Opioid mediated activity and expression of mu and delta opioi receptors in isolated human term non-laboring myometrium.

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Abstract

The existence of opioid receptors in mammalian myometrial tissue is now widely accepted. Previously enkephalin degrading enzymes have been shown to be elevated in pregnant rat uterus and a met-enkephalin analogue has been shown to alter spontaneous contractility of rat myometrium. Here we have undertaken studies to determine the effects of met-enkephalin on *in vitro* human myometrial contractility and investigate the expression of opioid receptors in pregnant myometrium. Myometrial biopsies were taken from women undergoing elective caesarean delivery at term. Organ bath experiments were used to investigate the effect of the met-enkephalin analogue [D-Ala 2, D-met 5] enkephalin (DAMEA) on spontaneous contractility. A confocal immunofluorescent technique and real time PCR were used to determine the expression of protein and mRNA respectively for two opioid receptor subtypes, mu and delta. DAMEA had a concentration dependent inhibitory effect on contractile activity ($1X10^{-7}$M to $1X10^{-4}$M; 54% reduction in contractile activity, $P<0.001$ at $1X10^{-4}$M concentration). Mu and delta opioid receptor protein sub-types and their respective mRNA were identified in all tissues sampled. This is the first report of opioid receptor expression and of an opioid mediated uterorelaxant action in term human non-laboring myometrium *in vitro*.

Key Words: human myometrium, contractile activity, pregnancy, endogenous opioid peptides, opioid receptors

1. **INTRODUCTION**

The endogenous opioid system consists of a family of structurally related small endogenous peptides acting through multiple opioid receptors (mu, delta and kappa) as neurotransmitters, autocrine or paracrine factors and hormones. The original endogenous opioid peptide families are enkephalins, dynorphins and endorphins. Most of the knowledge that has been accumulated about the expression, release and function of the endogenous peptides derives from studies of the central and peripheral nervous systems and the neuroendocrine system. Here they have a role in regulating a wide variety of physiological functions, which include but are not limited to nociception, the control of respiration, thermoregulation, the immune response and hormone secretion (Bodnar, 2008).

The existence of both endogenous peptides and opioid receptors in non-neural peripheral sites in animal (Tang et al., 1982; Zhu and Pintar, 1998; Barron, 2000; Denning et al., 2008) and human tissues (Sastry et al., 1980; Belisle et al., 1988; Agirregoitia et al., 2006; Rittner et al., 2008), is now widely accepted. These include placental (Sastry et al., 1980; Belisle et al., 1988) and uterine tissue (Zhu and Pintar, 1998) where they have been implicated in the regulation of female reproduction (Sastry et al., 1980; Belisle et al., 1988; Amed et al., 1989; Cemerikic, 1991; Zhu and Pintar, 1998). In mouse uterus, all three opioid receptor genes and endogenous opioid peptide precursors have been detected after implantation of the embryo with their expression pattern pointing toward a role in adaptation to pregnancy, in the regulation of uterine motility (Sastry et al., 1980). Binding sites for an opioid receptor antagonist have been identified in rat uterine membranes and are subject to down-regulation during gestation (Baraldi et al., 1985).

Met-enkephalin is a member of the enkephalin family of endogenous peptides. DAMEA ([DAla2, D-met 5] enkephalin), an analogue of met-enkephalin significantly increases the duration of spontaneous contractions in rat myometrium in late gestation (Adjroud, 1985). There is evidence that the pregnant rat uterus contains higher concentrations of enkephalinase, an enkephalin degrading enzyme, than a non-pregnant uterus, and that enkephalinase levels decrease the day before parturition commences (Ottlecz et al., 1991). Enkephalinase therefore, may act as a regulator of uterine motility by controlling levels of met-enkephalin in pregnancy.

Opioid receptors are coupled to inhibitory G proteins. Opioid receptor activation leads to decreased cyclic adenosine monophosphate (cAMP) formation and modification of ion channel activities resulting in reduced calcium influx and stimulation of potassium efflux (Zollner, 2006). These mechanisms suggest that opioids could directly affect the contractile response of human myometrium through the modulation of myocyte calcium fluxes and in decreasing cAMP generation, which at term promotes uterine contractility (Lopez-Bernal, 2003) and decreased progesterone responsiveness (Smith, 2007).

The effect of met-enkephalin on contractile parameters of human term myometrium has not previously been explored. Furthermore it is not known whether opioid receptors exist in human pregnant myometrium. The aim of this study was twofold. Firstly to investigate the effect of the met-enkephalin analogue DAMEA on spontaneous human pregnant myometrial contractility and secondly to systematically investigate the presence of mu and delta opioid receptors by immunohistochemical and PCR analysis in term non-labouring human myometrium.

2. MATERIALS AND METHODS

2.1. Subjects and Preparation of Tissues:

All biopsy specimens of human pregnant myometrial tissue were obtained from women undergoing elective lower segment cesarean delivery. Ethical approval for the study was obtained from the Research Ethics Committee of the Coombe Women and Infants University Hospital and all patients gave written consent. Criteria for inclusion consisted of a singleton gestation between 38 – 40 weeks who were not in labour. Exclusion criteria included 1) a history of intravenous opioid drug abuse 2) history of a chronic pain condition or history of regular opioid analgesia intake 3) the use of prostaglandins to induce labour 4) history of ruptured membranes or 5) a diagnosis of human immunodeficiency virus, hepatitis B or C or pre-existing diseases such as diabetes mellitus/ pre-eclampsia or renal disease. Indications for caesarean delivery included breech presentation and prior caesarean section. All patients received antacid prophylaxis with 30 ml of 0.3 M sodium citrate and 400 mg cimetidne orally, prior to spinal anesthesia with 2.0 – 2.4 ml 0.5% hyperbaric bupivacaine, with 20 – 25 μg of intrathecal fentanyl and 100 – 150 μg of intrathecal morphine. Table 1 shows the demographic data for all the patients used in this study.

The myometrial biopsy was excised from the midline of the upper margin of the lower uterine segment incision (inner myometrial layer) following delivery of the baby and placenta. All specimens were rinsed in Ringer's lactate solution ensuring all traces of blood were removed and that the specimen was free of placental tissue. For tissue bath experiments the biopsies were placed in a sterile container and refrigerated at 4ºC until used, which was within 2 to 12 h of collection. For immunoflouresence studies each specimen was embedded with Tissue-Tek O.C.T Compound (Sakura Finetek, Alphen van den Rijn, Netherlands) before being snap frozen in liquid nitrogen and subsequently stored at -80˚C. For mRNA studies each specimen was placed in RNAlater (Ambion, Austin, TX) and refrigerated at 4˚C for between 1 and 3 days before the RNA extraction process.

2.2. Contractile Analysis:

Uterine biopsies were dissected into at least 4 longitudinal muscle strips 12 X 5 X 1 mm under a naked eye, ensuring fibrous tissue, serosa or blood vessels were not included. Isometric tension recordings were obtained from an eight-chamber organ bath (either 10/15 ml, water jacketed) system (Myobath, World Precision Instruments Inc. Sarasota, Florida). The organ baths contained Krebs-Henseleit physiological salt solution (NaCl 118 mmol/l, D-glucose 11.1 mmol/l, $NaHCO_3$ 24.9 mmol/l, $MgSO_4$ 1.2 mmol/l, KCl 4.7 mmol/l, KH_2PO_4 1.2 mmol/l, and $CaCl_2$ 2.5 mmol/l, pH 7.4) and were aerated with a gas mixture of 95% oxygen and 5% CO_2 and maintained at 36.7ºC. A resting tension of 1g was initially applied, and subsequently re-applied as necessary over the first 30-40 min until a steady tension was achieved as previously described (Fanning et al., 2008). During this equilibration period, the Krebs solution was changed every 10 min. When spontaneous contractions became regular (within 60-90 min) experiments were carried out as follows: (1) a 30 min control period followed by cumulatively increasing concentrations of DAMEA (Sigma-Aldrich, St Louis, MO) in 1 log molar increases every thirty min from $1X10^{-8}$M to $1X10^{-4}$M (n=7) and (2) a 30 min control period followed by the addition of naloxone ($1X10^{-5}$M; Sigma-Aldrich; Yoo et al., 2001) and then 30 min later by cumulatively increasing concentrations of DAMEA in 1 log molar increases every thirty min from $1X10^{-8}$M to $1X10^{-4}$M (n=7). A time matched control strip, exposed only to Krebs solution was run in parallel from each separate *n* to ensure tissue viability for the duration of the experiment. Strips exposed

to DAMEA were also pre-treated once off with the peripheral enkephalinase inhibitor thiorphan ($1X10^{-4}$M; Adjroud, 1985) for 30 min prior to the addition of DAMEA (Sigma-Aldrich) to inhibit tissue enkephalinases. Prior to these experiments, cumulative concentration-effect curves were determined in myometrial biopsies from other women for both naloxone ($1X10^{-9}$ to $3X10^{-4}$M, *n*=3), thiorphan ($1X10^{-7}$ to $1X10^{-4}$M, *n*=7) and alcohol (diluent for thiorphan) (0.1% w/v n=3), which established that these agents had no effect on myometrial contractility. For each experiment Krebs-Henseleit solution was prepared freshly each day. All stock solutions of drugs were prepared according to the supplier's instructions. Thiorphan ($1X10^{-2}$M) was diluted in alcohol to a final bath concentration of 0.1% w/v. DAMEA ($1X10^{-2}$M) and naloxone ($1X10^{-1}$M) were diluted in distilled water. The organ baths and tubing were siliconised. All stock solutions were stored at -20˚C. Drugs were diluted further immediately before each experiment from the stock solution using Krebs solution. Following completion of the experiment the weight of each muscle strip was recorded to ensure weight and size equality.

2.3. RNA extraction, reverse transcription and polymerase chain reaction

RNA was isolated from myometrial biopsies using TRIzol solution (Invitrogen, Carlsbad, CA) according to manufacturers' instructions. A QiagenTissueLyser II (Qiagen, Germantown, MD) was used to disrupt biopsies using acid washed glass beads for two 30 second periods at a frequency of 30 Hz. All RNA samples were subsequently treated with DNase 1 with the RNeasy Mini Kit (Qiagen) to exclude contamination by genomic DNA (gDNA). Total RNA concentrations were determined using a Nanodrop and the integrity was checked by gel electrophoresis. Two micrograms of total RNA was reverse transcribed into complimentary DNA (cDNA) using an oligo dT primer and the Maloney murine leukaemia virus reverse transcriptase enzyme (MMLV-RT, Invitrogen) according to the manufacturer's instructions. These samples of myometrium were all taken from the mid-upper lip of the lower segment uterine incision and were from the inner myometrial layer.

2.4. PCR

Real-time PCR was conducted using SYBR Green master mix (Applied Biosystems, Foster City, CA) on an ABI 7300 thermocycler (Applied Biosystems). Duplicate reactions were analysed in 10 µL volumes containing 40 ng of cDNA and 0.3 µM of upper and lower primers. The primers chosen to amplify the mu and delta ORs and glyceraldehyde 3- phosphate dehydrogenase (GAPDH) mRNA transcripts were based on previous publications (Cheng et al., 2006; Philippe et al., 2006; Zocco et al, 2010). Human cerebral cortex cDNA was used as a positive control (First Choice Human Brain RNA, Ambion). Mu and Delta OR Ct values were normalised relative to the amount of constitutionally expressed GAPDH and normalised Ct values plotted. A deflection from the baseline before the 35th cycle of amplification was considered significant (Williams et al., 2008).

2.5. Immunoflourescence

Following a period of optimising the staining technique on rat spinal cord, 12µm sections of frozen tissue from each myometrial biopsy were fixed in 70% alcohol for 25 min at room temperature. Sections for incubation with the anti—mu opioid receptor antibody were subsequently placed in 0.1% (w/v) Triton X-100 (Sigma-Aldrich) diluted in phosphate buffered saline (PBS) for 25 min. All other slides were placed in PBS for 25 min. Specimens were then incubated with either the anti-mu (1:1000 dilution; Abcam, Cambridge, MA) or anti-delta

primary antibody (1:250; Affinity BioReagents Rockford, IL) overnight at 4˚C; duplicate slides were prepared for each sample and for each antibody. Each antibody was diluted in a solution containing 1% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich) and 1% (w/v) normal goat serum (NGS) (DAKO, Glostrup, Denmark). Samples were then incubated with a goat anti-rabbit immunoglobulin (IgG) conjugated to Alexa-Fluor 488 (Molecular Probes Inc, Carlsbad, OR) secondary antibody at room temperature for 3 h. Each slide was then immersed into propidium iodide solution (0.1% w/v) (Sigma-Aldrich) for 3-5 seconds to counter stain nuclei and mounted with Citiflour (Agar Scientific, Stansted, UK) and imaged. The same procedure was followed for the negative controls with the exception of the addition of the primary antibody, which was omitted. Images were captured on a Zeiss LSM Pascal 5 and associated software (Zen 2007 Light, Carl Zeiss Edition, Carl Zeiss AG, Oberkochen, Germany) at a magnification of 40 X and over a field of 230 x 230 μm.

2.6. Data Analysis

The mechanical response of tissues was measured by the calculation of the integral of selected areas for specified 20-min periods with the use of Powerlab software chart (version 3.0) (AD Instruments Pty Ltd, Bella Vista, NSW, Australia). This measurement of contractile activity incorporates frequency, amplitude, and duration of contractions (area under the curve). Basal contractile activity was calculated for the 20 min prior to the addition of any drug and was taken as 100%. Responses to cumulatively increasing DAMEA concentrations were compared with a concurrent time matched control and to the contractile activity calculated in the pre-treatment period for each strip. The cumulative concentration responses were analysed by non-linear regression analysis and cumulative IC_{50} values were obtained. The IC_{50} represents the molar concentration of the agonist that provoked a response halfway between the baseline and maximal inhibitory response. The pIC_{50} represents the $-\log_{10}(IC_{50})$ value. All data are expressed as mean± S.E.M. and the *n* in the text refers to the number of independent experiments performed using tissues from separate donors.

Statistical significance of drug effects relative to time matched controls was compared using a Paired Students *t* test. Comparisons of the contractile effect of DAMEA in the absence and presence of naloxone were performed by using repeated measures analysis of variance (ANOVA) followed by Bonferroni posttests to determine significant differences among data groups. All statistical analyses were performed using GraphPad Prism 5 statistical software (GraphPad Software Inc, La Jolla, CA) and $P < 0.05$ was accepted as statistically significant. EBImage software (Pau et al., 2010) was used to quantify intensity of fluorescence. The images were converted to greyscale images, whereby the fluorescence intensity value for each pixel was given an arbitrary unit between 0 and 1. These images were converted to threshold images as depicted in Fig. 5B and 5D. The threshold was set as 0.1% of the mean pixel intensity within a 200 x 200 pixel area of the image. Each pixel above this threshold was converted to a white pixel from which the mean fluorescence intensity was calculated (Watters et al., 2011). This analysis was performed by an independent blinded observer. Raw data from the EBImage software analysis were analysed using an unpaired two tailed Student's t-test.

3. RESULTS

3.1. Tissue samples

Demographic details of all women who participated in the study are shown in Table 1. In total five of the 32 myometrial strips obtained from eight women for the in vitro contractility experiments either failed to contract or contract within 90 min were excluded. The mean contractile activity during the control/ basal activity period was 2243.0 ± 893.0 g/s (range 1254 - 4425) with a mean peak-to-peak interval of 347.7 ± 92.9 s and a contraction rate of 5.8 contractions per hour.

3.2. In vitro myometrial contractility

The addition of DAMEA exerted a significant concentration dependent inhibitory effect on contractile activity compared to time matched control ($P<0.05$; Fig. 1A, B and C). The uterorelaxant effect of DAMEA was observed from $1X10^{-7}M$ ($P<0.001$). This effect was reversed in the presence of naloxone at all concentrations ($P<0.05$; Fig. 1D). The pIC_{50} values are given in Table 2.

3.3. RT-PCR

Using Real-time PCR of myometrial cDNA obtained from the inner layer of myometrium of the lower uterine segment myometrial biopsies, a Ct value of 34.09 and 32.99 was observed for the mu and delta opioid receptors respectively (n=4). Ct values normalised to GAPDH are depicted in Fig. 2.

3.4. Immunoflourescence

Confocal microscopy of sections of the inner layer of myometrium from lower uterine segment biopsies of term non-labouring women (n=5) using specific mu and delta opioid receptor antibodies, demonstrated the presence of both mu and delta opioid receptor protein (Fig. 3A and 3B respectively). The pattern of fluorescent staining observed was associated with the cell membrane and within the cytoplasm of individual myocytes. Nuclei were counterstained red. The negative controls for both mu and delta opioid receptor protein lacked any fluorescent staining (Fig. 4A and 4B respectively). Analysis of the confocal images with EBImage software showed a significant difference in the level of fluorescent staining detected for both mu opioid receptor staining ($P< 0.001$ Fig. 5A and 5C) and for delta opioid receptor staining in the myometrium ($P< 0.01$ Fig. 5B and D) compared to the corresponding negative controls.

4. DISCUSSION

Endogenous peptides have been implicated in the regulation of female reproduction at several sites. This includes the regulation of hormone release in the hypothalamic-pituitary axis (Goodman et al., 1995; Richter et al., 2001; Pimpinelli et al., 2006; Brunton and Russell, 2008) and placenta (Belisle et al., 1988; Ahmed et al., 1989; Cemerikic et al., 1991), in pain modulation during pregnancy and labor (Dabo et al., 2010), and in the regulation of uterine motility (Zhu and Pintar, 1998). We characterised the effect of a met-enkephalin analog DAMEA on spontaneous human myometrial contractility. DAMEA was observed to have an utero-relaxant effect. We have also established evidence of both mu and delta OR mRNA and OR protein expression in term human myometrium.

Met-enkephalin has previously been observed to have varying effects on uterine smooth muscle. In non-pregnant rat myometrium met-enkephalin was observed to have no effect on spontaneous contractions and a stimulant effect on KCl-depolarised uteri (Ohia and Laniyonu 1989) and no effect on the constancy of isometric developed tension in uterine strips of ovariectomised rats (Faletti et al 1992). DAMEA has been shown to significantly increase the duration but not amplitude of spontaneous contractions in rat myometrium in late gestation, an effect that was blocked by the addition of naloxone (Adjroud, 1985). The effects of non-endogenous opioids on myometrial contractility have also produced conflicting results to date. In humans fentanyl and pethidine have been shown to inhibit uterine contractility albeit at supra-clinical concentrations, while morphine had no reported effects (Yoo et al., 2001). To date no studies have elucidated the molecular mechanisms responsible for opioid mediated effects on myometrial contractility but nitric oxide, β adrenoreceptor activity and prostaglandins do not appear to have a role (Yoo et al., 2001). Plasma levels of met-enkephalin do not change in pregnancy (Newnham et al., 1983) and plasma levels in labor have not been published. However, met-enkephalin is synthesised and secreted by human placental villus tissue (Sastry et al., 1980) and it may be the change or rate of change in the local placental/uterine concentration that has the potential to alter the myometrial contractile response in pregnancy. These changes may not be reflected in plasma measured levels.

The mechanism by which opioid receptor activation alters the contractile response of myometrium is not known but can be postulated through several mechanisms. Opioid receptors interact with various ion channels in the membrane through inhibitory G proteins (G_i) including calcium and potassium channels, leading to suppression of calcium influx and membrane hyperpolarisation respectively (Zollner, 2006). Opioid receptor activation in the myometrium could also lead to increased contractile activity through cAMP accumulation by the inhibition of adenyl cyclase and by stimulating phospholipase C, increasing intracellular calcium (Zollner, 2006). We observed a uterorelaxant effect, the exact mechanism of which still remains to be determined.

We have provided evidence of opioid receptor mediated activity in term human non-laboring myometrium. This is further supported by evidence of mu and delta opioid receptor mRNA and protein expression in a human tissue model. However, *in vitro* myometrial contractility investigations do have limitations. There is a complex interplay of hormonal, chemical and mechanical signals in the pregnant uterus many of which may be attenuated or abolished in the *in vitro* setting. It is possible that any one of these pathways may amplify the effect of met-enkephalin *in vivo*. It is possible that not all tissue enkephalinases were inhibited by thiorphan. We only investigated the affect of met-enkephalin on contractility in tissue from the lower uterine segment of the uterus and we cannot rule out regional differences in contractility. With regards to the IHC analysis, we are unable to comment on the precise cellular location of opioid receptor protein which appears to be both in the cytoplasm and at the cell membrane of myocytes from the lower uterine segment. The cytoplasmic staining may represent the synthesised receptor as it is being cycled to the cell membrane where it becomes functionally active once inserted into the cell membrane. Work by Gray and colleagues studying the distribution of opioid receptors in the rat central nervous system with a similar technique found a comparable pattern of staining (Gray et al., 2006).

There are areas for future research arising out of this work. Most obviously the mechanism of the utero-relaxant effect remains to be determined specifically differentiating which opioid receptor subtype mediates the uterorelaxant effect. We investigated myometrial tissue of pregnant

women; it remains to be determined whether opioid receptor expression is a pregnancy related phenomenon and whether the level of expression changes throughout gestation and or at the onset of parturition in response to autocrine and or paracrine factors. In conclusion, we have provided functional evidence of opioid mediated activity in term human non-laboring myometrium *in vitro*. DAMEA a met-enkephalin analogue produces a utero-relaxant effect which is reversed in the presence of an opioid receptor antagonist, naloxone. We have also demonstrated for the first time evidence of mu and delta opioid receptor mRNA and protein expression in term human pregnant myometrium. The exact clinical significance of this in terms of the regulation of myometrial contractility in human parturition requires further investigation.

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6. FIGURES

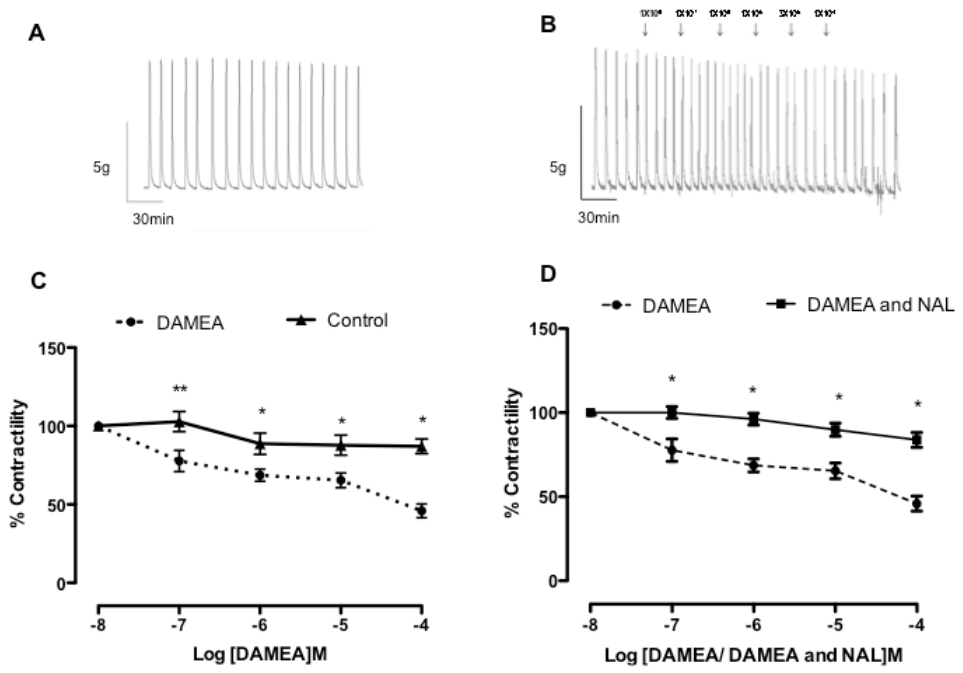

Fig. 1.
Effects of increasing concentrations of DAMEA with and without naloxone on spontaneous contractions in human myometrium. **A**. Sample control trace showing spontaneous contractions over a 6 hr period. **B**. Sample trace showing the effect of increasing the concentration of DAMEA on the contractility of human myometrium over an 6 hr period. The concentration of DAMEA was increased every 30 min. **C**. Concentration effect curve for DAMEA with time matched controls. There was a significant reduction in contractility at all concentrations of DAMEA from $1X10^{-7}$M to $1X10^{-4}$M (*n*=7). **D**. Concentration effect curves for DAMEA and DAMEA in the presence of naloxone $1X10^{-5}$M, demonstrating reversal of the uterorelaxant effect in the presence of naloxone (*n*=8). For C and D % basal contractility (AUC) is shown on the y axis and the concentration of DAMEA on the x axis. Each point represents the mean ± S.E.M. *P<0.001 and **P<0.01using repeated measures ANOVA and Bonferroni posttests.

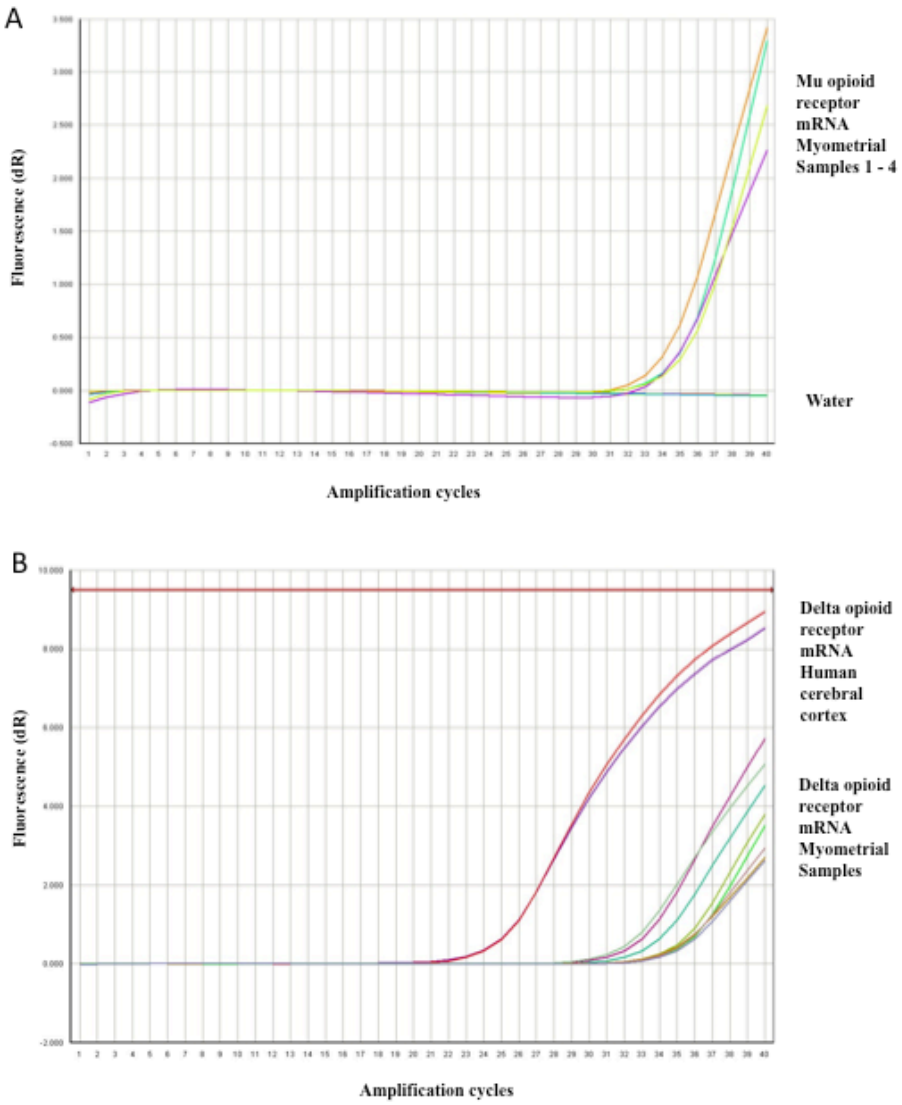

Fig. 2.
Real-time PCR curves from the inner myometrial layer of the lower uterine segment of human term pregnant uterus for (**A**) mu and (**B**) delta opioid receptors (OR). A deflection before the 35th cycle of amplification was considered significant. Mu and delta OR Ct values were normalised relative to the amount of constitutionally expressed GAPDH and normalised Ct values plotted. The mean Ct value for mu was 34.09 and for delta was 32.99.

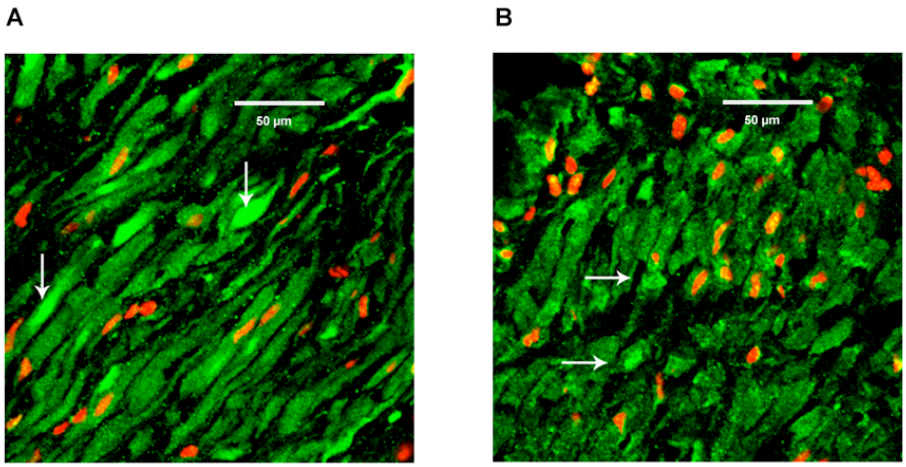

Fig. 3.
Representative examples of positive immunostaining of (**A**) mu and (**B**) delta opioid receptor protein in a 12 µm section taken from the inner myometrial layer of the lower uterine segment of human term pregnant uterus. A positive result was noted as a dot-like green fluorescence. (FITC conjugated secondary antibody; white arrows). Nuclei are counterstained red. Horizontal bar denotes 50 µm. Original magnification was 40 X over a field of 230 x 230 µm.

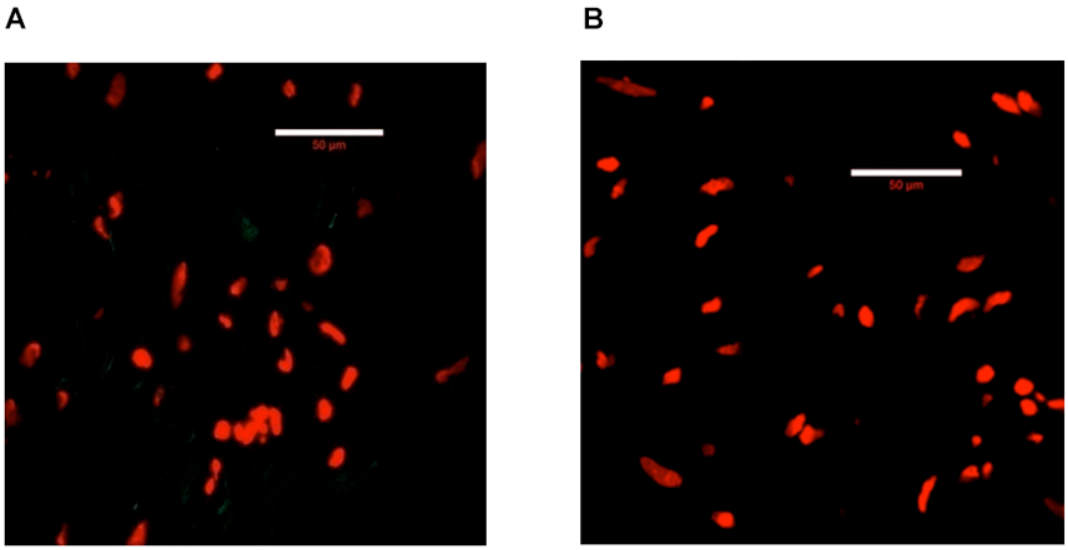

Fig. 4.
Representative examples of negative control 12 µm sections of myometrium taken from a full thickness lower uterine segment biopsy of human term pregnant myometrium for (**A**) mu opioid and (**B**) delta receptor subtypes, demonstrating the absence immunoflouresence. Nuclei are counterstained red. Horizontal bar denotes 50 µm. Original magnification was 40 X over a field of 230 x 230 µm.

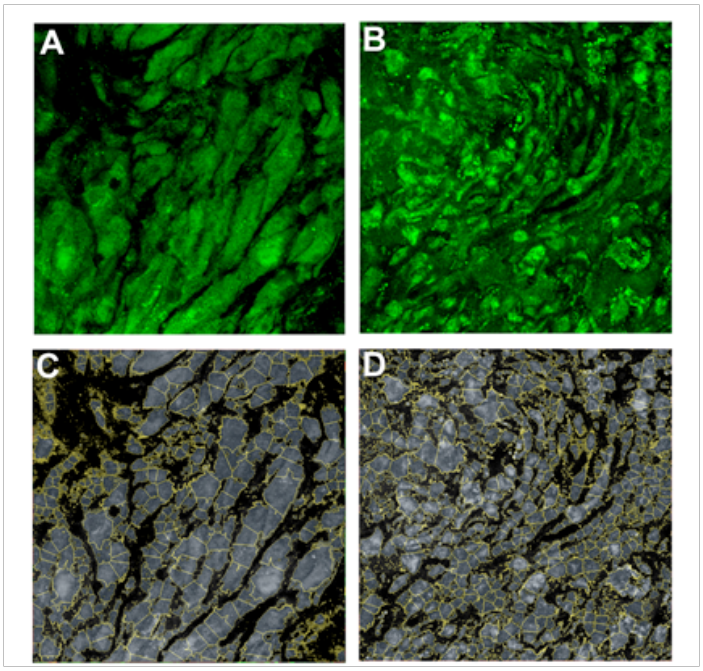

Fig. 5.
To quantify the intensity of fluorescence for mu opioid receptor (**A**) and for delta opioid receptor (**B**) in a 12 μm section of human term pregnant myometrium, EBImage software was used. The images were converted to greyscale images, whereby the fluorescence intensity value for each pixel was given an arbitrary unit between 0 and 1. These images were converted to threshold images (**C** and **D**). The threshold was set as 0.1% of the mean pixel intensity within a 200x200 pixel area of the image. Each pixel above this threshold was converted to a white pixel from which the mean fluorescence intensity was calculated.

	Water bath	IHC	PCR
Maternal age (years)			
20-25	0	0	1
26-35	6	2	1
>35	2	3	2
Ethnicity (%)			
Caucasian	100	100	100
Parity			
Nulliparous	1	1	1
Multiparous	7	4	3
Indications for Caesarean Section			
Breech presentation	1	1	1
Previous Caesarean section	7	4	3
Total Number (*n*)	8	5	4

Table 1. Demographic details of all patients recruited into the contractility, IHC Immunohistochemisrty and PCR polymersase chain reaction experimental protocol. Each *n* represents a different patient.

	IC_{50}	pIC_{50}	95% CI of pIC_{50}
DAMEA	9.2×10^{-8} +/- 0.3	7.0	7.6 - 6.4
DAMEA with Naloxone	5.8×10^{-6} +/- 0.5	5.2	6.3 - 4.2

Table 2 . IC_{50}, pIC_{50} and 95% CI values calculated using non-linear regression analysis from *n=7* for DAMEA and *n=8* for DAMEA and naloxone. IC_{50} represents the molar concentration of the agonist that provoked a response halfway between the baseline and maximal inhibitory response. The pIC50 represents the $-\log_{10}$ (IC50) value.