Neohormones in Milk

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Abstract (150 words)

Neohormone systems evolved specifically to regulate those mammalian traits, such as internal fertilization, pregnancy and lactation, which have proved to be central to the success, environmental independence, and adaptability of mammals as a vertebrate group. Neohormones such as oxytocin or relaxin are not only involved in the regulation of mammary gland development and function, but are also significant components of milk itself. Particularly for the latter hormone, it has been shown for the pig that relaxin in the first milk is taken up by the gastrointestinal tract of the offspring, enters the neonatal circulation and can have specific physiological and epigenetic effects on target organs such as the female reproductive system. Nevertheless, there are large gaps in our knowledge and understanding of such lactocrine systems especially in regard to other neohormones, species, and neonatal organ systems.

Introduction

The evolutionary success of mammals has been largely due to the prolongation of early development within a physical environment which can be controlled to a large degree by the parent(s). For mammals this involved retention of the zygote, early embryo, and foetus within the mother until an advanced stage of development, when most organ systems are established and the resulting offspring is more or less independent. To achieve this, a series of sophisticated and interconnected evolutionary steps were essential (Table 1). Fertilization became internal, mostly within the oviduct. This in turn required the male to develop an intromittent organ, the penis, and more importantly behavioural changes to signal when an oocyte was mature and about to be released from an ovarian follicle. On the male side, there was a requirement for sperm storage within the epididymis, for behavioural modifications to ensure sexual congress at the appropriate time, and mechanisms to maintain sperm quiescence until ejaculation and then activation within the female tract. This in turn involved the evolution of exteriorized testes within a scrotum, presumably to allow sperm storage within the epididymis at a lower than abdominal temperature, such that upon ejaculation into the female tract the sperm are subject to a temperature jump of about 5^{o}C, inducing capacitation. On the female side, there was requirement for intimate association of the growing embryo with the mother in the form of placentation, and importantly processes for physiological adjustment by the mother to recognize that she was pregnant (maternal recognition of pregnancy), accommodation of massively increased fluid volume relationships (osmotic adjustment), and immunological adjustment so that the genetically heterozygous embryo is not rejected by the maternal immune system. There needed to be a

mechanism regulating when the foetus was sufficiently developed to be expelled from the uterus, as well as all of the physiological mechanisms (birth contractions) required for this and its immediate sequelae (birth of the placenta; involution of the uterus and prevention of excessive bleeding). Finally, all of these processes required the accompaniment of highly developed behavioural adaptations to maximize survival of mother and offspring.

Subsequent to pregnancy and birth, female mammals additionally added a further adaptation to offspring survival, namely the development of a mammary gland, lactation, and the provision of milk in response to birth and suckling.

Besides the enormous organogenetic adaptations just mentioned, all of these also required highly coordinated endocrine and paracrine mechanisms for their induction and regulation. Perhaps surprisingly, the endocrine responsibility for these mammalian-specific physiological enhancements resides mostly within a relatively small group of hormones, termed ‘neohormones’ (1,2). These hormones effectively superimpose their functions onto those well-established endocrine systems, such as the hypothalamo-pituitary-gonadal (HPG) axis, already present in reptiles and lower vertebrates. The best known of the neohormones are those such as human chorionic gonadotropin (hCG) and oxytocin. But also members of the relaxin family of small peptide hormones are essentially involved in neohormone functions. These include besides relaxin (H2-relaxin) itself, also the structurally related molecules H1-relaxin, insulin-like peptide 3 (INSL3), INSL4, and INSL6, all of which are encoded by separate genes within the human genome.

Besides for oxytocin and relaxin, very little is known about their roles in the context of lactation. This is also surprising given that lactation can be seen as a logical continuation of internal fertilization, pregnancy and birth, wherein all neohormones by definition appear to be involved. Part of the reason for this lack of information is that until recently, there have been no specific assays with which to measure the presence of the relaxin family of peptide hormones in milk, and the fact that these peptides exhibit a large degree of species-specific variation, which mostly precludes the application of an assay from one species to another. Secondly, for some of these molecules, they may have a well-established classic role in a different physiological context, which has effectively blinded the scientist to look elsewhere. In this review, we shall critically assess what is known about the expression and role of these neohormone peptides specifically in the context of milk and lactation.

Oxytocin

Of all the neohormones, the nonapeptide hormone oxytocin is perhaps the most associated with lactation, being responsible for the milk let-down reflex during suckling. Pulsatile oxytocin is released from the posterior pituitary in response to vagal stimulation following infant suckling, and leads to contraction of the myoepithelial cells surrounding the mammary alveoli. This aspect has been adequately covered in other reviews (e.g. 3) and will not be discussed further here. What is less known is that oxytocin can be present in human milk at concentrations of between 0.01 and 18.9 ng/mL (4,5) and that oxytocin injected into post-parturient sows appears to increase leakiness of the alveolar tight junctions thereby increasing their permeability to serum factors (6), including to oxytocin itself. In breast-feeding

women, nursing significantly increases the concentration of OT in the milk (4). In cows, where milk yield may be enhanced by injections of oxytocin, concentrations of between 10 and 15 pg/mL are reported even after pasteurization (7), confirming what has been suggested elsewhere that oxytocin is quite stable in milk. There is almost no information on the impact in the neonate of oxytocin ingested with milk. One study in young rats (PND10-PND35) showed that oxytocin delivered by gavage could impact both body weight and ovarian physiology (8). Moreover, recent studies treating newborn monkeys with nebulized oxytocin do suggest that oxytocin delivered to the newborn in the perinatal period can have significant and positive impacts on the brain and behaviour (9,10). Together, such results do imply that oxytocin in milk, if available to the newborn in sufficient concentration, might have a positive effect on behaviour and/or physiology.

Human chorionic gonadotrophin (hCG)

Human chorionic gonadotrophin (hCG) is a paralogue of the pituitary gonadotrophin Luteinizing Hormone (LH) and is normally associated only with the early embryo in pregnancy, being part of human maternal recognition of pregnancy. It is assumed that there is none left in the circulation at the end of pregnancy and during lactation. However, in one case study of a woman who became pregnant while still breast-feeding her previous child, hCG was indeed measured in her breast milk (512 pmol/L) compared to 200,000 pmol/L in serum (11). Thus even though hCG is a large dimeric glycoprotein, sufficient appears to enter the milk during lactation to be measurable. Moreover, hCG has a much longer biological half-life compared to its

paralogue LH. Whether there are any effects in the infant is not known, though this is a concentration which could potentially activate the specific LH/hCG receptors, for example, in the neonatal gonads.

Relaxin

Most information is available for the hormone relaxin in milk. For humans, where there are three relaxin genes, this is the H2-relaxin principally made in and secreted from the ovary. Relaxin is synthesized initially as a single chain 14-18kD bioactive pro-hormone and is usually further processed within the Golgi and secretory vesicles to give rise to the normal 6kD heterodimeric hormone with a structure very like that for insulin (12). Depending on the species, relaxin is made in larger or smaller quantities by the ovary and in particular by the corpus luteum of pregnancy, whence it is secreted into the bloodstream to yield serum concentrations, for example, in the pig or rat, as high as 100 ng/ml (13) or 150ng/ml (14), respectively. Other species have usually much less relaxin in the blood. Importantly, relaxin is also expressed within the mammary gland itself (human (15); guineapig (16)), with immunohistochemistry identifying it in the human in the epithelial cells of the ducts and alveoli (17). Moreover, application of a neutralizing antibody in the rat showed that in this species almost all the relaxin in milk derives locally from within the mammary gland (18).

There are numerous studies on the effects of relaxin on mammary gland development and physiology, acting via locally expressed specific relaxin receptors

(RXFP1) present in the parenchyma or on myoepithelial cells (19,20). However, studies using relaxin knockout mice emphasize that a key effect is less on gland development and milk production but rather on nipple enlargement (21) and in relaxin receptor deleted mice, there appears to be no evident mammary phenotype at all (22). This would tend to support the observations in animals (pigs, rats) where circulating relaxin alone has been eliminated (23,24). However, earlier studies in pigs emphasized that it is less relaxin on its own that is important for breast development, rather than when it acts synergistically with estrogens and/or progesterone (25).

Irrespective of its source within the mammary gland or elsewhere in the body, relaxin has been identified in the milk from dog (26), human (27), rat (18), pig (28) and alpaca (29), ranging in concentration from 0.1 to 50ng/ml depending on the species and on the time during lactation. In porcine milk, relaxin appears to be present mostly as the 18kD bioactive pro-form rather than as the more usual 6kD mature heterodimer (28,30); whether this is true also for other species is not known. Relaxin, however, cannot be found in milk from cows or sheep since in these species the gene encoding relaxin has been naturally deleted from their genomes (31). Where it has been measured, maximal relaxin concentration appears to occur in the first milk (colostrum), during the first 24-48 hours after birth, and declines progressively thereafter.

Importantly, at least in some species, there seems to be sufficient relaxin entering the stomachs of neonates in these first days of life for this to be able to cross the rather permeable gut lining and be present in physiologically relevant amounts in the neonate circulation (28). This has been especially shown for relaxin in the neonate

pig where, when nursed, these showed a circulating concentration of 180pg/ml, but undetectable relaxin when fed a milk-replacer or prior to suckling (28). Together such data support the “lactocrine hypothesis”, whereby milk presents important and developmentally relevant signalling molecules to the neonate in addition to fulfilling nutritional requirements (32). Numerous experiments confirm that naturally nursed animals support different developmental patterns of gene expression in the neonate compared to animals which have been fed with milk-replacer (33). Exogenously applied relaxin in replacer-fed pigs appears to decrease the expression specifically of the relaxin receptor RXFP1 in both uterus and cervix of the female neonates, thus impacting on the development of the female reproductive tract (34,35), and it is suggested that there is a similar impact also on early testicular development (33). In the pig at birth the uterus is developing rapidly, particularly in terms of the estrogen-dependent growth of glandular tissue. Relaxin and its receptor RXFP1 appear to be intimately involved in this process (36), explaining why an absence of relaxin in milk might have significant epigenetic consequences on uterine development. Other possible impacts of lactocrine relaxin in this or any other species have not been specifically investigated, though are likely.

INSL3 and other neohormones

Of the remaining neohormones of the relaxin family of peptides, only for insulin-like peptide 3 (INSL3) do we have any information about its involvement in mammary gland physiology. Like the closely related hormone relaxin, INSL3 is expressed both as mRNA and protein within the tubule-alveolar epithelial cells of human breast tissue and not in stromal cells (37). Therefore analogously to relaxin one would

expect INSL3 to be detectable also in milk, though to date this has not been looked at for any species. In regard to other neohormones such as INSL4 and INSL6, there is no information available and cursory examination of the GEO microarray database does not suggest significant gene expression within mammary tissues.

Research outlook

It is clear from this survey that we still know very little about the physiological function of neohormones in milk for any species. The pioneering work on relaxin in the pig strongly supports the validity of the lactocrine hypothesis whereby hormones particularly in the first neonatal milk have an important role to play in establishing the epigenetic developmental landscape in the newborn mammal (32,33). From an evolutionary perspective it is very likely that other neohormone members will have similarly important roles. We know that the neonatal period represents an important and sensitive developmental window when the young mammal is particularly prone to the disruptive effects of environmental endocrine-acting chemicals, some of which are transported from the mother via milk (38). To date only impacts of milk-borne relaxin on the neonatal development of the female reproductive system have been investigated. Given that hormones like relaxin might also have impacts on other physiological systems which are developing rapidly in the young mammal, such as the cardiovascular (39), musculo-skeletal (40), or immune (41) systems, it is likely that the epigenetic importance of lactocrine neohormones is still widely underestimated. Another aspect which will need exploring is the mode of secretion and transport to the neonate. Whilst neohormones are mostly conventionally

secreted through the regulated pathway into extracellular fluids, there is no reason why in milk an apocrine process might not be used, employing exosomes released from the mammary epithelium. Moreover, this might explain why relaxin in porcine milk is largely present as the unprocessed bioactive pro-form. Apocrine secretion offers the advantage that the enclosed molecules are better protected against enzymatic attack in the neonate gastrointestinal tract. Also their mode of absorption through the gastrointestinal tract will require study; for example, one cannot assume that in older animals there is no transport of such peptide hormones across the gut lining. In fact, it has been shown that there are mechanisms which even allow the transport of molecules like INSL3 across the blood-testis barrier (42), foetal membranes (43), and the placenta (44). It is to be hoped that this nascent field of research on lactocrine neohormones will soon grow considerably.

Summary

Neohormones and their specific receptors have evolved to address all of the novel physiological and behavioural mechanisms that were essential for the emergence of mammals from lower vertebrates. Such new systems include internal fertilization and pregnancy, the development of a scrotal testis, and lactation, in addition to behavioural changes. The key molecules known to be involved in such neohormone functions include oxytocin as well as the relaxin-like family of peptide hormones. Although milk production is a typical mammalian trait, role of milk as an endocrine medium has been only sporadically investigated. Oxytocin, hCG and relaxin have all been identified as physiologically relevant neohormones in milk from various

species. Especially for relaxin in the pig, it has been clearly demonstrated that milk-borne relaxin is taken up by the neonate and exerts significant developmental and epigenetic effects particularly on the neonate reproductive system. However, the field is characterized by large gaps in our knowledge about different species and target organs, and different hormones, and it is to be hoped that in the next years this deficit can in part be remedied.

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Table 1

Reproductive physiology and behaviour acquired with mammalian emergence and regulated by neohormone systems.

- Viviparity and uterine accommodation of the embryo (placentation)
- Internal fertilization and its coordination
- Appropriate male and female sexual behaviour
- Maternal recognition of pregnancy
- Adjustment of cardiovascular function, as well as electrolyte and fluid balance in pregnancy
- Adjustment of maternal immune tolerance to accommodate genetically different sperm and embryo
- Thermoregulation and a constant core temperature
- Regulation of the birth process and postnatal uterine involution and regeneration
- Perinatal analgesia
- Breast development and lactation
- Appropriate maternal behaviour
- Scrotal testes, testicular descent, and reduced scrotal temperature
- Post-testicular sperm maturation, storage and capacitation as an adaptation to internal fertilization
- Post-reproductive survival to provide extended care for offspring

Research Agenda

- More information is required on the levels of different neohormones in milk from a range of species, including human, and their ability to withstand processing treatments.

- Information is required on the time-course of neohormone expression in milk. whether in the first milk or after more prolonged lactation.

- There is need for details on the biochemical format of bioactive neohormones in milk and its impact on hormone half-life.

- What is the physiological and epigenetic relevance of neohormones in milk for the neonate, including information on mode of transport from the gastro-intestinal tract?

- What is the mode of production and/or secretion from the mammary gland for any species, whether simple or apocrine secretion, or whether made locally or sequestered from the blood?