Title: METHOD FOR RESTORING REPRODUCTIVE FUNCTION

Abstract: A method of restoring or enhancing reproductive function in a subject that is reproductively impaired, the method comprising administering to said subject an melanocortin-3 (MC3-R) and/or melanocortin-4 receptor (MC4-R) agonist in an amount sufficient to restore or enhance reproductive function.
METHOD FOR RESTORING REPRODUCTIVE FUNCTION

FIELD OF THE INVENTION

The present invention relates generally to the field of endocrinology and, particularly, to methods of regulating the fertility of a subject. More particularly, the present invention relates to methods of enhancing or restoring reproductive function in a subject who is reproductively impaired.

BACKGROUND TO THE INVENTION

Loss of reproductive function in women is often correlated with amenorrhea. Amenorrhea in women may be the result of excessive exercise, or may be due to other reasons such as psychological stress, prior use of oral contraceptives or anorexia nervosa (Marshall, 2001). It is well established that amenorrhea of extremely lean women is due to extremely low fat and hypothalamic dysfunction, and adipose tissue, once thought to be only an energy source, has now been recognised as having a significant role in the regulation of female reproduction. It is also well recognised that significant decreases in body weight can result in lower circulating levels of reproductive hormones, a condition known as hypogonadotrophic hypogonadism. Moreover, it has been established that women suffering from hypogonadotrophic hypogonadism show reduced concentrations of serum luteinising hormone (LH) and follicle stimulating hormone (FSH). Collectively, LH and FSH are "gonadotropins", being hormones that stimulate the gonads.

LH is a glycoprotein produced in both men and women from the anterior pituitary gland in response to luteinising hormone-releasing hormone (LHRH or GnRH) released by the hypothalamus, which in women has an important role in fertility and, particularly, in the stimulation of ovulation. The secretion of GnRH from the hypothalamus is pulsatile and this causes pulsatile secretion of LH. The secretion of FSH is also controlled by GnRH, but the pulsatile pattern of secretion of this hormone is not as clear as for LH, because it has a longer half-life in plasma and may be secreted in the absence of GnRH. Control of the serum level of LH concentration in healthy menstruating women is subject to the complex ovulatory cycle and, more specifically, depends upon a sequence of hormonal events along the gonado-hypothalamic-pituitary axis. That is, control of the serum level of LH is affected by the levels of progesterone and oestradiol that vary across the ovulatory
cycle. Rising FSH levels stimulate the production of ovarian follicles (which contain the eggs, or ova). One or more follicles burst at the time of ovulation to discharge ova into the fallopian tubes of the female reproductive tract. The ovulatory cycle is divided into different phases depending upon the secretion of GnRH, the gonadotropins and ovarian hormones. The "luteal phase" is characterised by low levels of GnRH and gonadotropins and high levels of progesterone, secreted from the corpus luteum of the ovary. During the luteal phase, GnRH and LH are secreted at low frequency. The corpus luteum forms from the ovarian follicle after ovulation. When the corpus luteum regresses, progesterone levels fall and the "follicular phase" begins. This is a phase of the cycle dominated by rising levels of oestrogen, secreted by one or more growing ovarian follicles. The pulse frequency of GnRH and LH increases in the follicular phase. In particular, rising levels of oestrogen, in the absence of progesterone, exerts a "positive feedback" effect on the brain and pituitary, leading to a surge in the secretion of LH and FSH (at mid-cycle in women). This surge in gonadotropin secretion is the trigger for ovulation.

Not surprisingly then, losses or abnormalities in the control of LH and FSH secretion can lead to a reduction in reproductive function (ie infertility) in women and other associated problems (eg osteoporosis). Perturbation of LH secretion (leading to reduced serum concentrations of LH) in women is often associated with renal failure, cirrhosis, hyperthyroidism, over-exercise leading to loss of body weight and severe starvation or chronic malnutrition (such as chronic malnutrition resulting from anorexia nervosa). In particular, the condition of hypothalamic amenorrhea is a condition in which ovulation and failure of the regular ovulatory cycle is due to reduction in the secretion of GnRH from the hypothalamus. This leads to the loss of production and secretion of the gonadotropins and loss of ovarian function.

As a consequence of the observed association between chronic malnutrition or excessive exercise in women and reduction in LH secretion and infertility, considerable research has been undertaken to understand how body weight may affect reproductive function and to elucidate the factors that may be involved. Amongst this research, there has been a number of investigations made into the possible role of the factor, leptin. Leptin is a 167 amino acid, fat-derived, hormone that is a product of the ob gene, which is believed to act as a satiety factor, limiting food intake. Leptin acts on the brain to signal metabolic function. Leptin levels in serum are proportional to the amount of white fat and therefore acts as a "barometer" of relative adiposity. Leptin also acts on cells in the brain that
regulate food intake and energy expenditure. The control of homeostasis is a very complex process, involving various types of brain cells and systems and there is neuronal communication between the appetite regulating systems and the neuroendocrine systems. The latter comprises the cells of the hypothalamus that regulate the endocrine system, by production and secretion of releasing factors specific for each system. Thus, GnRH regulates the reproductive system by acting on the gonadotropes of the pituitary gland and thyrotropin releasing hormone (TRH) act on thyrotropes of the pituitary to regulate the thyroid axis etc. In animals of low body weight, due to food restriction, reproductive function is compromised, such that the levels of GnRH and gonadotropin secretion are reduced. In this condition, levels of circulating leptin are reduced. Treatment of such animals with leptin can restore reproductive function, strongly suggesting that low leptin levels of animals of low body weight signal reduced metabolic stores to the brain, causing the reproductive system to down-regulate. The infusion of leptin "fools" the animals to "think" that they have adequate metabolic stores. Similar effects have been observed in women suffering from hypothalamic amenorrhea, when given exogenous leptin.

Whilst leptin provides a means of restoring reproductive function in women suffering from hypothalamic amenorrhea, due to low body weight, it is known that administration of leptin may have adverse effects. For example, as a large protein hormone, continued administration may lead to the production of antibodies. Further, leptin also has peripheral effects on a range of tissues/organs, such as the ovary, so effects of parenteral administration are not specifically related to the neuroendocrine control of reproduction. In addition, leptin is a relatively large polypeptide and is therefore expensive to produce in commercial quantities (eg by recombinant technology).

In addition to leptin, there has been a number of hormonal and other treatments proposed for restoring reproductive function in women. For example, in the case of hypothalamic amenorrhea, one proposed treatment involves the subject wearing a pump that delivers GnRH in a pulsatile mode. Clearly this is quite cumbersome and there are also attendant problems of protracted infusion (eg infection). Accordingly, there remains a need for identifying improved and/or alternative methods for restoring reproductive function in reproductively impaired women.
SUMMARY OF THE INVENTION

The present invention is based on the surprising finding that the administration of an agonist of a melanocortin receptor can restore or enhance reproductive function in animals of low body weight, particularly in such animals showing reduced gonadotropin secretion.

Thus, in a first aspect, the present invention provides a method of restoring or enhancing reproductive function in a subject that is reproductively impaired, the method comprising administering to said subject an melanocortin-3 and/or melanocortin-4 receptor (ie MC3-R and/or MC4-R) agonist in an amount sufficient to restore or enhance reproductive function.

In a second aspect, the present invention provides the use of an MC3-R and/or MC4-R agonist in the preparation of a medicament for restoring or enhancing reproductive function in a subject that is reproductively impaired.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the experimental design for the Experiment described in Example 1.

Figure 2 shows the effect of the non-receptor specific melanocortin agonist, MTII, on the secretion of LH in OVX ewes of low body weight.

Figure 3 shows the LH secretory profile of OVX ewes of low body weight receiving either aCSF or MTII.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of restoring or enhancing the reproductive function in a subject that is reproductively impaired, the method comprising administering to said subject an MC3-R and/or MC4-R agonist to the subject in an amount sufficient to restore or enhance reproductive function.

The present invention is particularly suitable for women that are reproductively impaired, but is also suitable for reproductively impaired men. As indicated above, men also secrete luteinising hormone (LH). LH stimulates testosterone production in the Leydig cells in the testes of men, where spermatogenesis takes place, and testosterone is also necessary for libido and secondary sexual characteristics such as muscle bulk. An increased serum LH level in men markedly increases intratesticular testosterone levels, which, in combination
with an increased serum FSH level, enhances sperm production. In men with low reproductive function as a result of low testosterone levels, it is anticipated that administration of an MC3-R and/or MC4-R agonist will result in an increase in testosterone production and subsequent restoration or enhancement of reproductive function.

Further, it is anticipated that the present invention may also be useful in restoring or enhancing reproductive function in male and female non-human animals. Thus, as used herein, the term "subject" also extends to non-human animals and particularly, non-human mammals such as companion animals (eg dogs, cats and the like) and livestock (eg horses, cows, sheep and the like).

Reproductively impaired subjects which can be treated with the present invention include infertile and sterile subjects.

Reproductively impaired women who can be treated with the present invention will typically suffer from a deficiency in the hormonal system that supports ovulation, conception and/or maintenance of pregnancy. Reproductively impaired men who can be treated with the present invention will typically suffer from a deficiency in the hormonal system that renders the subject incapable of impregnating women. The hormonal deficiency suffered by these male and female subjects will typically occur in the gonado-hypothalamic-pituitary axis resulting from dysfunction within the brain to reduce the secretion of GnRH.

Preferably, subjects treated with the present invention will be reproductively impaired through chronic malnutrition, hypogonadotropic hypogonadism or excessive exercise, although the present invention generally has utility for any form of idiopathic hypothalamic hypogonadism.

The melanocortin receptors are members of the G-protein coupled receptor class, which display seven transmembrane regions as a hallmark, and interact with a group of peptides (ie "melanocortin peptides") all of which are derived from the processing of pro-opiomelanocortin (POMC) by the converting enzymes pro-convertase 1 and 2 as well as carboxypeptidase E. These processed peptides include adrenocorticotropic hormone (ACTH), alpha melanocyte-stimulating hormone (α-MSH), β-MSH, γ-MSH, β-lipotrophin
and the opioid, β-endorphin. To date, five melanocortin receptors, referred to as MC1-R, MC2-R, MC3-R, MC4-R and MC5-R, have been identified.

MC1-R has been found predominantly in skin, and when stimulated, it acts to regulate pigmentation and reduce a number of inflammatory processes (Schioth, 2001). MC2-R is present in the adrenal gland and has a high affinity for ACTH and will not bind to other melanocortin peptides (Schioth, et al 1996). α-MSH acts upon MC3-R and MC4-R which are both predominantly expressed within the central nervous system. They exist in various regions and nuclei of the brain. MC4-R, however, is found only in the central nervous system, and its distribution is varied and overlapping in areas including the cortex, brainstem, spinal cord, and hypothalamus. Within the hypothalamus of the sheep, both the MC3-R and MC4-R can be found in the medial pre-optic area, paraventricular nucleus, dorsal medial hypothalamus, ventral medial hypothalamus and the arcuate nucleus (Iqbal, et al 2001). MC5-R is expressed in peripheral tissue and while it has been linked to exocrine gland function, there is yet to be any function of it linked to metabolic homeostasis.

As used herein, the term "MC3-R and/or MC4-R agonist" refers to any agent which is capable of interacting with and activating a melanocortin-3 and/or melanocortin-4 receptor. The term therefore encompasses, inter alia, the natural melanocortin peptides which interact with either or both of these receptors (eg α-MSH) and agents which are analogues thereof. The MC3-R/MC4-R agonist used in the present invention include those that may restore or enhance serum LH levels to within a range considered normal for a healthy subject. It will be appreciated that the normal range for a healthy subject will vary depending on the age and/or sex of the individual, the stage of the menstrual cycle for females, whether the female is pregnant or postmenopausal, or whether the female is taking/being administered with contraceptives (see Table 1).

Preferably, the MC3-R/MC4-R agonist is an α-MSH, β-MSH or γ-MSH analogue, and particularly an MC3-R/MC4-R agonist comprises the amino acid sequence:

His-D-Phe-Arg-Trp (SEQ ID NO:1).

More preferably, MC3-R/MC4-R agonist is an cyclic α-MSH analogue.
Even more preferably, the MC3-R/MC4-R agonist is the cyclic α-MSH analogue known as melanotan II (MTII) having the amino acid sequence:

Ac-Nle-Asp-His-D-Phe-Arg-Trp-Lys-NH₂ (amide bridge: Asp3-Lys8)(SEQ ID NO:2).

or a related peptide or peptide-mimetic showing at least 75%, and more preferably at least 85%, amino acid sequence identity. Examples of such related peptides or peptide-mimetics are provided by US Patent No 6,613,874, WO01/00224, WO01/13112, WO03/006620 and WO02/064091 (the entire disclosures of which are to be regarded as incorporated herein by reference). Particularly suitable related peptides and peptide-mimetics are those incorporating one or more conservative amino acid substitutions of the amino acid sequence shown as SEQ ID NO:2. Particular conservative amino acid substitutions envisaged are: Nle for Met, Val, Ile, Leu, Phe or Ala; Asp for Glu, Asn or Glh; His for Lys or Arg; D-Phe for L-Phe; Arg for Lys or His; and Lys for Arg or His. In a further embodiment, the peptide of SEQ ID NO:2 has a terminal –OH at the carboxy terminus to provide:

Ac-Nle-cyclo(-Asp-His-D-Phe-Arg-Trp-Lys)-OH (SEQ ID NO:3).

Preferably, the MC3-R/MC4-R agonist is administered in an amount sufficient to restore the level of luteinising hormone (LH) to within a normal reference range (see Table 1). For women, the normal range will typically vary depending upon whether the particular subject is pre-pubertal, menopausal, or whether the subject is in the follicular, mid-cycle or luteal phase of the menstrual cycle. For men, the normal range will typically vary depending upon whether or not the particular subject is pre-pubertal.
Table 1 shows normal reference ranges for
serum LH

<table>
<thead>
<tr>
<th>Age</th>
<th>Male (mIU/mL)</th>
<th>Female (mIU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-23 mo</td>
<td>0.5-1.9</td>
<td>0.0-0.5</td>
</tr>
<tr>
<td>2-10 y</td>
<td>0.0-0.5</td>
<td>0.0-0.5</td>
</tr>
<tr>
<td>11-20 y</td>
<td>0.5-5.3</td>
<td>0.5-9.0</td>
</tr>
<tr>
<td>20-70 y</td>
<td>1.5-9.3</td>
<td>0.0-76.3</td>
</tr>
<tr>
<td>70-100 y</td>
<td>3.1-34.6</td>
<td>5.0-52.3</td>
</tr>
</tbody>
</table>

Follicular: 1.9-12.5
Midcycle: 8.7-76.3
Luteal: 0.5-16.9
Pregnant: 0.0-1.5
Postmenopausal: 5.0-52.3
Contraceptives: 0.7-5.6

The MC3-R/MC4-R agonist may be administered by routes of administration conventionally used for the delivery of drugs including oral, intranasal, transdermal, subcutaneous, intradermal, intravenous, intramuscular, and intraperitoneal administration.

The MC3-R/MC4-R agonist may be administered in any suitable form including injectable formulations, tablets, suspensions, implants, solutions, emulsions, capsules, powders, syrups and water compositions. The MC3-R/MC4-R agonist may also be administered with any pharmaceutically acceptable (e.g., a buffered aqueous carrier, preferably a saline or citrate buffered carrier, vehicle, adjuvant, additive or diluent).

The present invention therefore also provides the use of an MC3-R/MC4-R agonist in the preparation of a medicament for restoring or enhancing reproductive function in a subject that is reproductively impaired.

The specific dose amount of an MC3-R/MC4-R agonist administered in accordance with this invention will, of course, be determined by the particular circumstances surrounding the case including, for example, the type of MC3-R/MC4-R agonist to be administered, the route of administration, the state of being of the subject, and the severity of the reproductive impairment being treated.
In a preferred embodiment, the MC3-R/MC4-R agonist is administered at a dose of about 0.5 μg/kg of body weight to about 75 μg/kg of body weight.

In a preferred treatment regimen, a subject is administered multiple doses of a MC3-R/MC4-R agonist over a period of days or weeks. Administration of the MC3-R/MC4-R agonist to the subject can also preferably be a controlled release delivery over a period of days or weeks.

The treatment may be continuous over a period of preferably about 1 to 2 weeks.

In order to achieve a continuous treatment, the MC3-R/MC4-R agonist is preferably formulated for controlled release of the agonist (eg a depot administration) or for sustained release of the agonist. Preferably, the MC3-R/MC4-R agonist is formulated in a long-acting form having a relatively long plasma half-life.

The delivery of MC3-R/MC4-R agonist in small continuous doses is preferred. The advantage of such a continuous treatment is that long term treatment of a reproductive impaired subject is possible and controlled continuous treatment reduces the incidence of side effects in the subject. Therefore, the MC3-R/MC4-R agonist may be formulated in an osmotic dosage form that can be delivered at a controlled and continuous rate over time.

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following non-limiting examples.

**EXAMPLE 1**

**Materials and Methods**

**Animals**

The experiments used Corriedale ewes weighing between 50-60kg (n= 4) and 30-40kg, considered lean (n=13) and were conducted at the applicant's animal research facility located at Werribee in Victoria, Australia (38° Latitude). Animals weighing between 50-60kg were fed lucerne chaff and water *ad libitum* but were otherwise kept at pasture. Lean animals of weight 30-40kg were restricted to a low feed diet and were fed an average of 500g of lucerne chaff/hay per day which was fed in a feed lot. During the blood sampling
period, all animals were kept in individual pens and exposed to continuous light, but were otherwise were kept outdoors. All animals were conditioned to housing pens one night prior to experimental sampling.

Surgery

Each sheep was first ovariectomised under sterile conditions at least two weeks prior to lateral ventricular surgery. They were fasted 24 hours prior to both surgical procedures.

Surgical preparation of Animals

Surgical procedures were all carried out under a general anaesthetic. Each ewe initially received an intravenous injection of Bothamal (7.0ml Pentothal; Pharm Tech Pty Ltd, West Pymble, Sydney, NSW, Australia) by needle puncture into the jugular vein in order to induce general anaesthesia. An endotracheal tube was then introduced in order to allow the animal to breathe freely without any obstruction to airflow and to allow inhalation of anaesthesia. Each ewe then received a 5ml intramuscular antibiotic injection of IIium Oxytet (Troy Laboratories Pty Limited, Smithfield, NSW, Australia), along with an intravenous injection of Rimadyl (Carprofen 50mg/ml, 1ml/12.5kg bodyweight; Pfizer Animal Health, West Ryde, NSW, Australia) by needle puncture in the jugular vein which acts as a non-steroidal anti-inflammatory drug. Following this, each animal was then placed on an operating table and restrained with adjustable leg ropes. A custom made wine box, with vinyl covering and bean bag filling was used as a head cushion rest. This custom box was placed at the front of the surgical table to provide proper head support to the animals and was fastened down to the surgical table with Tawel clamps. Once securely fastened to the surgical table, each animal received a mixture of 3-5% gaseous halothane (Rhone Merieux Australia, West Footscray, Vic, Australia) and artificial air, to maintain the anaesthesia for the duration of the surgical procedure. The wool surrounding the surgical sites at the head and neck were clipped and cleaned using Betadine Surgical Scrub (7.5% w/v PVP– iodine surgical scrub, Apex Laboratories Pty Ltd., Somersby, NSW, Australia) and 70% ethanol solution (Yarraville Distillery, Yarraville, Vic, Australia). The animal was then covered in surgical drapes leaving only the surgical site exposed. A subcutaneous injection of 10ml of Adrenaline (0.25ml of 1:1000 adrenaline in 100ml of Saline; AstraZeneca Pty Ltd., North Ryde, NSW, Australia) was injected under the skin of the head to restrict blood flow and help reduce bleeding prior to commencement of
surgery. Following placement of lateral ventricular cannula, the surgical site was sprayed with an antibiotic, Terramycin (Pinkeye aerosol, oxytetracycline hydrochloride 2.0mg/g; Pfizer Animal Health, West Ryde, NSW, Australia).

**Ovariectomy**

Ovariectomies were performed at least 6 weeks prior to the commencement of the experiment under standard sterile conditions and general anaesthetic. A midline abdominal incision was made next to the midline and blind dissection was used to part muscle and fat layers. Surgery commenced with a midline incision made in the peritoneum. The uterus was located and the ovarian blood vessels were clamped and ligated just below the ovary. The ovary above the clamp was exteriorised and removed with the use of a scalpel. The peritoneum and external skin layers were sutured. Bleeding was controlled with electro cauterisation.

**Lateral Ventricular surgery for cannula placement**

Once anaesthesia was induced and the surgical site was cleaned, a midline incision was made on top of the sheep’s head between the two poles and was cut rostrally for 4-5 cm. Electro cauterisation was employed for hemostasis. With the bone visible, a periosteal elevator was used to clear the periosteum from the skull. Using a surgical drill, a hole was made 1 cm latero-caudal of the bregma. A second hole was drilled 3 cm rostral of the bregma and a jig-post screwed in. Silastic Laboratory tubing (Dow Corning Corporation, Midland, USA) (0.025 in diameter) was cut to a length of 250mm. A guide tube with obturator was connected to surgical jig in order to guide LV tubing in to the ventricle. The silastic tubing was introduced in 18mm from dura and it was observed to see whether CSF would flow out. The cannula guide tube was then carefully removed to leave LV line in position. The drapes were then held back with the use of clips and 2ml of radio-opaque dye (Omniopaque 300mg/ml, Nycomed Australia, Pty, Ltd, Chatswood, NSW, Australia) was injected into the line and the lateral ventricle. An X-Ray cassette was placed at the opposite lateral side of the ewes head than the X-Ray machine (Atomscope HF200) which was moved into position and a lateral X-ray was taken. Once the X-ray had developed, the exact positioning of the LV line was visualised and determined whether or not it needed further readjustment. A second X-Ray was then taken to confirm its position after the dye had cleared from the LV (5 minutes). Next small amounts of subcutaneous tissue were cut
and glued using superglue over the top of the cannula. Small amounts of Gelfoam (Pharmacia & Upjohn, Kalamazoo, USA), a sterile absorbable gelatine sponge were also placed and glued at the site of the LV line and the bone. A suture was used to stitch up the dura (wax coated Softsilk braided silk 30' 75cm Black coated), and again to stitch up the skin (wax coated Softsilk braided silk 18' 45cm black coated) with the use of arthrotic needle taking care not to remove or alter the position of the cannula.

Post-Operative Care

Following surgery, the animals were placed in a small paddock and were carefully monitored over a period of 24 hours. They were then released into a larger paddock and their well being was monitored daily, along with the surgical site for signs of infection. No infections were found.

Venous cannulation

One day prior to experiment, one external jugular vein of each ewe was catheterised. This procedure was achieved by holding the ewe in a sitting position and clipping the wool over the jugular vein. This area was then disinfected with 70% ethanol (Yarraville Distillery, Yarraville, Vic, Australia) and a local anaesthetic, Xylocaine (lignocaine hydrochloride 12.32mg; Astra Pharmaceuticals Pty Ltd, N. Ryde, NSW, Australia). This was sprayed over the jugular vein and a small skin incision was then made with a scalpel blade. A cannula (12G x 9cm, Dwellcath, Tuta Laboratories, Lane Cove, NSW, Australia) was inserted into the jugular vein and stitched to the skin using silk suture. The catheter was then flushed with heparinized sterile saline (100 units/ml) and closed with a three-way stopcock (Connecta, Becton Dickinson, Helsringborg, Sweden).

Preparation and insertion of infusion needles

One day prior to the experiment, 0.85m of single lumen polyethylene tubing was connected to a 19 gauge infusion needle. A piece of stainless steel tubing (23 gauge) was connected at the other end of each polyethylene tubing line. The tubing lines and needles had been preconditioned and sterilised with 70% ethanol (Yarraville Distillery, Yarraville, Vic, Australia) and UV light exposure the previous night. Once sterilised, each tubing line was connected to a plastic 1ml syringe at the 19 gauge infusion needle, and then fitted with either artificial CSF or the treatment drug. The syringe was placed in a battery operated
mini pump (Graseby MS 16A infusion pump, Graseby Medical Ltd., Gold Coast, Australia) which were set to infuse at a rate of 110 µl per hour. The pumps were secured to the side of the pen in a plastic case.

The polyethylene tubing line was fastened to the wool of the ewe with elastic bands and leucoplast tape in order to ensure that no loose tubing could tear off. Finally the tubing was connected to the LV line. This was achieved through removal of a 20 gauge 1cm blunt end already connected to the LV line, and replacing it with the 23 gauge stainless steel tubing from the polyethylene tubing line. The connection was then fastened with leucoplast tape.

*Artificial cerebrospinal fluid and drug treatment solutions*

The drug treatments were all diluted in artificial CSF (aCSF: 150mM NaCl, 1.2mM, CaCl2, 1mM MgCl2, 2.8mM KCL) to ensure solution availability throughout the infusion period.

*Procedure for Experiment*

This experiment involved the use of 6 low body weight ewes and was of a cross over design. This involved sampling in week 1 with 3 ewes receiving MTII and 3 ewes receiving artificial aCSF. Following this in week 2, those ewes that were previously infused with artificial CSF then received MTII and vice versa. The experiment design is shown in Figure 1.

*Blood sampling*

Blood sampling for the experiments was carried out as follows.

Every 10 minutes, 4ml of jugular venous blood was collected via the indwelling catheters connected to manometer lines. The blood samples were drawn through the catheter using a syringe attached to a three-way stopcock. Heparinised saline was initially removed from the manometer line by drawing back approximately 5ml into the flushing syringe. A blood sample was then taken with the sample syringe and the sampling line was refilled with heparinised saline (50 units/ml). The blood samples were placed into a collection tube containing the anticoagulant lithium heparin (Sarstedt Australia, Technology Park, SA).
Blood tubes were centrifuged at 4°C for 10 minutes at a rate of 300rpm. The plasma was then poured into storage tubes and stored at -20°C for later use in hormone assays.

During the sampling period, the experiments involved blood sampling over two treatment days. Blood sampling commenced at 0900 hours. Samples of 4ml were taken every 10 minutes. At 1200 hours infusion pumps were turned on and sampling continued until the conclusion of the experiment at 1500.

Radioimmunoassay

Levels of Luteinising Hormone (LH) in plasma samples were determined using the radioimmunoassay protocol described by Lee, et al (1976). All samples were assayed in duplicate. The LH assay was a 3 day procedure. Included in the assay were the following tubes, "total counts", used to measure tracer activity, "non-specific binding" and "zero binding" tubes and "four quality control" tubes each with differing LH concentrations. For each assay, the concentration of LH in the quality controls fell within the expected range for this assay. All samples were assayed in 100μl duplicates. The sensitivity of the assays was 0.16ng/ml and the intra-assay coefficient of variation was less than 10% over the range of 2.0 – 9.9 ng/ml.

Day one of the assay consisted of adding 200μl of assay buffer (0.5 % egg white in 0.02M PBS, pH 7.4) to each of the quality control, zero binding and sample tubes. Each ovine LH "standard tube" (NIH-S18-oLH, with concentrations of 0.5, 1, 2, 3, 5, 10, 20, 50 ng/ml) received 100μl of buffer and 100μl of Hypophysectomised sheep plasma to make up a standard curve of samples. To the sample tubes, 100μl of the plasma sample was added.

In order to prepare an oLH antibody solution for addition to sample tubes, a 100μl aliquot of oLH antibody (NIDDK-anti-oLH-I) was diluted to a concentration of 1:700 000 through the addition of 70ml of normal rabbit serum 1:2000, (1.89% EDTA in 0.02M PMS pH 7.4). 200μl of this solution was then added to all tubes with the exception of the non-specific binding and total count tubes. In order to correct the volume for the non-specific binding tubes, 200μl of normal rabbit goat serum was added. In order to produce LH radioactive tracer, ovine LH (NIH-NIDDK, AFP8614B) was iodinated using idogen 1,3,4,6-tetrachloro-3α,6α-diphenylglcouril (Sigma St.Louis, MO, USA) to label the purified oLH with $^{125}$I (NEN Life Science Products, Boston, Massachusetts, USA).
according to the protocol by Salacinski PR et al (1981). This iodinated hormone was stored in a concentrated form at 4°C and diluted as tracer in buffer when needed. The tracer was prepared through the dilution of 5μl of ¹²⁵I-oLH in 20ml of assay buffer to give an activity of approximately 15 000 counts per minute per 100μl. 100μl of tracer was added to each tube. Once addition of all appropriate reagents was complete, every tube was vortexed to ensure proper mixture of the reagents. The tubes were then covered with a layer of foil and incubated for 20-24 hours at 32°C. The second day of the assay (or 14 hours following incubation), involved the addition of 200μl of secondary goat anti-rabbit antibody (Werribee GAR24/2, 24/1). This solution was made up to 200ml, with 0.02M PBS. This was added to all tubes except for the total counts tubes. All tubes were then vortexed again and incubated for 20-24 hours at 32°C with foil covering all tubes.

The third day of the assay required all tubes except for the total count tubes to be centrifuged for 30 minutes at rate of 3000 rpm to allow for the separation of the bound and unbound hormone. Following this, the tubes were immediately placed on ice and the supernatant aspirated. Every tube was then counted for 2 minutes using a Gamma counter containing the Multicalc® operation (1470 Wizard; Wallac Oy, Finland). The data of counts detected by the counter was then computed to levels of LH in ng/ml with the use of an in-house RIA program by Lee, et al (1976).

Statistical analysis

Hormonal levels of LH were characterised by the pulse amplitude, the pulse frequency, the mean baseline concentration, the response of 1st pulse and the mean concentration. These characterisations were based on previous secretory profile of LH described by Scott CJ et al (1992). A pulse was defined as having occurred when the assay value of a given sample exceeded the assay value of the previous sample by at least three times the standard deviation (SD) of the previous sample. The baseline concentration was the lowest hormone value preceding the consistent rise in sample concentration. The pulse amplitude was calculated as the difference between the pulse peak and the baseline concentration and the inter-pulse interval was the average time (min) in minutes, between two successive peaks. The corresponding means placed under statistical analysis, which consisted of a repeated measures analysis of variance using SPSS (SPSS Inc., Chicago, Illinois). The between-subjects factor was the type of treatment the ewe received and the within subjects
factor was the time that the sampling took place, pre or post treatment. The pulse frequency was testing using a non parametric chi squared test.

Results

The effect of the non-receptor specific melanocortin agonist, MTII, on the secretion of LH in OVX ewes of low body weight is shown in Figure 2 where central infusion of MTII resulted in significant (p < 0.05) increases in the secretion of LH. This increase in secretion is due to an elevation in the mean (± SEM) plasma concentration (p < 0.041), the mean (± SEM) baseline concentration (p < 0.03), the response of first pulse with infusion (p < 0.03) and the mean LH pulse amplitude (p < 0.036). No effect was seen in OVX ewes of low body weight receiving aCSF (Figure 2).

An example of the LH (ng/ml) secretory profile of a control ewe (C-736, #265) receiving aCSF and a treatment ewe (C-736, #363) receiving MTII is shown in Figure 3, both ewes are OVX ewes of low bodyweight. The first three hours of blood sampling represent the pre-treatment period whereas the last three hours of blood sampling represent the post-treatment period. Treatment with MTII showed a clear increase in LH in the post-treatment period.

Discussion

The results described above demonstrate that the melanocortin system is involved in the hypothalamic mechanism responsible for the inactivation of the reproductive axis in the lean ovariectomised ewe. Without being limited by theory, the applicants consider that a decreased stimulatory tone on GnRH neurons by the melanocortin system may be responsible for the hypogonadotropic hypogonadism seen in the lean ovariectomised ewe.
Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

All publications mentioned in this specification are herein incorporated by reference. Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia or elsewhere before the priority date of each claim of this application.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.
REFERENCES


CLAIMS

1. A method of restoring or enhancing reproductive function in a subject that is reproductively impaired, the method comprising administering to said subject an melanocortin-3 (MC3-R) and/or melanocortin-4 receptor (MC4-R) agonist in an amount sufficient to restore or enhance reproductive function.

2. A method according to claim 1, wherein the subject is of low body weight.

3. A method according to claim 1 or 2, wherein the subject shows reduced gonadotropin secretion prior to treatment.

4. A method according to any one of claims 1 to 3, wherein the agonist is administered in an amount sufficient to restore the level of luteinising hormone to within the normal range for the subject's sex, age and reproductive cycle.

5. A method according to any one of claims 1 to 4, wherein the subject is a female.

6. A method according to any one of claims 1 to 5, wherein the MC3-R/MC4-R agonist is administered to the subject in multiple doses over a period of 1 to 2 weeks.

7. A method according to any one of claims 1 to 6, wherein administration of the MC3-R/MC4-R agonist to the subject is by controlled release delivery over a period of 1 to 2 weeks.

8. A method according to any one of claims 1 to 7, wherein the MC3-R/MC4-R agonist is administered at a dose of about 0.5 µg/kg of body weight to about 75 µg/kg of body weight.

9. A method according to any one of claims 1 to 8, wherein the MC3-R and/or MC4-R agonist is selected from the group consisting of α-MSH, β-MSH, γ-MSH and analogues thereof.

10. A method according to any one of claims 1 to 8, wherein the MC3-R and/or MC4-R agonist is α-MSH or an analogue thereof.
11. A method according to any one of claims 1 to 8, wherein the MC3-R and/or MC4-R agonist comprises a cyclic α-MSH or analogue thereof.

12. A method according to any one of claims 1 to 8, wherein the MC3-R and/or MC4-R agonist comprises the amino acid sequence of His-D-Phe-Arg-Trp (SEQ ID NO:1).

13. A method according to any one of claims 1 to 8, wherein the MC3-R and/or MC4-R agonist comprises:

   (i) an amino acid sequence of Ac-Nle-Asp-His-D-Phe-Arg-Trp-Lys-NH₂ (amide bridge: Asp3-Lys8)(SEQ ID NO:2);
   (ii) an amino acid sequence having at least 75% amino acid identity to SEQ ID NO:2;
   (iii) an amino acid sequence having at least 85%, amino acid identity to SEQ ID NO:2; or
   (iv) the amino acid sequence of Ac-Nle-cyclo(-Asp-His-D-Phe-Arg-Trp-Lys)-OH (SEQ ID NO:3).

14. A method according to any one of claims 1 to 13, wherein the subject is reproductively impaired due to one or more of sterility, GnRH hormonal dysfunction, chronic malnutrition, hypogonadotropic hypogonadism, excessive exercise, hypothalamic amenorrhea and idiopathic hypothalamic hypogonadism.

15. Use of an MC3-R and/or MC4-R agonist in the preparation of a medicament for restoring or enhancing reproductive function in a subject that is reproductively impaired.
FIGURE 1

WEEK 1

aCSF

MTII

WEEK 2

aCSF

MTII
Treatment: aCSF
1.

SEQUENCE LISTING

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# INTERNATIONAL SEARCH REPORT

## A. CLASSIFICATION OF SUBJECT MATTER

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According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, DWPI. Keywords: melanocortin, MC3-R, MC4-R, melanotan H, reproduce+, fertile+, α-MSH, β-MSH, γ-MSH

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of Box C  [X] See patent family annex

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

Document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Document member of the same patent family

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Date of mailing of the international search report: 16 JAN 2006

Mailing address of the ISA/AU:

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PCT/ISA/210 (second sheet) (April 2005)
## INTERNATIONAL SEARCH REPORT

**DOCUMENTS CONSIDERED TO BE RELEVANT**

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# INTERNATIONAL SEARCH REPORT

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

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