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

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Short title: Effects of a GJA1 mutant on parturition

Summary sentence: The G60S mutant of the gap junction protein GJA1 (Cx43) has a dominant-negative effect on wild-type Cx43 function in the mouse myometrium, severely reducing gap junctional coupling leading to impaired parturition.

Key words: connexin43, gap junction, myometrium, intercellular communication, oculodentodigital dysplasia, ODDD

Topic category: Female reproductive tract

**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 of Cx43 mediated gap junctional intercellular communication (GJIC) for 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 (*Gjal<sup>Jrt</sup>*) in the gene encoding Cx43 that displays features of the human genetic disease, oculodentodigital dysplasia (ODDD). We found that Cx43 level, specifically the phosphorylated species of the protein, is significantly reduced in the myometrium of the mutant mice (*Gjal<sup>Jrt</sup>/+*) as revealed by western blotting and immunostaining. Patch-clamp electrophysiological measurements demonstrated that coupling between myometrial smooth muscle cells (SMCs) is reduced to <15% of wildtype, indicating that the mutant protein acts dominantly on its wildtype 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 contraction 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 was observed in some of the mutant mice. We conclude that the *Gjal<sup>Jrt</sup>* mutation has a dominant-negative effect on Cx43 function in the myometrium, severely reducing GJIC leading to impaired parturition.

## 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 *Gjal* 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 neurological 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 as compared to wildtype Cx43.

Furthermore, when co-expressed, the mutants typically act to inhibit the function of wildtype Cx43 [16-18].

In 2005, a line of mutant mice (*Gja1<sup>Jrt/+</sup>*) 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, Cx43<sup>G60S</sup> 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 myometrium function. Given the important role of Cx43 in synchronizing myometrial contraction, we hypothesized that the Cx43<sup>G60S</sup> mutant affects parturition by inhibiting gap junctional coupling between the myometrial smooth muscle cells.

## 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 *Gja1<sup>Jrt/+</sup>* mice were generated at the Centre for Modeling Human Disease, University of Toronto, and were kindly provided by Dr. Janet Rossant. The original mice were on a mixed C57BL/6J and C3H/HeJ background [19] and were backcrossed to C57BL/6J for up to 4 generations. Genotypes were determined by polymerase chain reaction (PCR) as previously described [19]. Mice were housed under controlled lighting (12 h light, 12 h dark) and temperature (21–24 °C) conditions.

### *Animal Treatment and Tissue Preparation*

Sexually mature 6- to 8- week-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 proestrus stage were selected for experiment. Females were injected intraperitoneally with 5 IU equine chorionic gonadotropin (eCG) (Sigma-Aldrich Canada, Oakville, ON cat. no. G4877) or subcutaneously with 5 µg 17β-estradiol (Sigma-Aldrich E2758) dissolved in sesame oil. They were killed by cervical dislocation following CO<sub>2</sub> anesthesia 40 hours after eCG treatment or 12 hours after estradiol treatment. For pregnancy testing, one female and one wildtype 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's solution for 12 hours. For western blotting, the uterine horns were placed on ice cold glass slides and opened longitudinally. The endometrium was carefully scraped off using a spatula. Alternatively, whole uteri were prepared for western blotting. The tissue was homogenized in single-detergent lysis buffer (50 mM Tris-Cl pH = 8.0, 150 mM NaCl, 0.02% sodium azide, 100 µg/ml PMSF, 1 µg/ml aprotinin, 1% Triton X-100) supplemented with 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub> and protease inhibitor cocktail (1 tablet per 10 ml buffer; Sigma-Aldrich). For recording contraction, a 1 x 0.5 cm longitudinal strip of uterus was cut from the middle section of the uterine horns and immediately mounted in an organ bath.

### ***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 (mM): NaCl, 120; KCl, 4.6;  $\text{KH}_2\text{PO}_4$ , 1.2;  $\text{MgSO}_4$ , 1.2;  $\text{CaCl}_2$ , 1.5;  $\text{NaHCO}_3$ , 20, and glucose 11. KRB solution, pH 7.4, was maintained at 37 °C and gassed continuously with a mixture of 95%  $\text{O}_2$ –5%  $\text{CO}_2$ . 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 Pty Ltd) 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 by pipette to the bottom of the chamber in increasing order. There was a 10 min interval 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 Smooth Muscle Cells***

Primary cultures of myometrial smooth muscle cells (SMCs) were prepared as described previously [25]. Briefly, uteri were placed in Hanks basic salt solution (HBSS) (Invitrogen Canada, Burlington, ON) 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 at 37°C with agitation (100 rpm) for 30 minutes 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 was 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 for 3–4 times. The first incubation solution was discarded and the remaining solutions were collected in a 50 ml Falcon tube. The dissociated cells were collected by centrifugation (200 x g, 10 min), and the cell pellets were resuspended in sterile, phenol red-free Dulbecco's modified Eagle's 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 pre-plated in a 60 mm culture dish for 45 minutes at 37°C. The supernatant that contained the slowly adhering SMCs was collected and plated on 12 mm glass coverslips coated with type I collagen (BD Biosciences 354236, Mississauga, ON). 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 3 times with phosphate-buffered saline (PBS) before blocking with 2% bovine serum albumin (BSA, Sigma-Aldrich) in PBS for 1 hour. Cx43 was detected using a rabbit polyclonal antibody (1:500) (Sigma-Aldrich C6219); in addition to western blotting (see Results), the specificity of this antibody was verified by showing the absence of immunostaining in mouse tissue lacking Cx43. The SMCs were labeled with a monoclonal mouse antibody against  $\alpha$ -actin (1:800, Sigma-Aldrich A2547); this antibody has been characterized previously and its specificity demonstrated by western blotting and immunostaining of tissues expressing other actin isoforms but lacking  $\alpha$ -smooth muscle actin

[26]. Appropriate Alexa594 or Alexa488 conjugated anti-mouse and anti-rabbit secondary antibodies were used at a 1:200 dilution (Invitrogen A11005, A11012, A11001, A11008). 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 80% methanol/20% acetone for 20 minutes 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 [27]. Briefly, pipettes were made from borosilicate glass capillaries using a two-stage pipette puller (PP-83; Narishige, Tokyo, Japan). The intracellular pipette solution contained (in mM) 70 KCl, 65 CsCl, 5 NaCl, 2 EGTA, 2.5 MgCl<sub>2</sub>, 5 TEA-Cl, and 10 HEPES, pH 7.3, and pipettes had a resistance of 3–5 M $\Omega$ . Cells on coverslips were transferred to a 2 ml recording chamber mounted on the stage of an inverted microscope (Olympus IMT-2). Cells were bathed in solution containing (in mM) 130 NaCl, 5 KCl, 1.2 MgCl<sub>2</sub>, 1.5 CaCl<sub>2</sub>, 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).

### ***Western Blot Analysis***

Protein concentrations were determined using bicinchonic acid (BCA) assay (Pierce Biotechnology Inc., Rockford IL). Fifty  $\mu$ g 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 (1:1,000, Sigma-Aldrich C6219), or a rabbit anti-Cx26 antibody (1:500, Invitrogen 71-0500), or a rabbit anti-oxytocin receptor antibody (1:500, Abcam Ab13076, Cambridge, MA), or a mouse anti-GAPDH antibody (1:10,000, Chemicon MAB374, Temecula, CA). Following three washes with Tris-buffered saline with Tween 20 (5 min each at room temperature), infrared fluorescent-labeled secondary antibodies (IRDye 800 anti-rabbit, Rockland Immunochemicals 611132122, Gilbertsville, PA; Alexa680 anti-mouse, Invitrogen A21057) were incubated at room temperature for 1 h and 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  $\pm$  s.e.m., 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. Comparison of more than two groups was carried out with one-way ANOVA followed by a Tukey test. 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 *Gjal<sup>Jrt/+</sup>* Females

Similar to what was reported in other tissues [19, 21], western blot analysis demonstrated that the abundance of Cx43 protein is significantly reduced in the *Gjal<sup>Jrt/+</sup>* uteri ( $0.089 \pm 0.004$ ,  $n = 7$  for *Gjal<sup>Jrt/+</sup>* vs.  $0.16 \pm 0.01$ ,  $n = 6$  for wildtype;  $P < 0.01$  by two-way ANOVA) (Fig. 1A, B). In particular, the phosphorylated P1 and P2 forms of Cx43 were significantly reduced ( $0.070 \pm 0.003$ ,  $n = 7$  for *Gjal<sup>Jrt/+</sup>* vs.  $0.13 \pm 0.01$ ,  $n = 6$  for wildtype;  $P < 0.01$  by two-way ANOVA). In contrast, Cx26 expression level is similar between the two genotypes ( $0.38 \pm 0.05$  for *Gjal<sup>Jrt/+</sup>* vs.  $0.33 \pm 0.05$  for wildtype,  $n = 8$  for each group;  $P > 0.05$  by unpaired t-test) (Fig. 1A, C), indicating that the *Gjal<sup>Jrt</sup>* mutation does not extend its effect to another member of the connexin family co-expressed in the uterus. Similarly, immunofluorescent labeling demonstrated that there are fewer Cx43 gap junction plaques present in the myometrium of the *Gjal<sup>Jrt/+</sup>* females, although Cx43 is expressed at relatively low levels in both groups (Fig. 1D).

### Impaired Coupling Among Myometrial SMCs in *Gjal<sup>Jrt/+</sup>* Females

To test the effect of reduced Cx43 expression on gap junctional intercellular communication (GJIC), coupling levels among primary cultured myometrial SMCs were measured by single patch clamp as described previously [27]. Immunolabeling with SMC-specific  $\alpha$ -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 *Gjal<sup>Jrt/+</sup>* SMCs was reduced (Fig. 2A). The estimated conductance among SMCs isolated from *Gjal<sup>Jrt/+</sup>* females was  $2.2 \pm 0.4$  nS ( $n = 5$ ), significantly lower than that of wildtype SMCs ( $13.9 \pm 2.0$  nS,  $n = 6$ ;  $P < 0.001$  by unpaired t-test) (Fig. 2B), indicating that the *Gjal<sup>Jrt</sup>* mutant dominantly inhibits the function of co-expressed wildtype Cx43.

### Elevated Estrogen Can Not Rescue Impaired Cx43 Expression in *Gjal<sup>Jrt/+</sup>* Females

To assess whether the *Gjal<sup>Jrt</sup>* mutation affects the normal response of Cx43 to hormonal regulation, both wildtype and *Gjal<sup>Jrt/+</sup>* females were treated with eCG, which elevates serum estradiol. As shown in Figures 3A and 3B, uterine Cx43 expression increased in both wildtype and *Gjal<sup>Jrt/+</sup>* females in response to eCG ( $2.8 \pm 0.3$  fold increase in wildtype vs.  $3.0 \pm 0.1$  in *Gjal<sup>Jrt/+</sup>*,  $n = 5$  in each group). However, eCG treatment did not rescue the impaired phosphorylation observed in the *Gjal<sup>Jrt/+</sup>* mice, indicated by a  $59.7 \pm 7.3$  % loss of phosphorylated Cx43 species in the eCG-treated *Gjal<sup>Jrt/+</sup>* mice compared with their wildtype littermates (Fig. 3C). Myometrial Cx26 expression was also significantly upregulated after eCG treatment in both wildtype and *Gjal<sup>Jrt/+</sup>* mice ( $n = 4$  for each group;  $P < 0.01$ , two-way ANOVA) but there was no significant difference between the two genotypes (no treatment,  $0.37 \pm 0.03$  for *Gjal<sup>Jrt/+</sup>* vs.  $0.32 \pm 0.05$  for wildtype; eCG treatment,  $0.51 \pm 0.05$  for *Gjal<sup>Jrt/+</sup>* vs.  $0.53 \pm 0.01$  for wildtype;  $n = 4$  for each group;  $P > 0.05$ , two-way ANOVA) (Fig. 3D). This result confirms that the *Gjal<sup>Jrt</sup>* mutation specifically impaired Cx43 expression. To avoid different responses to eCG between groups, some mice (both wildtype and *Gjal<sup>Jrt/+</sup>*) 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



wildtype myometrium after eCG treatment (not shown), there were still fewer plaques present in the eCG-treated *Gjal<sup>Jrt/+</sup>* myometrium (Fig. 3E).

### Reduced Uterine Contraction in *Gjal<sup>Jrt/+</sup>* 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 dose-related excitatory responses induced by oxytocin. Full dose-response curves demonstrated that oxytocin evoked a significantly greater tension response in the wildtype uterine strips compared with the strips isolated from the *Gjal<sup>Jrt/+</sup>* females ( $n = 3$  for each group;  $P < 0.01$  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 (Fig. 4D & E) ( $P > 0.05$ , two-way ANOVA).

### Impaired Uterine Cx43 Expression in Late Gestational *Gjal<sup>Jrt/+</sup>* 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 wildtype uteri at 18.5 days pc ( $0.40 \pm 0.02$  for pregnant animals vs  $0.14 \pm 0.01$  for nonpregnant controls;  $P < 0.001$  by two-way ANOVA), especially the highly phosphorylated P1 and P2 species ( $2.5 \pm 0.2$  fold increase vs. nonpregnant controls;  $P < 0.001$  by two-way ANOVA) (Fig. 5A, B). In the *Gjal<sup>Jrt/+</sup>* uteri, although Cx43 expression also increased significantly ( $1.8 \pm 0.2$  fold,  $P < 0.05$  vs nonpregnant controls by two-way ANOVA), the elevation was not as great as that observed in the wildtype ( $2.9 \pm 0.3$  fold). Consequently, on gestational day 18.5, there was a  $58.0 \pm 7.4$  % reduction of total Cx43 protein level and a  $63.2 \pm 2.7$  % reduction of Cx43 P1/2 species in the *Gjal<sup>Jrt/+</sup>* uteri compared with wildtype littermates (Fig. 5B, C). Consistent with the protein expression profile, immunolabeling demonstrated extensive Cx43 expression in the wildtype myometrium on gestational day 18.5, whereas little Cx43 signal was observed in the *Gjal<sup>Jrt/+</sup>* myometrium (Fig. 5E). In contrast, Cx26 expression was significantly downregulated at 18.5 days pc in both wildtype and *Gjal<sup>Jrt/+</sup>* uteri (wildtype  $0.14 \pm 0.02$  for pregnant animals vs  $0.28 \pm 0.04$  for nonpregnant controls; *Gjal<sup>Jrt/+</sup>*  $0.12 \pm 0.02$  for pregnant animals vs  $0.30 \pm 0.03$  for nonpregnant controls;  $P < 0.001$  by two-way ANOVA,  $n = 9$  for each group) (Fig. 5A& D). There was no significant difference between wildtype and *Gjal<sup>Jrt/+</sup>* groups under both conditions, indicating that the *Gjal<sup>Jrt</sup>* mutation does not affect Cx26 expression in the uteri.

### Prolonged Gestation and Impaired Parturition of *Gjal<sup>Jrt/+</sup>* Females

After mating with wildtype males, the pregnancy rate of *Gjal<sup>Jrt/+</sup>* females was significantly lower than their wildtype littermates. This has been characterized elsewhere [28]. The *Gjal<sup>Jrt/+</sup>* females that become pregnant had a gestation period of  $19.7 \pm 0.3$  days ( $n = 18$ ), which is significantly longer ( $P < 0.05$  by unpaired t-test) than their wildtype littermates ( $18.8 \pm 0.2$  days,  $n = 24$ ) (Fig. 6A). Furthermore, when six of the *Gjal<sup>Jrt/+</sup>* mothers were sacrificed at 2 pm on the day of birth, dead pups ( $1.4 \pm 0.9$  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 *Gjal<sup>Jrt/+</sup>* females ( $4.8 \pm 0.7$  for *Gjal<sup>Jrt/+</sup>* females vs  $0.7 \pm 0.3$  for wildtype;  $n = 6$  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 germ line mutation in the gene encoding Cx43. *Gjal<sup>Jrt</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 wildtype Cx43, severely limiting Cx43 mediated intercellular communication [1]. Consistent with these findings, we demonstrated that Cx43 expression level is also significantly less in the *Gjal<sup>Jrt</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 *Gjal<sup>Jrt</sup>/+* SMCs, demonstrated by an ~85% reduction of intercellular coupling as compared with wildtype littermates. Thus the Cx43<sup>G60S</sup> mutant is functionally dominant to co-expressed wildtype Cx43. Similar effects have been observed in other cell types from the same *Gjal<sup>Jrt</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, since in most tissues of the ODDD mice, such as mammary gland and cardiac tissue, expression of other co-expressed connexins did not change [27, 30]. Our results concur with these findings since Cx26 expression is constant in the uteri of the *Gjal<sup>Jrt</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 *Gjal<sup>Jrt</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 *Gjal<sup>Jrt</sup>* mutation affects this regulatory mechanism. Our data demonstrated that, similar to what was observed in their wildtype littermates, total Cx43 expression in *Gjal<sup>Jrt</sup>/+* females increased in response to estrogen. However, total Cx43 level was still significantly reduced in these mice due to their lower baseline level. More importantly, the nonphosphorylated form of Cx43 was the major species that responded to increased estrogen in the *Gjal<sup>Jrt</sup>/+* uteri, resulting in a more pronounced reduction in the phosphorylated species of Cx43 in eCG-treated *Gjal<sup>Jrt</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 *Gjal<sup>Jrt</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 post-translational 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 *Gjal<sup>Jrt</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 *Gjal<sup>Jrt</sup>/+* mice have significantly weaker contractions and reduced response to oxytocin as compared with strips from wildtype littermates. 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 *Gjal<sup>Jrt</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 *Gjal<sup>Jrt</sup>/+* myometrial SMCs was severely diminished, arguing that the compensation from other co-expressed 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 prostaglandin F<sub>2α</sub> 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 arrayed 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 *Gjal<sup>Jrt</sup>/+* females, as was also found in myometrium-specific Cx43 knockout mice [13].

Finally, the impaired parturition observed in *Gjal<sup>Jrt</sup>/+* 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 section rates may not have been recognized. Therefore, further clinical assessments of ODDD patients at the time of delivery are warranted.

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## FIGURE LEGENDS

### FIG. 1. Reduction of Cx43 expression in the myometrium of *Gjal<sup>Jrt/+</sup>* females (A)

Representative western blots showing expression of Cx43 and Cx26 in uteri from nonpregnant wildtype and *Gjal<sup>Jrt/+</sup>* females relative to GAPDH. (B) Quantification of western blots demonstrated that the abundance of total Cx43 (Cx43) and the phosphorylated P1 and P2 forms of Cx43 (Cx43-P1/2) are significantly reduced in the *Gjal<sup>Jrt/+</sup>* uteri ( $P < 0.01$ ). (C) Quantification of western blots demonstrated that the abundance of Cx26 in the myometrium from *Gjal<sup>Jrt/+</sup>* mice is not significantly different ( $P > 0.05$ ) from that of wildtype littermates. (D) Representative micrographs showing uterine tissue from wildtype and *Gjal<sup>Jrt/+</sup>* females double immunolabeled for Cx43 (green) and  $\alpha$ -actin (red). CM, circular muscle layer; LM, longitudinal muscle layer; scale bar = 20  $\mu$ m.

### FIG. 2. Impaired coupling between myometrial SMCs in *Gjal<sup>Jrt/+</sup>* females (A)

Representative micrographs showing primary cultured smooth muscle cells (SMCs) isolated from wildtype and *Gjal<sup>Jrt/+</sup>* females double immunolabeled for Cx43 (green) and  $\alpha$ -actin (red); scale bar = 20  $\mu$ m. (B) The estimated conductance among SMCs isolated from *Gjal<sup>Jrt/+</sup>* females was significantly lower than that of wildtype SMCs ( $P < 0.001$ ).

### FIG. 3. Elevated estrogen can not rescue impaired Cx43 expression in *Gjal<sup>Jrt/+</sup>* females (A)

Representative western blots showing expression of Cx43 and Cx26 in nonpregnant uteri from wildtype and *Gjal<sup>Jrt/+</sup>* females treated with eCG. (B) Cx43 expression increased in both wildtype and *Gjal<sup>Jrt/+</sup>* females in response to eCG. However, *Gjal<sup>Jrt/+</sup>* females have significantly lower Cx43 expression level in both control ( $P < 0.01$ ) and eCG treated groups ( $P < 0.001$ ). (C) Phosphorylated P1 and P2 forms of Cx43 (Cx43-P1/2) are significantly reduced in the *Gjal<sup>Jrt/+</sup>* uteri in both control ( $P < 0.1$ ) and eCG treated groups ( $P < 0.001$ ). (D) Uterine Cx26 expression was significantly upregulated after eCG treatment in both wildtype and *Gjal<sup>Jrt/+</sup>* mice ( $P < 0.01$ ) and there was no significant difference between the two genotypes ( $P > 0.05$ ). (E) Representative micrographs showing uterine tissue from eCG treated wildtype and *Gjal<sup>Jrt/+</sup>* females double immunolabeled for Cx43 (green) and  $\alpha$ -actin (red). CM, circular muscle layer; LM, longitudinal muscle layer; scale bar = 20  $\mu$ m.

### FIG. 4. Reduced uterine contraction in *Gjal<sup>Jrt/+</sup>* females (A)

Sample traces showing activity of uterine strips taken from nonpregnant, wildtype and *Gjal<sup>Jrt/+</sup>* females in response to oxytocin. The dotted line indicates baseline tension at 0 gram. (B, 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 *Gjal<sup>Jrt/+</sup>* females showed a significantly weaker tension response compared with wildtype strips ( $P < 0.001$  for both graphs). (D) Representative western blots showing expression of oxytocin receptor (OTR) in uterine myometrium from wildtype and *Gjal<sup>Jrt/+</sup>* females. (E) The abundance of OTR in myometrium from *Gjal<sup>Jrt/+</sup>* mice is not significantly different ( $P > 0.05$ ) from that of wildtype littermates in both the control group and the eCG-treated group.

### FIG. 5. Impaired Cx43 expression in *Gjal<sup>Jrt/+</sup>* late gestational uteri (A)

Representative western blots showing Cx43 and Cx26 expression in nonpregnant and gestational day 18.5 (G18.5) uteri isolated from wildtype and *Gjal<sup>Jrt/+</sup>* females. (B) The abundance of total Cx43 is significantly reduced in both the nonpregnant ( $P < 0.05$ ) and gestational 18.5 day *Gjal<sup>Jrt/+</sup>*

myometrium ( $P < 0.001$ ). **(C)** The phosphorylated P1 and P2 forms of Cx43 (Cx43-P1/2) are significantly reduced in both the nonpregnant ( $P < 0.05$ ) and gestational 18.5 day *Gjal<sup>Jrt/+</sup>* myometrium ( $P < 0.001$ ). **(D)** Uterine Cx26 expression was significantly downregulated in gestational 18.5 day uteri in both wildtype and *Gjal<sup>Jrt/+</sup>* mice ( $P < 0.001$ ) and there was no significant difference between the genotypes ( $P > 0.05$ ). **(E)** Representative micrographs showing uterine tissue from gestational day 18.5 wildtype and *Gjal<sup>Jrt/+</sup>* females double immunolabeled for Cx43 (green) and  $\alpha$ -actin (red). Nuclei were stained with Hoechst dye (blue). CM, circular muscle layer; LM, longitudinal muscle layer; Scale bar = 20  $\mu$ m.

**FIG. 6. Prolonged gestation and impaired parturition of *Gjal<sup>Jrt/+</sup>* females** **(A)** The gestation period of *Gjal<sup>Jrt/+</sup>* females is significantly longer ( $P < 0.05$ ) than their wildtype littermates. Each dot represents an individual mouse and the horizontal line represents the mean for each group. **(B)** In addition to producing significantly smaller litters, *Gjal<sup>Jrt/+</sup>* females gave birth to some stillborn pups and retained significantly more placentas in their uteri than did wildtype females ( $P < 0.001$ ).

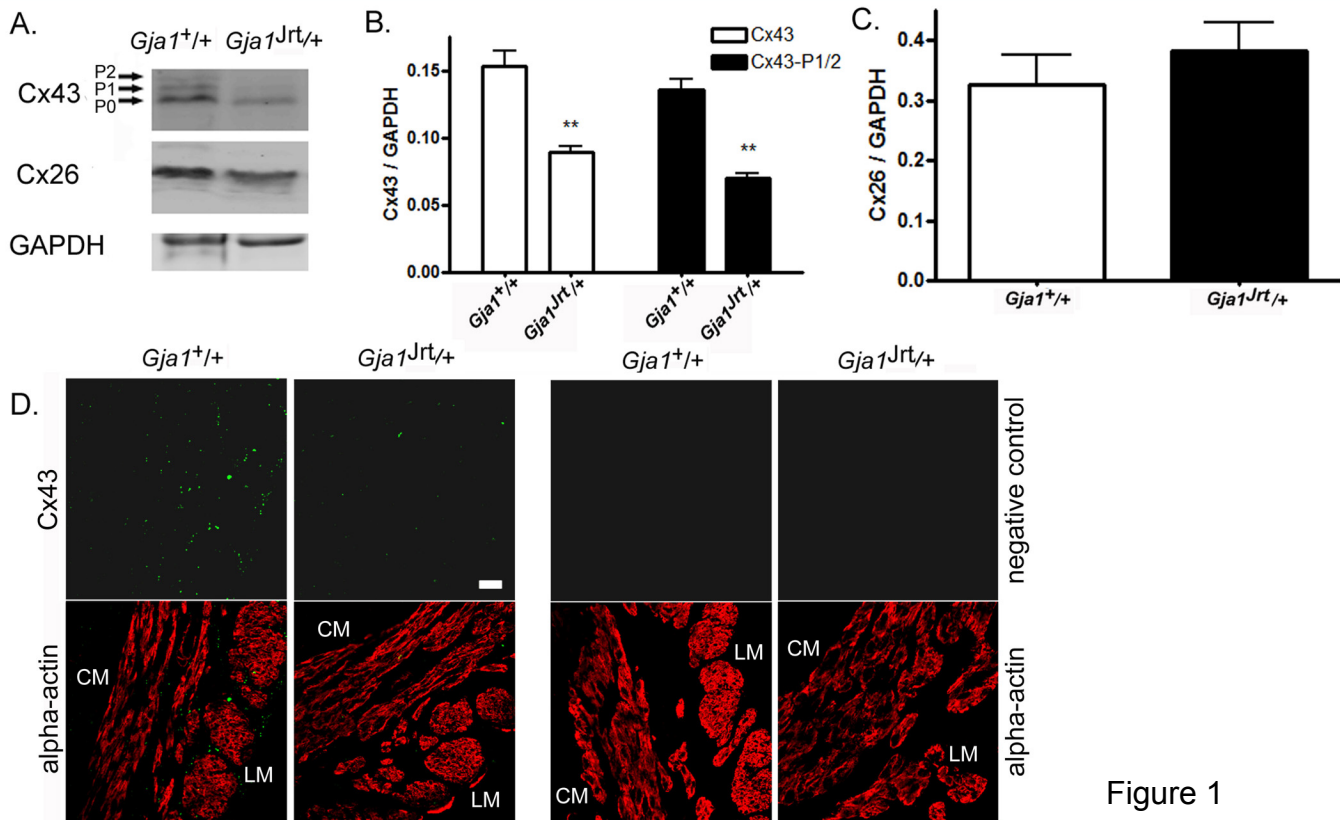


Figure 1



Figure 2

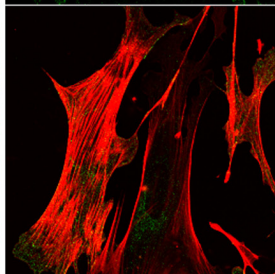
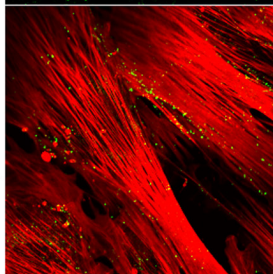
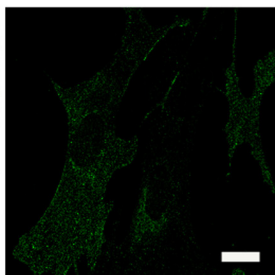
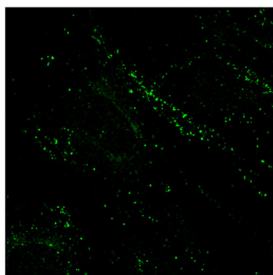
*Gja1*<sup>+/+</sup>

*Gja1*<sup>Jrt/+</sup>

A.

Cx43

alpha-actin



B.

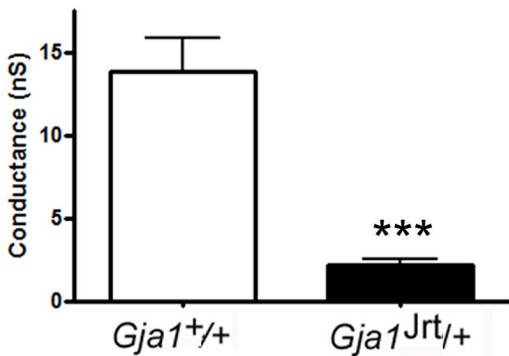


Figure 3

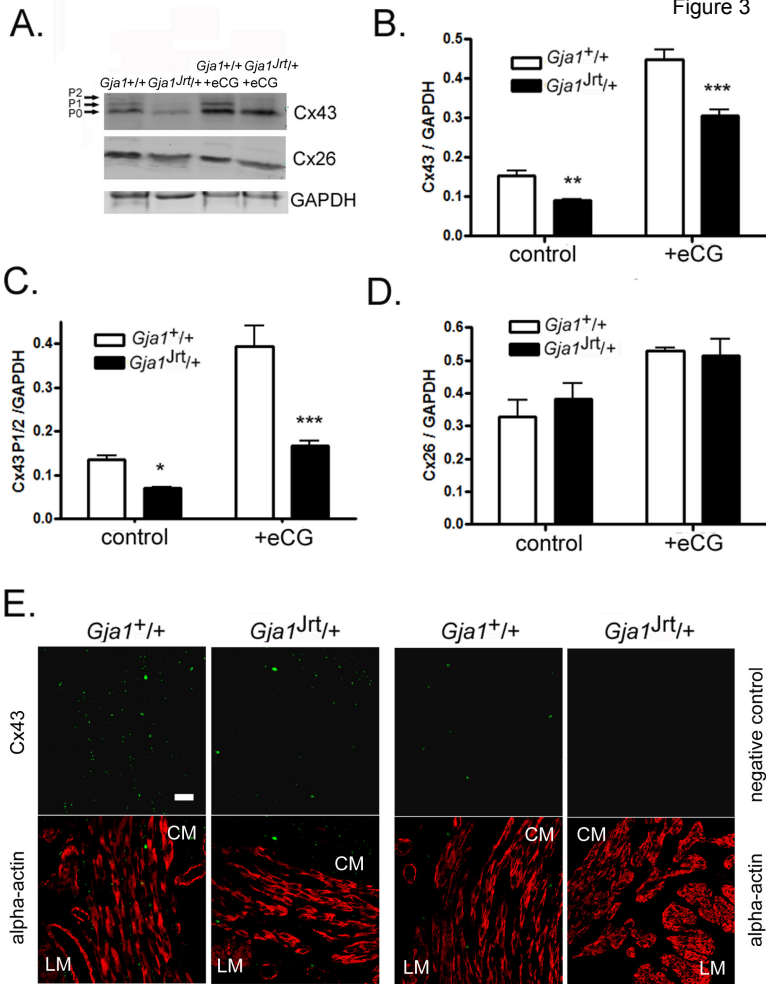


Figure 4

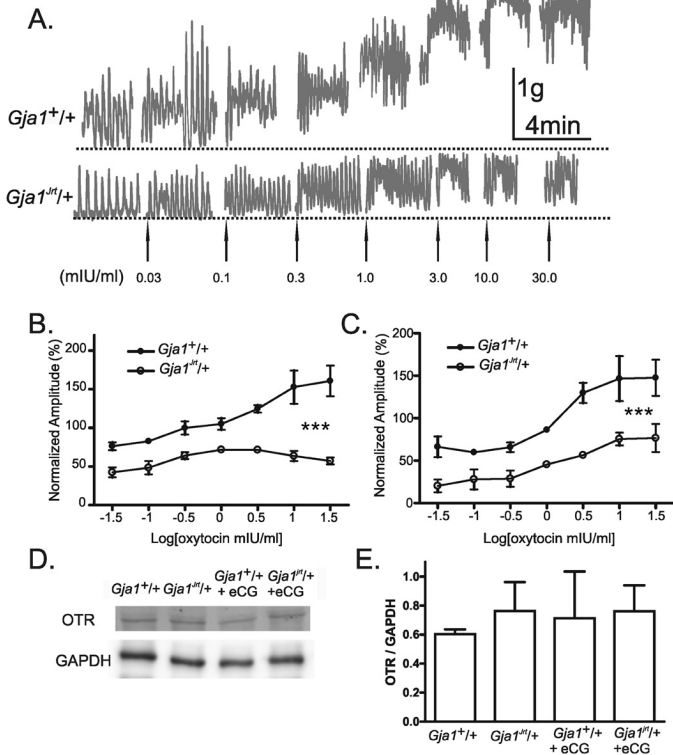
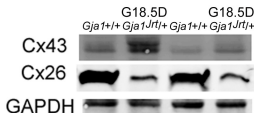
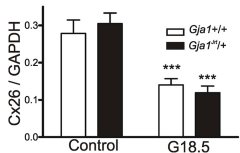


Figure 5

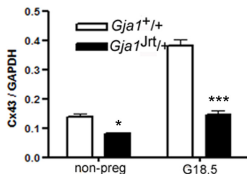
A.



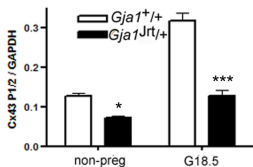
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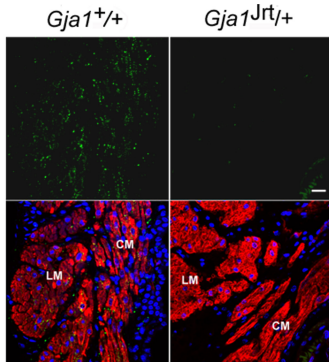
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C.



E.



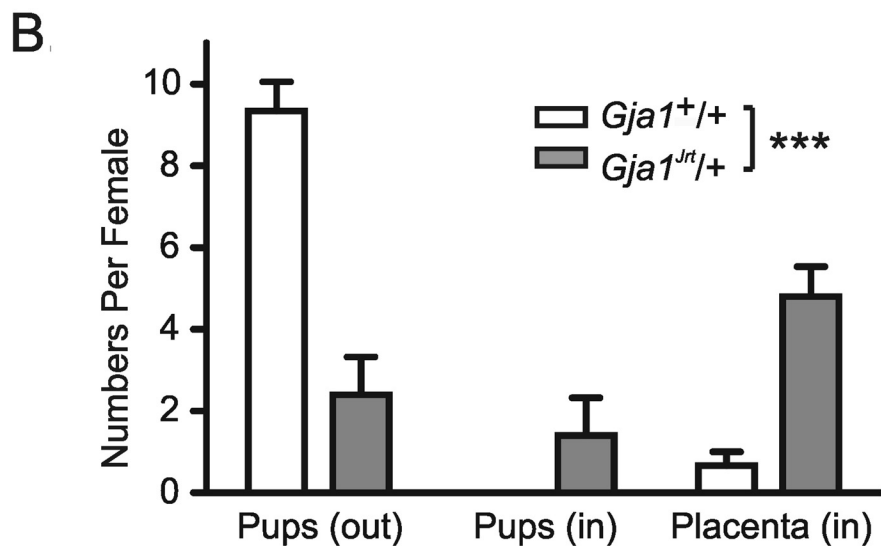
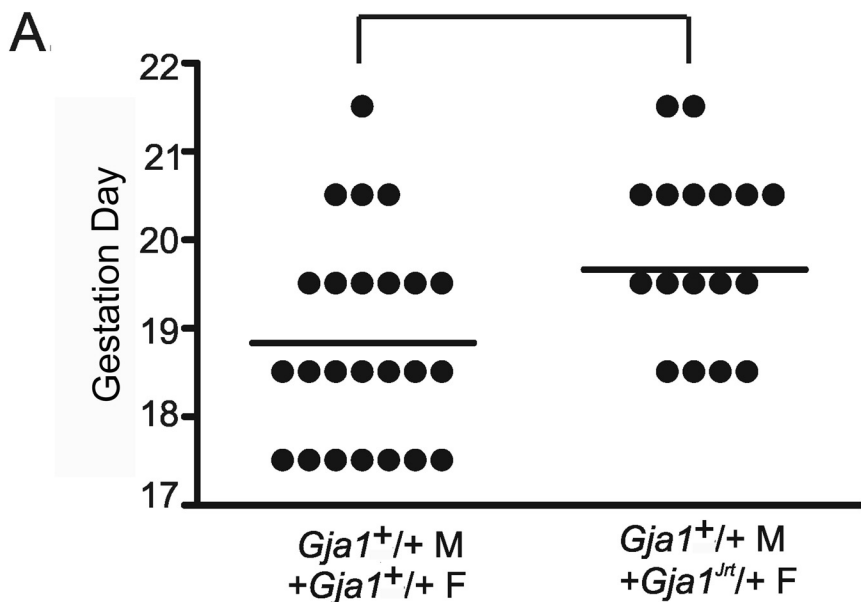


Figure 6