhibiting gap junctional coupling between the myometrial smooth muscle cells (SMCs).

### MATERIALS AND METHODS

#### Mouse Breeding and Genotyping

All animal experiments were approved by the Animal Use Subcommittee of the University Council on Animal Care at the University of Western Ontario.

The Gja1Jrt/− mice were generated at the Centre for Modeling Human Disease, University of Toronto (Toronto, ON, Canada), and were kindly provided by Dr. Janet Rossant (Hospital for Sick Children, Toronto, ON, Canada). The original mice were on a mixed C57BL/6J and C3H/HeJ background [19] and were backcrossed to C57BL/6 for up to four generations. Genotypes were determined by PCR as described previously [19]. Mice were housed under controlled lighting (12L:12D) and temperature (21°C–24°C) conditions.

#### Animal Treatment and Tissue Preparation

Sexually mature 6- to 8-wk-old females were used. In nonpregnant females, the stage of the estrus cycle was determined by microscopic examination of vaginal smears [22], and those at the proestrus stage were selected for experiment. Females were injected intraperitoneally with 5 IU of equine chorionic gonadotropin (eCG; catalogue no. G4877; Sigma-Aldrich Canada, Oakville, ON, Canada) or subcutaneously with 5 μl of 17β-estradiol (E2758; Sigma-Aldrich) dissolved in sesame oil. They were killed by cervical dislocation following CO2 anesthesia 40 h after eCG treatment or 12 h after estradiol treatment. For pregnancy testing, one female and one wild-type male were housed together to mate. The presence of a vaginal plug was taken as evidence of mating, and the morning of plug detection was termed Day 0.5 of gestation. Pregnant females were killed at Day 18.5 of gestation.

For immunostaining, the uterine horns were removed from the body and fixed in Bouin solution for 12 h. For Western blotting, the uterine horns were placed on ice-cold glass slides and opened longitudinally. The tissue was homogenized in single-detergent lysis buffer (50 mM Tris-Cl, pH 8.0; 150 mM NaCl; 0.02% sodium azide; 100 μM/ml PMSF; 1 μM/ml aprotinin; and protease inhibitor cocktail (one tablet per 10 ml of buffer; Sigma-Aldrich). For recording contraction, a 1 × 0.5 cm longitudinal strip of uterus was cut from the middle section of the uterine horns and immediately mounted in an organ bath.

### FIG. 1. Reduction of Cx43 expression in the myometrium of Gja1Jrt/+ females.

A) Representative Western blots showing expression of Cx43 (~43 kDa for P0) and Cx26 (~26 kDa) in uteri from nonpregnant wild-type and Gja1Jrt/+ females relative to GAPDH (~35 kDa). B) Quantification of Western blots demonstrated that the abundance of total Cx43 (Cx43) and the phosphorylated 1 and 2 forms of Cx43 (Cx43-P1/2) are significantly reduced in the Gja1Jrt/+ uteri (P < 0.05). C) Quantification of Western blots demonstrated that the abundance of Cx26 in the myometrium from Gja1Jrt/+ mice is not significantly different (P > 0.05) from that of wild-type littermates. D) Representative micrographs showing uterine tissue from wild-type and Gja1Jrt/+ females double immunolabeled for Cx43 (green) and α-actin (red). CM, circular muscle layer; LM, longitudinal muscle layer. Bar = 20 μm.
Isometric Recording of Uterine Contraction

Uterine strips were placed longitudinally in a 12-ml organ bath containing Krebs-Ringer-Bicarbonate (KRB) solution with the following composition (in mM): 120 NaCl, 4.6 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 1.5 CaCl₂, 20 NaHCO₃, and 11 glucose. KRB solution, pH 7.4, was maintained at 37°C and gassed continuously with a mixture of 95% O₂/5% CO₂. Each uterine strip was placed under a resting force of 1 g and allowed to equilibrate for 1 h before exposure to drugs. The contractile responses of the strips were recorded isometrically with a tension transducer (MLT 0201/D) connected to a Powerlab bridge (AD Instruments Pty. Ltd.). Chart for Windows 5 software (AD Instruments) was used to display and analyze the tension changes in the tissue. Oxytocin (Sigma-Aldrich), ranging in final concentration from 0.03 to 30 mIU/ml, was applied at 10 min intervals between each application of oxytocin. At the end of each experiment, the superfusate was changed from KRB solution to distilled water to induce a large contraction of the tissue (hypotonic shock) [23, 24], which was used as a reference contraction (100%).

Isolation and Culture of Myometrial SMCs

Primary cultures of myometrial SMCs were prepared as described previously [25]. Briefly, uteri were placed in Hanks basic salt solution (Invitrogen Canada, Burlington, ON, Canada) supplemented with 2.25 mmol/l HEPES (buffer A) and cut into small pieces. Then, the tissues were washed three times with buffer B (buffer A without calcium and magnesium) and digested by 0.1% trypsin (37°C) with agitation (100 rpm) for 30 min by the addition of 1 mg/ml collagenase type II, 0.15 mg/ml deoxyribonuclease I, 0.1 mg/ml soybean trypsin inhibitor, 1 mg/ml bovine serum albumin (all from Sigma-Aldrich), and 10% fetal bovine serum (FBS; Invitrogen) in buffer B. After incubation, the mix was gently pipetted to aid enzymatic digestion. Equal amounts of buffer B supplemented with 10% FBS were added, and the mixture was passed through a cell strainer and stored on ice. Fresh enzyme mix was added to the remaining undigested tissue, and the incubation-aspiration process was repeated three to four times. The first incubation solution was discarded, and the remaining solutions were collected in a 50-ml Falcon tube (Becton Dickinson Labware, Franklin Lakes, NJ). The dissociated cells were collected by centrifugation (200 × g, 10 min), and the cell pellets were resuspended in sterile, phenol red-free Dulbecco modified Eagle medium (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). To selectively enrich for uterine SMCs, the cell mixture was preplated in a 60-mm culture dish containing (in mM): 70 KCl, 65 CsCl, 5 NaCl, 2 ethylene glycol tetraacetic acid, 2.5 MgCl₂, 5 tetrathylammonium chloride, and 10 HEPES, pH 7.3. The supernatant that contained the slowly adhering SMCs was collected and plated on 12-mm glass coverslips coated with type I collagen (354236; BD Biosciences, Mississauga, ON, Canada). All experiments were carried out on Day 2 or 3 of culture.

Immunofluorescent Staining

Fixed uterine tissues were embedded in paraffin and sectioned continuously at a thickness of 5 µm. Sections were then deparaffinized and washed three times with PBS before blocking with 2% bovine serum albumin (Sigma-Aldrich) in PBS for 1 h. Cx43 was detected using a rabbit polyclonal antibody (1:500; C6219; Sigma-Aldrich); in addition to Western blotting (see Results), the specificity of this verification was confirmed by observing the absence of immunostaining in mouse tissue lacking Cx43. The SMCs were labeled with a monoclonal mouse antibody against α-actin (1:800; A2547; Sigma-Aldrich); this antibody has been characterized previously, and its specificity has been demonstrated by Western blotting and immunostaining of tissues expressing other actin isoforms but lacking α-smooth muscle actin [26]. Appropriate Alexa594- or Alexa488-conjugated anti-mouse and anti-rabbit secondary antibodies were used at a 1:200 dilution (A11005, A11012, A11001, A11008; Invitrogen). Parallel sections were stained with secondary antibody alone (negative control). Nuclei were labeled with Hoechst 33342 (1:1000 dilution; Molecular Probes). Slides were imaged on a Zeiss (Thornwood, NY) LSM 510 META confocal microscope. For immunostaining on cultured SMCs, cells grown on glass coverslips were fixed with ice-cold methanol/20% acetone for 20 min before immunolabeling as described above.

Patch-Clamp Recording

Single-electrode, whole-cell, patch-clamp recording was used to measure SMC membrane capacitance and gap junctional conductance as described previously [27]. Briefly, pipettes were made from borosilicate glass capillaries using a two-stage pipette puller (PP-83; Narishige, Tokyo, Japan). The dissociated cells were collected in a 50-ml Falcon tube (Becton Dickinson Labware, Franklin Lakes, NJ). The dissociated cells were collected by centrifugation (200 × g, 10 min), and the cell pellets were resuspended in sterile, phenol red-free Dulbecco modified Eagle medium (Invitrogen Canada, Burlington, ON, Canada) supplemented with 2.25 mmol/l HEPES (buffer A), and 10 min), and the cell pellets were resuspended in sterile, phenol red-free Dulbecco modified Eagle medium (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). To selectively enrich for uterine SMCs, the cell mixture was preplated in a 60-mm culture dish containing (in mM): 70 KCl, 65 CsCl, 5 NaCl, 2 ethylene glycol tetraacetic acid, 2.5 MgCl₂, 5 tetrathylammonium chloride, and 10 HEPES, pH 7.3. The supernatant that contained the slowly adhering SMCs was collected and plated on 12-mm glass coverslips coated with type I collagen (354236; BD Biosciences, Mississauga, ON, Canada). All experiments were carried out on Day 2 or 3 of culture.

Western Blot Analysis

Protein concentrations were determined using a bicinechonic acid assay (Pierce Biotechnology Inc., Rockford, IL). A total of 50 µg of protein was separated by electrophoresis on 12% SDS-PAGE gels and transferred to a nitrocellulose membrane. The membrane was incubated overnight at 4°C with a rabbit anti-Cx43 antibody (1:1000; C6219; Sigma-Aldrich), a rabbit anti-Cx26 antibody (1:500; 71–0500; Invitrogen), a rabbit anti-oxytocin receptor antibody (1:500; Ab13076; Abcam, Cambridge, MA), or a mouse anti-GAPDH antibody (1:10000; MAB374; Chemicon, Temecula, CA). Following three washes with Tris-buffered saline containing (in mM) 30 NaCl, 5 KCl, 1.2 MgCl₂, 1.5 CaCl₂, 10 glucose, and 10 HEPES, pH 7.4. Voltage clamp for whole-cell recordings was carried out with an Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA). A single SMC within a confluent culture on a 12-mm glass coverslip was voltage-clamped. A depolarization voltage pulse (10 mV, 120-ms duration) was used to generate a transient capacitive current. The peak current and the steady-state current were measured. Currents were high-cut filtered at 10 kHz and digitized at 100 kHz. The estimated conductance between the patched cell and its surrounding cells was calculated. Data acquisition and analysis were performed using the Digidata 1200A interface and pClamp6 software (Axon Instruments).

FIG. 2. Impaired coupling between myometrial SMCs in Gja1+/+ females. A) Representative micrographs showing primary cultured SMCs isolated from wild-type and Gja1+/+ females double immunolabeled for Cx43 (green) and α-actin (red). Bar = 20 µm. B) The estimated conductance among SMCs isolated from Gja1+/+ females was significantly lower than that of wild-type SMCs (P < 0.0001).

of an inverted microscope (Olympus IMT-2). Cells were bathed in solution containing (in mM) 130 NaCl, 5 KCl, 1.2 MgCl₂, 1.5 CaCl₂, 10 glucose, and 10 HEPES, pH 7.4. Voltage clamp for whole-cell recordings was carried out with an Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA). A single SMC within a confluent culture on a 12-mm glass coverslip was voltage-clamped. A depolarization voltage pulse (10 mV, 120-ms duration) was used to generate a transient capacitive current. The peak current and the steady-state current were measured. Currents were high-cut filtered at 10 kHz and digitized at 100 kHz. The estimated conductance between the patched cell and its surrounding cells was calculated. Data acquisition and analysis were performed using the Digidata 1200A interface and pClamp6 software (Axon Instruments).
immunoblots were processed and quantified using the Odyssey infrared-imaging system (Licor). The relative intensities of Cx43 and Cx26 bands were determined by normalizing to GAPDH.

Data Analysis and Statistics

The data are expressed as mean ± SEM, with "n" denoting the number of independent experiments. Comparison of two groups was carried out using a two-tailed unpaired t-test, with a P value below 0.05 indicating significance. Two-way ANOVA and Bonferroni test were used to compare two groups under different experimental conditions. Statistical significance is indicated in the graphs with a single symbol (*) for P < 0.05, double (**) for P < 0.01, and triple (***) for P < 0.001.

RESULTS

Reduced Cx43 Expression in the Myometrium of Gja1<sup>+/+</sup> Females

Similar to what was reported in other tissues [19, 21], Western blot analysis demonstrated that the abundance of Cx43 protein was significantly reduced in the Gja1<sup>+/+</sup> uteri (0.07 ± 0.01, n = 3 for Gja1<sup>+/+</sup> vs. 0.17 ± 0.04, n = 3 for wild-type; P < 0.05 by two-way ANOVA; Fig. 1, A and B). In particular, the phosphorylated P1 and P2 forms of Cx43 were significantly reduced (0.029 ± 0.004, n = 3 for Gja1<sup>+/+</sup> vs. 0.070 ± 0.025, n = 3 for wild-type; P < 0.05 by two-way ANOVA). In contrast, Cx26 expression level is similar between the two genotypes (0.33 ± 0.03 for Gja1<sup>+/+</sup> vs. 0.29 ± 0.02 for wild-type, n = 3 for each group; P > 0.05 by unpaired t-test; Fig. 1, A and C), indicating that the Gja1<sup>+</sup> mutation does not extend its effect to another member of the connexin family coexpressed in the uterus. Similarly, immunofluorescent labeling demonstrated that there are fewer Cx43 gap junction plaques present in the myometrium of the Gja1<sup>+/+</sup> females, although Cx43 is expressed at relatively low levels in both groups (Fig. 1D).

Impaired Coupling among Myometrial SMCs in Gja1<sup>+/+</sup> Females

To test the effect of reduced Cx43 expression on GJIC, coupling levels among primary cultured myometrial SMCs were measured by single patch clamp as described previously.
[27]. Immunolabeling with SMC-specific α-actin antibody indicated that the purity of the SMCs was more than 90% in our preparation. Consistent with immunostaining of uterine tissue, the number of Cx43 gap junction plaques among the Gja1Jrt/þ SMCs was reduced (Fig. 2A). The estimated conductance among SMCs isolated from Gja1Jrt/þ females was 2.2 ± 0.4 nS (n = 5), significantly lower than that of wild-type SMCs (13.9 ± 2.0 nS, n = 6; P < 0.001 by unpaired t-test; Fig. 2B), indicating that the Gja1Jrt mutant dominantly inhibits the function of coexpressed wild-type Cx43.

Elevated Estrogen Cannot Rescue Impaired Cx43 Expression in Gja1Jrt/+ Females

To assess whether the Gja1Jrt mutation affects the normal response of Cx43 to hormonal regulation, both wild-type and Gja1Jrt/+ females were treated with eCG, which elevates serum estradiol. As shown in Figure 3, A and B, uterine Cx43 expression increased in both wild-type and Gja1Jrt/+ females in response to eCG (2.2 ± 0.2-fold increase in wild-type vs. 2.3 ± 0.4-fold in Gja1Jrt/+, n = 5 in each group). However, eCG treatment did not rescue the impaired phosphorylation observed in the Gja1Jrt/+ mice, indicated by a 62% ± 5% loss of phosphorylated Cx43 species in the eCG-treated Gja1Jrt/+ mice compared with their wild-type littermates (Fig. 3C).

Myometrial Cx26 expression was also significantly upregulated after eCG treatment in both wild-type and Gja1Jrt/+ mice (n = 4 for each group; P < 0.01, two-way ANOVA), but there was no significant difference between the two genotypes (no treatment, 0.31 ± 0.08 for Gja1Jrt/+ vs. 0.32 ± 0.06 for wild-type; eCG treatment, 0.47 ± 0.03 for Gja1Jrt/+ vs. 0.49 ± 0.06 for wild-type; n = 4 for each group; P > 0.05, two-way ANOVA; Fig. 3D). This result confirms that the Gja1Jrt mutation specifically impaired Cx43 expression. To avoid different responses to eCG between groups, some mice (both wild-type and Gja1Jrt/+) were treated with estradiol directly. Similar changes were observed in those mice (data not shown).

The consequence of reduced Cx43 phosphorylation on gap junction formation was evaluated by immunostaining. In contrast to an obvious increase of Cx43 gap junction plaques observed in the wild-type myometrium after eCG treatment (data not shown), there were still fewer plaques present in the eCG-treated Gja1Jrt/+ myometrium (Fig. 3E).

Reduced Uterine Contraction in Gja1Jrt/+ Females

To assess the effect of impaired GJIC on uterine contraction, spontaneous and oxytocin-induced contractile responses were quantitatively evaluated. As shown in Figure 4A, uterine strips from both groups displayed spontaneous contractions and

FIG. 4. Reduced uterine contraction in Gja1Jrt/+ females. A) Sample traces showing activity of uterine strips taken from non-pregnant wild-type and Gja1Jrt/+ females in response to oxytocin. The dotted line indicates baseline tension at 0 g. B and C) The oxytocin dose-response curve is expressed as a percentage of a reference contraction induced by hypotonic shock in control animals (B) and eCG-treated animals (C). In both cases, uterine strips isolated from Gja1Jrt/+ females showed a significantly weaker tension response compared with wild-type strips (P < 0.001 for both graphs). D) Representative Western blots showing expression of oxytocin receptor (OTR; ~ 55 kDa) in uterine myometrium from wild-type and Gja1Jrt/+ females. E) The abundance of OTR in myometrium from Gja1Jrt/+ mice is not significantly different (P > 0.05) from that of wild-type littersmates in both the control group and the eCG-treated group.
dose-related excitatory responses induced by oxytocin. Full dose-response curves demonstrated that oxytocin evoked a significantly greater tension response in the wild-type uterine strips compared with the strips isolated from the Gja1\textsuperscript{Jrt/+} females (n = 3 for each group; P < 0.001 by two-way ANOVA; Fig. 4B). A similar difference was present in uterine strips isolated from animals treated with eCG (n = 3 for each group; P < 0.001 by two-way ANOVA; Fig. 4C). Western blotting failed to detect any difference in expression of oxytocin receptors between the two genotypes (P > 0.05, two-way ANOVA; Fig. 4, D and E).

**Impaired Uterine Cx43 Expression in Late Gestational Gja1\textsuperscript{Jrt/+} Females**

To determine whether Cx43 expression is impaired in vivo during gestation, we examined the Cx43 expression profile in late-gestational uteri (n = 5 for each group). Consistent with previous studies [3, 6], we found a significant increase in Cx43 expression in wild-type uteri at 18.5 days postcoitum (0.49 ± 0.07 for pregnant animals vs. 0.19 ± 0.01 for nonpregnant controls; P < 0.001 by two-way ANOVA), especially in the highly phosphorylated P1 and P2 species (3.1 ± 0.3-fold increase vs. nonpregnant controls; P < 0.001 by two-way ANOVA; Fig. 5, A and B). In the Gja1\textsuperscript{Jrt/+} uteri, although Cx43 expression also increased significantly (2.0 ± 0.1-fold, P < 0.001 vs. nonpregnant controls by two-way ANOVA), the elevation was not as great as that observed in the wild-type (2.5 ± 0.2 -fold). Consequently, on Gestational Day 18.5, there was a 63% ± 3% reduction of total Cx43 protein level and a 74% ± 2% reduction of Cx43 P1/2 species in the Gja1\textsuperscript{Jrt/+} uteri compared with wild-type littermates (Fig. 5, B and C). Consistent with the protein expression profile, immunolabeling demonstrated extensive Cx43 expression in the wild-type myometrium on Gestational Day 18.5, whereas little Cx43 signal was observed in the Gja1\textsuperscript{Jrt/+} myometrium (Fig. 5E). In contrast, Cx26 expression was significantly downregulated at 18.5 days postcoitum in both wild-type and Gja1\textsuperscript{Jrt/+} uteri (wild-type, 0.20 ± 0.03 for pregnant animals vs. 0.33 ± 0.02 for nonpregnant controls; Gja1\textsuperscript{Jrt/+} 0.17 ± 0.03 for pregnant animals vs. 0.41 ± 0.07 for nonpregnant controls; P < 0.001 by two-way ANOVA, n = 9 for each group; Fig. 5, A and D). There was no significant difference between wild-type and Gja1\textsuperscript{Jrt/+} groups under both conditions, indicating that the Gja1\textsuperscript{Jrt} mutation does not affect Cx26 expression in the uterus.

**Prolonged Gestation and Impaired Parturition of Gja1\textsuperscript{Jrt/+} Females**

After mating with wild-type males, the pregnancy rate of Gja1\textsuperscript{Jrt/+} females was significantly lower than their wild-type littermates. This has been characterized elsewhere [28]. The Gja1\textsuperscript{Jrt/+} females that became pregnant had a gestation period of 19.7 ± 0.3 days (n = 18), which was significantly longer (P < 0.05 by unpaired t-test) than their wild-type littermates (18.8 ± 0.2 days, n = 24; Fig. 6A). Furthermore, when six of the Gja1\textsuperscript{Jrt/+} mothers were killed at 1400 h on the day of birth, dead pups (1.4 ± 0.9 per female) were found retained in the uteri of three of them (Fig. 6B). In addition, significantly more placentas were found retained in the uteri of the Gja1\textsuperscript{Jrt/+} females (4.8 ± 0.7 for Gja1\textsuperscript{Jrt/+} females vs. 0.7 ± 0.3 for wild-type; n = 6 for each group; P < 0.01 by unpaired t-test), indicating that parturition is impaired in the mutant females.

**DISCUSSION**

Significant upregulation of Cx43 and enhanced connectivity within the myometrium just before the onset of labor indicate that Cx43-mediated intercellular communication may be critical for synchronized smooth muscle contraction required
for normal parturition. This notion was confirmed by the fact that parturition was delayed in mice specifically lacking Cx43 in their myometrium [13]. Therefore, we hypothesized that parturition would also be compromised under pathological conditions when Cx43 function is impaired in mice that carry a germline mutation in the gene encoding Cx43. Gja1<sup>−/−</sup> mutant mice, which have a phenotype resembling human ODDD, provided us with a model to study how a disease-linked Cx43 mutant can affect myometrial function.

Several in vitro studies have demonstrated that ODDD-linked Cx43 mutations exert dominant-negative effects on wild-type Cx43, severely limiting Cx43-mediated intercellular communication [1]. Consistent with these findings, we demonstrated that Cx43 expression level is also significantly lower in the Gja1<sup>−/−</sup> myometrium, especially the phosphorylated P1 and P2 species, indicating that the mutant expression interferes with Cx43 reaching its normal phosphorylation status. In general, phosphorylation of Cx43 correlates with its assembly into functional gap junctions at the cell surface [1]. Thus, our finding of reduced Cx43 phosphorylation in the mutant myometrium is consistent with the immunostaining results showing that very few Cx43-containing gap junction plaques are present in the junctional membranes between myometrial smooth muscle cells. Reduced Cx43 expression and aberrant phosphorylation severely impaired gap junction assembly between Gja1<sup>−/−</sup> SMCs, demonstrated by an approximately 85% reduction of intercellular coupling compared with wild-type littersmates. Thus, the Cx43<sup>Gal7S</sup> mutant is functionally dominant to coexpressed wild-type Cx43. Similar effects have been observed in other cell types from the same Gja1<sup>−/−</sup> mice, including osteoblasts [21], mammary epithelium [29], cardiac myocytes [30], and ovarian granulosa cells [28], as well as cardiac myocytes isolated from mice carrying the human ODDD-linked mutations G138R and I130T [31, 32], indicating that ODDD-linked Cx43 mutants interfere with normal Cx43 function in a variety of cell types. This negative effect seems to be specific to Cx43, because in most tissues of the ODDD mice, such as mammary gland and cardiac tissue, expression of other coexpressed connexins did not change [27, 30]. Our results concur with these findings, because Cx26 expression is constant in the uteri of the Gja1<sup>−/−</sup> mice. Collectively, these findings indicate that Cx43 may not directly and promiscuously interact with other connexin family members in these tissues. However, Cx26 was downregulated in the epidermis of adult Gja1<sup>−/−</sup> mice [33], suggesting possible cross-talk between Cx43 and Cx26 in the epidermis.

Given the fact that myometrial Cx43 expression is under tight hormonal control, it was of importance to determine whether the Gja1<sup>−/−</sup> mutation affects this regulatory mechanism. Our data demonstrated that, similar to what was observed in their wild-type littermates, total Cx43 expression in Gja1<sup>−/−</sup> females increased in response to estrogen. However, total Cx43 level was still significantly reduced in these mice because of their lower baseline level. More importantly, the nonphosphorylated form of Cx43 was the major species that responded to increased estrogen in the Gja1<sup>−/−</sup> uteri, resulting in a more pronounced reduction in the phosphorylated species of Cx43 in eCG-treated Gja1<sup>−/−</sup> females. Similar changes were observed in late-gestational uteri, when estrogen increases in vivo in association with parturition, indicating that Cx43 expression is still under hormonal regulation in the Gja1<sup>−/−</sup> uteri. Despite this, the highly phosphorylated species of Cx43 were not rescued by the elevated estrogen level in mutant mice. These findings are consistent with the hypothesis that impaired gap junctional coupling among myometrial SMCs of the mutant females is primarily caused by aberrant posttranslational modification and/or trafficking of Cx43.

Consistent with previous work with conditional knockout mice specifically lacking Cx43 in the myometrium [13], parturition defects were observed in the Gja1<sup>−/−</sup> mice. These included prolonged gestation as well as retained intrauterine fetuses and placentas. Correspondingly, our in vitro experiments showed that uterine strips isolated from Gja1<sup>−/−</sup> mice have significantly weaker contractions and reduced response to oxytocin compared with strips from wild-type littersmates. This provides further confirmation that a certain level of Cx43-mediated GJIC within the myometrium is critical for effective contraction.

Although parturition is impaired in Gja1<sup>−/−</sup> females, mutant mice could still deliver their pups. Given the presence of multiple connexins in the myometrial SMCs [2], it is possible that other connexin isoforms may compensate for the reduced function of Cx43. However, our data demonstrated that ionic coupling among Gja1<sup>−/−</sup> myometrial SMCs was severely diminished, arguing that the compensation from other coexpressed connexins, if any, was limited. On the other hand, it was suggested that in addition to GJIC, coordination of SMC contractions is also provided by paracrine release of prosta-
glandin F₂α and local release of calcium [34]. Another factor that must be considered is that myometrial cells, like the SMCs of the intestine and bladder, may have their contractions coordinated by the activity of interstitial cells arrays between and coupled via gap junctions with SMC bundles [35]. Although Cx43 contributes to these putative interstitial cell junctions [35], the presence of other connexins has not been explored. These various alternative pathways for coordination of myometrial contraction may explain why parturition was impaired but not abolished in Gja1+/− females, as was also found in myometrium-specific Cx43 knockout mice [13].

Finally, the impaired parturition observed in Gja1+/− females raises a concern that human ODDD females may have potential delivery problems. Given the highly variable length of human delivery, problems like prolonged delivery or increased caesarean delivery rates may not have been recognized. Therefore, further clinical assessments of ODDD patients at the time of delivery are warranted.

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