ROLE OF CONNEXIN 43 IN GT1-7 CELL COMMUNICATION DURING EPISODIC GONADOTROPIN-RELEASING HORMONE (GnRH) SECRETION

Raghavaulu Deepthi Koppula

Role of connexin 43 in GT1-7 cell communication during episodic gonadotropin-releasing hormone (GnRH) secretion

A Thesis Presented for

The Master of Science Degree

The College of Graduate Studies

Austin Peay State University

Raghavaulu Deepthi Koppula

August 2014

August, 2014

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Gilbert Pitts, Ph.D., Advisor

Sarah Lundin-Schiller, Ph.D., Committee Member

Karen Meisch, Ph.D., Committee Member

Accepted for the Graduate and Research Council

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Role of connexin 43 in gap junction communication between GT1-7 Cells during episodic release of gonadotropin releasing hormone (GnRH)

By

Raghavulu Deepthi Koppula

Submitted to the Department of Biology, Austin Peay State University

On August 2014 in partial fulfillment of the requirements

for the Degree of Masters of Science in Biology

ABSTRACT

Gonadotropin releasing hormone (GnRH) is a neurohormone and is secreted in pulses from the hypothalamus. Pulsatile secretion of GnRH is necessary in establishing and maintaining normal function of reproductive organs. These cells are dispersed in the hypothalamus and surprisingly they all release GnRH at the same time. The ability of a population of individually pulsing neurons to collectively release distinct episodes of GnRH would require some type of coordination between individual secreting cells of the population. From the recent study, we found that a gap junction protein, ~~C~~connexin 43 is necessary for synchronized secretory activity in GT1-7 cell cultures which are immortalized GnRH neurons. In the present study, a fluorescence microscope~~y~~ was used to record the- calcium ion influx in adjacent cells in GT1-7 cells before and after application of -connexin 43 specific blocker, -Gap26. Cells treated with Gap26 showed a significant difference ($p<0.05$) in calcium ion signaling from non-treated cells.

Thesis Advisor: Gilbert R. Pitts, Ph.D.

Title: Professor of Biology

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Acknowledgement

The contributions of many different people, in their different ways, have made this possible. I would like to extend my appreciation especially to the following.

Foremost, I would like to express my sincere gratitude to my advisor Dr. Gilbert R. Pitts for the continuous support of my research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in my research and writing of this thesis. I could not have imagined having a better advisor and mentor for my master's degree.

A very special thank you goes out to Dr. Sarah Lundin-Schiller, who supported and encouraged me throughout my project. Without her help, I would not have completed my thesis. She is always there when I need any help.

Many thanks, to Dr. Karen Meisch, for serving on my committee and providing the continuous support.

I would like to thank my husband and parents for their unconditional support, both financially and emotionally throughout my degree. In particular, the patience and understanding shown by my husband during entire course is greatly appreciated.

Chapter 1: Introduction

Gonadotropin releasing hormone (GnRH) is the master molecule that controls reproduction in vertebrates. It is secreted in a pulsatile manner by -GnRH neurons located in the hypothalamus (Weiner, R.I.,*et al.,* 1992; Terasawa, E.,*et al.,* 1995). GnRH is carried by the hypophyseal portal system to the anterior pituitary where it stimulates the release of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Fink, 2000). They are secreted into the general circulation and attach to receptors on the ovary, where they trigger ovulation and stimulate ovarian production of the hormones estrogen and progesterone. These female hormones cause monthly menstrual cycling and have multiple effects throughout the body. In particular, estrogen has profound effects on the skeletal system and is crucial to maintaining normal bone health. The gonadotropins are also secreted in a pulsatile fashion (Levine *et al.,* 1982; Pohl *et al.,* 1983). GnRH pulses vary in amplitude and frequency in males and females. In males GnRH is secreted at a constant amplitude and frequency whereas, in females GnRH amplitude and frequency peaks during ovulation (Clarke *et al.,* 1987). In order to secrete GnRH in a pulsatile manner there should be some sort of communication between the neurons. Much progress has been made over recent years, to understand how GnRH neurons communicate (Campbell *et al.,* 2007; Christian *et al.,* 2010; Bose *et al.,* 2010). However, the role of gap junctions has yet to be elucidated. *In vitro* studies using immortalized GnRH-secreting cell lines have found evidence for gap junctions, comprised of multiple different connexins, to be critically involved in the episodic release of GnRH from these cultures (Charles *et al.,* 1996; Matesic *et al.,* 1996; Vazquez-Martinez *et al.,* 2001; Bose *et al.,* 2010).

During GnRH release in GT1-7 cells, calcium ion (Ca^{+2}) influx increases. The spontaneous pulsatility in GnRH release was abolished in calcium deficient medium and was markedly

attenuated in the presence of nifedipine, an antagonist of voltage-sensitive Ca^{+2} -channels. The basal intracellular Ca^{+2} level of perifused GT1-1 cells cultured on cover slips was also dose-dependently reduced by nifedipine. Conversely, depolarization with high K^+ increased intracellular Ca^{+2} and GnRH release in an extracellular Ca^{+2}-dependent and nifedipine-sensitive manner. The dihydropyridine Ca^{+2} channel agonist Bay K 8644 increased basal and K^+-induced elevations of intracellular Ca^{+2} concentration and GnRH secretion. These findings demonstrate that pulsatile neuropeptide secretion is an intrinsic property of GnRH neuronal networks and is dependent on voltage-sensitive Ca^{+2} influx for its maintenance (Krsmanović, *et al*, 1992). The confirmation that hypothalamic neurons possess intrinsic pulsatile activity occurred with the development of GT1 cultures (Mellon *et al.,* 1992). Taken together, these observations imply that GnRH neurons show intrinsic pulsatility.

The ability of a population of individually pulsing neurons to collectively release distinct episodes of GnRH into the portal circulation would require some type of coordination or synchronization between individual secreting cells of the population , as the GnRH neurons are distributed all over hypothalamus. Until recently, very little information was available concerning the synchronization of GnRH neurons, this was due mainly to the difficulty of studying these GnRH cells that are few in number and scattered throughout the hypothalamus (Kawano *et al.,* 1981). Vazquez-Martinez and co-workers (Vazquez-Martinez *et al.,* 2001) demonstrated that pulsatile function among GT1-7 cells becomes synchronized with time in culture. They used FM1–43 dye to monitor the membrane turnover associated with exocytosis in single GT1–7 neurons and found the Ca^{+2} ~~calcium~~ oscillations (frequency, 1.4 ± 0.1/h; pulse duration, 17.3 ± 0.6 min) during time in culture became progressively synchronized among

neighboring cells. Voltage-gated Ca^{+2} -channels and gap junctional communication each played a major role in synchronized pulsatility.

Using GT1-7 cells culture, Bose *et al.*, -(2010) investigated whether the gap junction proteins connexin43 (Cx43) and/or connexin26 (Cx26) are involved in the synchronization of secretory pulses from the GnRH neurons with time. Their results revealed that ~~siRNA targeting Cx43 abolished the pulsatile release of GnRH in cultures of immortalized GT1-7 neuronal cells (Bose *et al.*, 2010) which suggests that~~ Cx43 is involved in gap junction communication between GT1-7 cells~~. Microinjection of Cx43 siRNA into day-3 cultures abolished synchronized GnRH secretory activity at day 7; whereas, Cx26 siRNA administration did not alter secretary activity at day 7. This indicates that Cx43~~ siRNA ~~abolished GnRH secretory activity but Cx26~~ siRNA ~~does not. Through these findings they demonstrated that Cx43, but not Cx26 is necessary for synchronized secretory activity in these GT1-7 cultures and raised the possibility that Cx43-related gap junctions may be important in GnRH neuronal coordination in the hypothalamus~~ (Bose *et al.*, 2010).

The goal of my thesis was to determine if immortalized GnRH cells (GT1-7) remain synchronized after blockade of connexin 43 and to observe the difference in Ca+2 ~~calcium~~ oscillations in cells with and without blockade of connexin 43.

GnRH pulse synchronization can be observed indirectly by measuring the intracellular Ca^{+2} oscillations in the cells. GnRH release requires depolarization stimuli followed by Ca^{+2} entry through voltage-sensitive Ca^{+2} channels (Terasawa *et al.*, 1999). Individual GnRH cells exhibit Ca^{+2} oscillations, which synchronize at intervals of nearly 50 min (Krsmanovic~~ LZ., *et al.*,~~ 1992; Ei Terasawa *et al.*, 1999). Therefore, investigation on intracellular free Ca^{+2} levels before and after blocking Cx43 with a Cx43 specific blocker would provide critical information on

synchronizing activity of immortalized GnRH neurons. Fura-2 AM is a widely used UV-excitable fluorescent Ca^{+2} indicatore that is employed here ~~we are using for this study~~ to analyze the GT1-7 intracellular Ca^{+2} -levels using fluorescence microscope.~~y.~~

Chapter 2. Literature Review

Introduction

Reproduction in both sexes is dependent upon a cascade of reactions initiated by the pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus (Terasawa *et al.*, 1995; Spratt *et al.*, 1987). The amino acid sequence of porcine GnRH is Glu-His-Trp-Gly-Leu-Arg-Pro-Gly-NH_2 (Baba *et al.*, 1971; Matsuo *et al.*, 1971). Burgus *et al.* (1972) characterized ovine GnRH and found its sequence to be identical with that of porcine GnRH. Pulsatile GnRH released into the hypophyseal portal system controls both LH and FSH secretion from anterior pituitary in a number of species, such as pigs (Schally *et al.*, 1971; Matsuo *et al.* 1971; Burgus *et al.*, 1972), rhesus monkeys (Belchetz *et al.*, 1978; Pohl *et al.*, 1983) and sheep (Clarke and Cummins, 1982; Levine *et al.*, 1982). Furthermore, the patterns of GnRH secretion in perfusates of the median eminence or portal circulation and LH secretion in the peripheral circulation and are in close temporal association in the ewe (Clarke and Cummins, 1982; Levine *et al.*, 1982).

The frequency of GnRH pulses is crucial for the continued release of GnRH as continuous GnRH administration to ovariectomized monkeys with hypothalamic lesions resulted in the cessation of gonadotropin secretion (Belchetz *et al.*, 1978). Furthermore, the frequency of GnRH pulses determines the ratios of LH and FSH that are secreted (Wildt *et al.*, 1981), with low-frequency pulses increasing FSH secretion and high-frequency pulses stimulating LH

secretion (Jayes *et al.*, 1997). These pulses are required for proper reproductive function (Spratt *et al.*, 1987; Crowley *et al.*, 1992).

Research has demonstrated that GnRH neurons contain an intrinsic pulse generator that determines the basic pattern of GnRH secretion. Cultures of immortalized GnRH cells ~~lines~~ release episodic pulses of GnRH *in vitro* suggesting that the pulse generator is either intrinsic to individual GnRH cells or is a property of a network of GnRH cells (Mellon *et al.*, 1992). These cultures consisted only of GnRH-expressing cells and were found to release GnRH in a rhythmic fashion (Martinez de la Escalera *et al.*, 1992). Martinez de la Escalera *et al.* (1992) used an immortalized GnRH cell line (GT1-1) to investigate the endogenous pattern of GnRH release. The GT1-1 cell line was derived from a GnRH-secreting tumor in a transgenic mouse induced by genetically targeted expression of the potent simian virus 40 oncogene encoding tumor antigen (Mellon *et al.*, 1990). Cells attached to cover slips were superfused in Sykes-Moore chambers with Locke's medium, Ca^{+2+2} free Locke's medium, or Opti-MEM for 2 hr, and samples were collected at 4-min intervals. Release of GnRH in 17 of 18 superfusion chambers was seen to be pulsatile when data were analyzed by cluster analysis. No significant differences were observed whether only one or both of the cover slips forming the chamber were coated with cells. GnRH pulses exhibited a mean interpulse interval of 25.8 +/- 1.5 min, a mean duration of 18.8 +/- 1.4 min, and a mean amplitude of 150.5 +/- 6.0% above preceding nadir. The removal of Ca^{+2+2} from the Locke's medium resulted in the progressive reduction of the amplitude and eventually in the absence of identifiable pulses. GnRH pulses reappeared after the return of Ca^{+2+2} to the medium. It was concluded that the GT1-1 cell line secretes GnRH in a rhythmic pattern. These findings support ~~suggest~~ that the pulsatile release of GnRH and the GnRH pulse generator may be an intrinsic characteristic of the GnRH neurons. ~~These observations imply that GnRH neurons~~

~~have intrinsic pulsatility with regard to GnRH secretion.~~ Since individual GnRH neurons contain pulse generators, there must be a mechanism that allows the pulse generators to be synchronized.

The present thesis investigates the hypothesis that electrical synapses, mediated by Cx43 play a role in the synchronization of immortalized GnRH cells. The following literature review begins by describing the pattern of GnRH release and the effects of GnRH in males and females. Next, I review the immortalized GT1-7 cells which serve as a model system to study the GnRH neurons and describe gap junction communication between GnRH neurons and role of connexins. Finally I describe ~~calcium~~ Ca^{+2} imaging using fluorescent microscopy ~~technique using fluorescent microscopy~~, which was used in the present study to determine the synchronization of GnRH release by GnRH neurons with time in cultures by tracing ~~the~~ Ca^{+2} influx into the cells.

Pattern of GnRH Release

GnRH is released into the hypophysial portal blood circulation in a pulsatile manner (Carmel *et al.*, 1976; Levine *et al.*, 1982, Urbanski *et al.*, 1988; Moenter *et al.*, 1992a; Moenter *et al.*, 1992b). Levine *et al.* (1982) measured GnRH levels in hypophyseal portal blood collected from ovariectomized ewes at 10 minute intervals for 3-7 hours. GnRH was clearly released in an episodic manner with an interpulse interval of 36-44 minutes and amplitude that fluctuated between 2 and 17pg/min. GnRH pulse frequency varies under different physiological conditions including the menstrual cycle, where frequency increases in the early follicular phase, causing a surge in gonadotropin secretion that drives ovulation (Crowley *et al.*, 1985). GnRH pulse frequency varies throughout reproductive life (Ferris *et al.*, 2006; Marshall *et al.*, 1993). Children exhibit relatively low GnRH pulse frequency; but it increases during puberty. In adult men,

GnRH pulse frequency is relatively constant (approximately 1 pulse per 2 hours); whereas, in women it varies throughout the menstrual cycle, with increased GnRH pulse frequency and amplitude driving the pre-ovulatory gonadotropin surge (Marshall *et al.*, 1993). GnRH pulse frequency can vary markedly between species with pulses every 1–6 h in women and with pulse intervals as low as 8 min in mice (Ferris *et al.*, 2006).

Like GnRH, LH secretion occurs in a pulsatile manner as was demonstrated in ovariectomized monkeys (Dierschke *et al.* 1970). Later studies showed this phenomenon also occurs in humans (Backstrom *et al.*, 1982) and rats (Gallo *et al.*, 1981). This LH pulse is produced by a corresponding GnRH pulse from the hypothalamus (Clarke *et al.*, 1982). In rhesus monkeys with hypothalamic lesions, constant infusion of exogenous GnRH fails to restore gonadotropin secretion, but administration of GnRH once per hour reestablished gonadotropin secretion (Belchetz *et al.*, 1978). This indicates that gonadotropin secretion depends on GnRH pulses. The frequencies of GnRH and LH pulses have been reported to change in a similar manner throughout the estrous cycle and post-partum period. An experiment was conducted by Wise *et al.*, (1990) in ewes to determine the relationship between hypothalamic release of GnRH and the onset of pulsatile secretion of LH during postpartum anestrus. Ewes were surgically fitted with cannula for collection of hypophyseal-portal blood at 3, 7, 14, and 21 days postpartum. Hypophyseal-portal and jugular blood samples were collected at 10-min intervals over a 6 to 7 h period. The number of GnRH pulses increased throughout the postpartum period (Day 3: 2.2 ± 0., Day 7: 3.6 ± 0.2, Day 14: 3.9 ± 0.4, and Day 21: 6.4 ± 0.4 pulses/6 h). Changes in pulsatile LH release paralleled changes observed in pulsatile GnRH release over Days 3, 7, 14, and 21 postpartum (0.83 ± 0.3, 2.8 ± 0.4, 2.9 ± 0.6, and 4.0 ± 1.1 pulses/6 h, respectively). In addition, GnRH pulse amplitude was higher at Day 21 than at Days 3, 7, or 14 postpartum. These findings

suggest that an increased GnRH pulse frequency promoted the onset of pulsatile LH release during postpartum anestrus in ewes. FSH secretion in ewes is composed of both basal and episodic modes (Padmanabhan *et al.*, 1997). Most FSH secretion occurs using the basal mode; while in the episodic mode, a GnRH pulse preceded each pulse of FSH in the pituitary venous drainage, but not in the peripheral circulation (Padmanabhan *et al.*, 1997; Van Cleeff *et al.*, 1995). However, another study reported that some FSH pulses in the portal circulation in ovariectomized ewes were not preceded by GnRH pulses (Padmanabhan *et al.*, 2003). Another study in sheep, reported finding both GnRH-associated and non-GnRH-associated pulses of FSH (Padmanabhan *et al.*, 2001a. When pulsatile GnRH secretion was blocked by treatment with a GnRH antagonist, pulsatile FSH secretion was still noted (Padmanabhan *et al.*, 2003; Van Cleeff *et al.*, 1995). Similarly, in ovariectomized rabbits, evidence for pulsatile FSH secretion was seen in peripheral circulation after blocking the action of GnRH (Pau *et al.*, 1991). Some researchers have suggested a hypothalamic-independent regulation of FSH secretion in the ewe (Padmanabhan and McNeilly, 2001). For example, in as early as 1986, Clarke *et al.*, proposed differential regulation of LH and FSH secretion by GnRH and that pulses of GnRH are not required to activate the release of FSH.

The pulsatile release of GnRH is crucial to the differential regulation of LH and FSH secretion. In a study in primates, it was shown that when GnRH pulse frequency was decreased from 1 pulse/hour to 1 pulse/3hours, serum FSH increased while LH decreased (Wildt *et al.*, 1981). Slower frequency of GnRH release favored the release of FSH secretion and higher frequency of GnRH release favored the release of LH secretion This association has been reported in other species such as rats (Dalkin *et al.*, 1989; Haisenleder *et al.*, 1991; Marshall *et al.*, 1993),

monkeys (Wildt *et al.*, 1981), cattle (Kesner *et al.*, 1982) and sheep (Clark *et al.*, 1984; Hamernik *et al.*, 1988).

Episodic patterns of GnRH release have also been observed *in vitro*. Isolated GnRH-EGFP (enhanced green fluorescent protein) neurons from juvenile mice consistently generated repetitive bursts of action potentials separated by periods of silence that could each last for minutes (Kuehl-Kovarik *et al.*, 2002). As GnRH neurons fire spontaneous bursts of action potentials, they show two types of bursting/oscillation that are driven by different mechanisms. The first type of GnRH neuron (1–2%) exhibits slow (~0.05Hz) spontaneous oscillations in membrane potential. The second type of GnRH neuron, exhibits irregular bursts (Chu *et al.*, 2006). Episodic increases in the firing rate of unidentified hypothalamic neurons have been associated with downstream markers of GnRH secretion (Nunemaker *et al.*, 2001). Whether episodic electrical activity is intrinsic to GnRH neurons, intrinsic to other "pulse generator" neurons that drive GnRH neurons, or a combination of these is unknown. To determine if GnRH neurons display episodic firing patterns in isolation from other cell types, immortalized GnRH neurons (GT1-7 cells) were cultured on multiple microelectrode arrays (Nunemaker *et al.*, 2001). Long-term, multi-site recordings of GT1-7 cells revealed repeated episodes of increased firing rate with an interval of 24.8 ± 1.3 (SE) min that were completely eliminated by the sodium channel blocker, tetrodotoxin. The pattern of electrical activity was comprised of active units that fired independently as well as coincidentally, suggesting the overall pattern of electrical activity in GT1-7 cells emerges as a network property. Application of the A-type potassium-channel antagonist, 4-aminopyridine (1 mM), increased both action potential firing rate and GnRH secretion, demonstrating the presence of A-type currents in these

cells and supporting the hypothesis that electrical activity is associated with GnRH release. Physiologically relevant episodic firing patterns are thus an intrinsic property of immortalized GnRH neurons and appear to be associated with secretion. The finding that overall activity is derived from the sum of multiple independent active units within a network may have important implications for the genesis of the GnRH secretory pattern that is delivered to the target organ (Nunemaker *et al.*, 2001). However, the short duration of the recordings in these studies (<30 min) precluded observation of episodic changes in firing rate on the same time scale as pulsatile hormone release from this cell line; i.e., approximately half hourly (Besecke *et al.* 1994;Krsmanovic 1993; Martinez de la Escalera *et al.* 1992; Pitts *et al.* 2001; Wetzel *et al.* 1992). Long-term, multi-electrode recordings of GT1-7 cells for 6hr, revealed repeated episodes of increased firing rate with an interval of 24.8 ± 1.3 (SE) min that were completely eliminated by tetrodotoxin (Moenter *et al.*, 2003). This pattern was comprised of active units that fired independently as well as coincidentally, suggesting the overall pattern of electrical activity in GT1-7 cells emerges as a network property (Moenter *et al.*, 2003).

~~GT1 cells are immortalized GnRH cells, produced by genetically-targeted tumorigenesis to specific hypothalamic neurons in transgenic mice using the promoter region of the gonadotropin-releasing hormone (GnRH) gene to express the SV40 T-antigen oncogene (Mellon *et al.* 1990). These cells are used widely for *in vitro* studies of GnRH neurons. GT1 cells release GnRH in a pulastile manner even though they are devoid of any external input, indicating that release of GnRH is an intrinsic property of GnRH neurons (Besecke *et al.* 1994; Charles and Hales 1995; Costantin and Charles 1999; Krsmanovic *et al.* 1992, 1993; Martinez de la Escalera *et al.* 1992; Pitts *et al.* 2001; Van Goor *et al.* 1999; Wetzel *et al.* 1992).~~

The ultradian, circadian, and yearly patterns of GnRH release differ dramatically between males and females. Nevertheless, the ultradian frequency of secretion is key to successful reproduction. The next few paragraphs describe how the pattern of GnRH secretion regulates male and female reproduction.

GnRH Regulation of Male Reproduction

In males, GnRH is secreted at a constant frequency (Haisenleder *et al.*, 1987; Katt *et al.*, 1985; Dalkin *et al.*, 1989) into hypophyseal portal system by cells that are located in the preoptic area and the adjacent sites within the rostral portion of the hypothalamus (Knobil, 1981; Merchenthaler *et al.*, 1984). GnRH stimulates the pulsatile release of LH and FSH from the anterior pituitary. LH acts on Leydig cells of testis (Figure 1), causing them to release testosterone. Testosterone inhibits release of GnRH and LH and stimulates spermatogenesis (Sheckter *et al.*, 1989), differentiation of male genitalia stimulates development of male secondary sexual characteristics at puberty, growth and development of internal/external genitalia bone growth and epiphyseal plate closure, muscle development, axillary, pubic, body hair, male pattern baldness, fat distribution, laryngeal growth, sebaceous glands, behavioral effects, libido, aggression (Clay *et al.*, 1988). FSH supports the function of Sertoli cells, which in turn support many aspects of sperm cell maturation. FSH production is inhibited by the hormone inhibin, which is secreted by Sertoli cells. (Griswold *et al.*, 1988; Kotsuji *et al.*, 1988) During fetal development (Figure 2), one surge of GnRH is secreted; the next surge is at mini puberty. Later, a sustained release of GnRH is observed in males (Bouvattier *et al.*, 2011). Thus, GnRH stimulates the release of FSH and LH from the anterior pituitary. During the GnRH surges in fetal development and mini puberty, FSH and LH were found at a higher amount in the circulation (Figure 2).

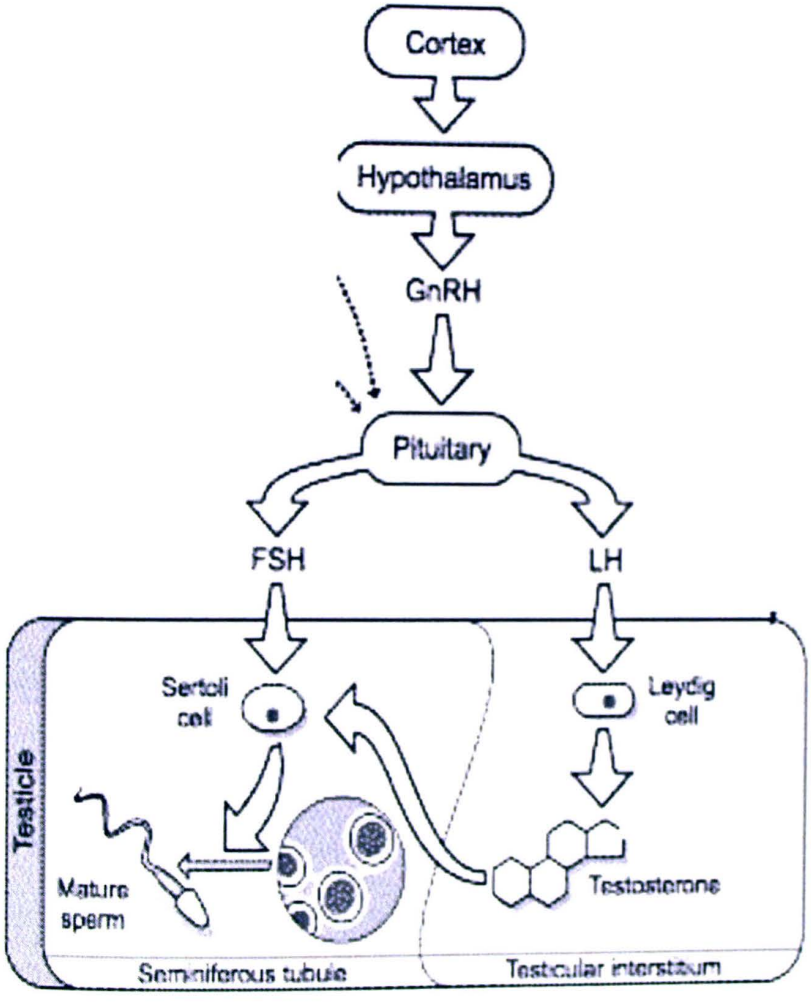

Figure 1. Effects of GnRH in the male reproductive system. GnRH stimulates the release of FSH and LH form the anterior pituitary. FSH promotes spermatogenesis and LH stimulates the release of testosterone form Leydig cells (modified from Kanis *et al.*, 1994).

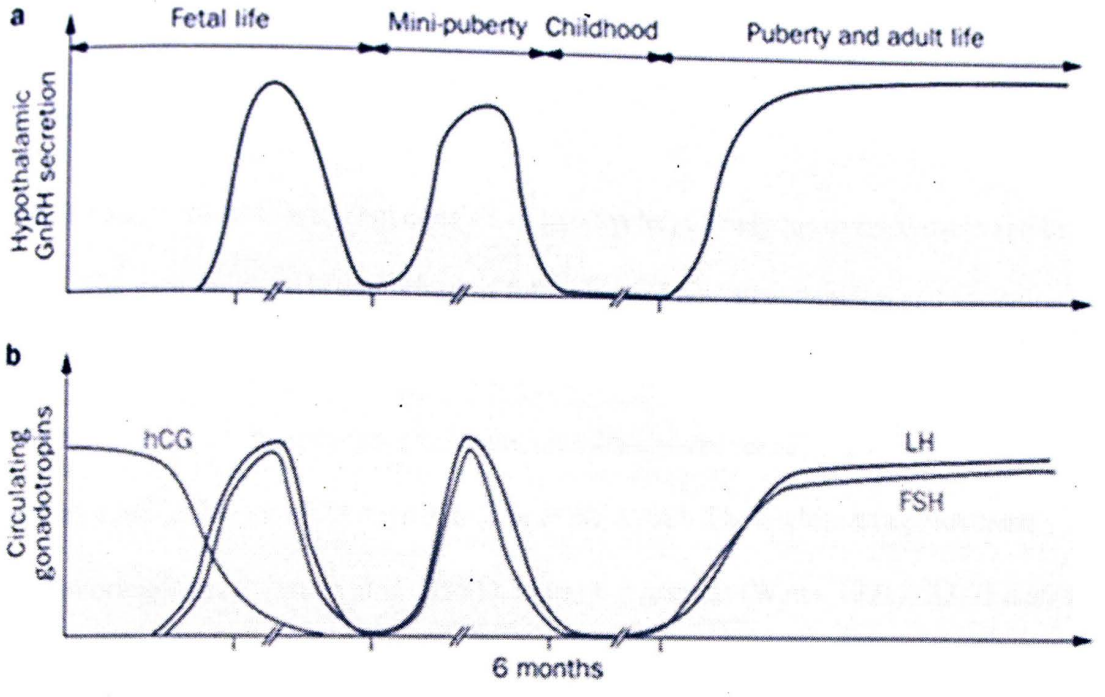

Figure 2. Schematic of the hypothalamic–pituitary–testicular axis during fetal and postnatal life in humans (Bouvattier *et al.*, 2011). During fetal, early neonatal and pubertal development, a) pulsatile hypothalamic secretion of GnRH stimulates b) pituitary gonadotropins FSH (Blue line) and LH (Red line) biosynthesis.

GnRH Regulation of Female Reproduction

Primates

The female reproductive system consists of two cycles, namely the ovarian cycle and the uterine cycle. The ovarian cycle is comprised of follicular and oocyte maturation and ovulation, while the uterine cycle is composed of the preparation and maintenance of the uterine lining. In humans, (Figure 3) these cycles occur concurrently and are coordinated over a 22–32 day cycle, with an average length of 28 days (Chiazze *et al.*, 1968). The cycles last approximately 26-32 days in orangutans (Masters at al., 1991),30 days in gorillas (Watts, 1991), 32-35 days in bonobos (Furuichi, 1992) and about 35days in chimpanzees (Lacreuse *et al.*, 2008).

a. Ovarian cycle

The ovarian cycle consists of the follicular and luteal phases. GnRH is secreted at a higher frequency and lower amplitude during the follicular phase of human ovarian cycle compared to the luteal phase (Reame *et al.*, 1984). Blood samples were obtained at 10 or 20 min intervals for 12 or 24 h at 7-day intervals during the same ovulatory cycle in eight normal women. Ovarian steroids showed the expected cyclical changes and mean plasma FSH concentrations showed an inverse relationship to estradiol, being low when estradiol was greater than 150 pg/ml. Sampling every 10 min revealed a constant LH pulse amplitude but LH pulse frequency increased (from 11.8 to 14.3 pulses/12 h) during the follicular phase. LH pulse frequency did not increase further in two women sampled during the LH surge, but pulse amplitude was markedly higher. During the luteal phase, LH pulse frequency was reduced to eight pulses/12 h but frequency was more variable between subjects than in the follicular phase. LH pulse amplitude showed striking

variation (0.8–29.4 milli-international units per milliliter (mlU/ml)) during the luteal phase of the cycle and large LH secretory episodes which lasted 1–3 h were irregularly interspersed among periods of low amplitude LH secretion. These data show that the frequency of LH pulses, and by inference GnRH secretion, varies during the ovarian cycle but the degree of change is less than reported in previous studies. This observation may explain the reported efficacy of fixed frequency GnRH regimes in inducing ovulation and cyclical ovarian function. (Reame *et al.*, 1984).

An increase in GnRH frequency and amplitude in mid-cycle favors the surge of LH that is necessary for ovulation and the beginning of the luteal phase. After ovulation, the ovary enters the luteal phase. The slowing of GnRH pulse frequency in the late luteal phase is an important change, favoring FSH synthesis and secretion; consequently, allowing FSH to rise in preparation for the next cycle (McCartney *et al.*, 2002). During the luteal-follicular transition, pulse frequency increases approximately 4.5-fold (Hall *et al.*, 1992). This increase is due to estrogen switching from negative feedback to positive feedback to the hypothalamus in mammals (Thomas *et al.*, 1997). At ovulation the follicle ruptures expelling the ovum into the fallopian tube. The remnants of the follicle are called the corpus luteum, it produces estrogen and progesterone (Hauger *et al.*, 1977). Progesterone facilitates regrowth of the uterine lining and inhibits further release of FSH and LH (Reame *et al.*, 1984). The inhibition of FSH and LH (Figure. 4) prevents additional oocytes and follicles from developing, while the progesterone is elevated. The elevated levels of progesterone will prepare uterus to accept a fertilized egg. If no fertilization occurs the corpus luteum will regress.

b. Uterine cycle

The uterine cycle consists of three phases, namely: menstrual, proliferative and secretory. During the menstrual phase, the functional layer of the uterine wall is sloughed off and discarded. Next, the maturing follicles release more estrogens causing the lining of the uterus to proliferate during the proliferative phase (Losos *et al.*, 2002). The estrogen also stimulates the cervix to produce less viscous cervical mucus (Weschler *et al.*, 2002). The secretory phase is the final phase of the uterine cycle. During this phase, the corpus luteum produces progesterone (Figure 4), which plays a vital role in making the endometrium receptive to implantation of the blastocyst and supportive of the early pregnancy (Lombardi *et al.*, 1998).

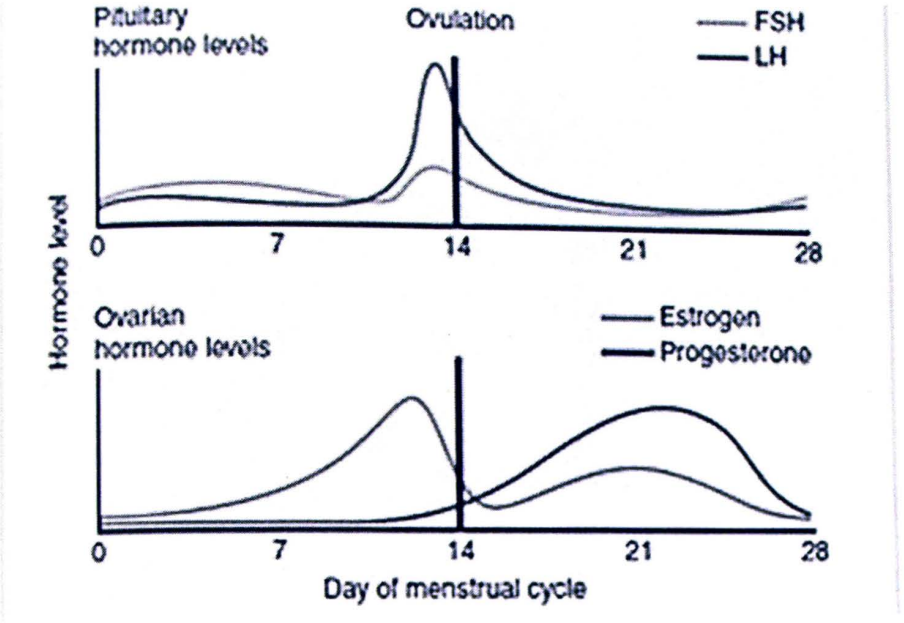

Figure 3. Pattern of hormone release during the human menstrual cycle (Gordon *et al.*, 2013).

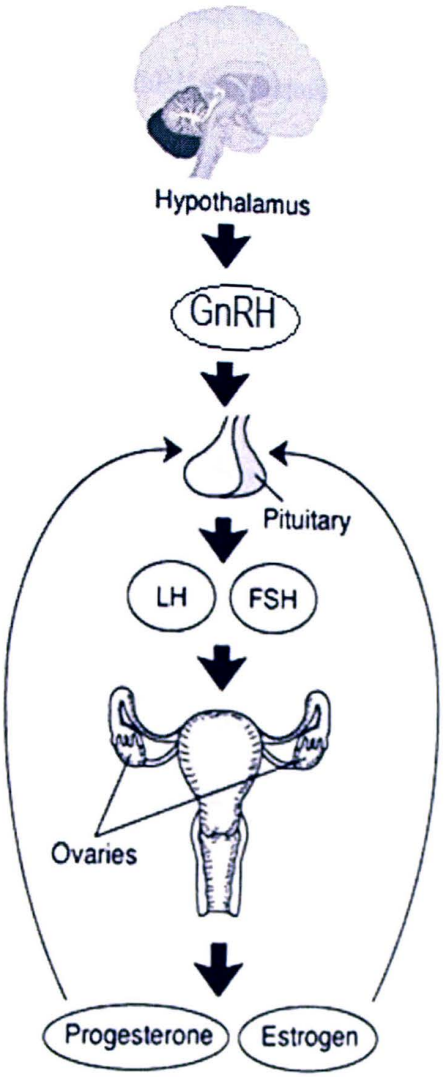

Figure 4. The female hypothalamic–pituitary–gonadal axis.(Kanis, 1994).

The hypothalamus produces and secretes GnRH into a system of blood vessels that link the hypothalamus and the pituitary gland. GnRH stimulates the pituitary gland by attaching to specific molecules (i.e., receptors). After the coupling of GnRH with these receptors, a cascade of biochemical events causes the pituitary gland to produce and secrete two hormones, LH and FSH.

Non-primate Species

Female cats, dogs, ewes, horses and rats have estrous cycles instead of menstrual cycles. The estrous cycle is composed of estrus, metestrus, diestrus and proestrus. Estrus refers to the stage when the female is receptive to the male; it is commonly referred to as heat. Ovulation occurs during this period. Metestrus begins with the cessation of estrus and lasts for about 3 days. During this period, the corpus luteum is formed (corpora luteam with multiple ovulations). Diestrus refers to the period in between the estrus phases when the female is not receptive to the male. Proestrus begins with the regression of the corpus luteum and drop in progesterone and lasts to the start of the next estrus. Anestrus refers to the complete absence of estrus.

The bovine estrous cycle lasts for 21 days (Figure 5) (Larson *et al.*, 1992). Progesterone plays an important role in estrous cycle. It inhibits FSH and LH release through negative feedback of the hypothalamus and anterior pituitary. A drop in progesterone removes the hypothalamus from its negative feedback inhibition. Two to three days after the drop in progesterone, estradiol reaches the threshold concentration and stimulates (through a positive feedback control on the hypothalamus) the large preovulatory surge of GnRH, FSH, and LH. The preovulatory surge of FSH stimulates more rapid growth of the follicle and greater secretion of estradiol. The sensitivity of the anterior pituitary to GnRH increases through up-regulation of the GnRH receptors by the more frequent pulses of GnRH (Rispoli *et al.*, 2005). Likewise, increasing

concentrations of FSH and estradiol up-regulate ovarian FSH and LH receptors (Foster at al., 1980; Bevers *et al.*, 1989).

Diestrus lasts 14 days in mares. During this phase GnRH is secreted at lower frequency; hence, LH (Figure 7) concentrations are lowest during the mid-luteal phase of estrous. LH levels rise only a few days before the onset of estrus and usually peak near the day of ovulation before dropping to previous levels over the next few days. The bi-modal pattern of the initial FSH surge starts late in estrus and peaks early in diestrus when the frequency of GnRH is lower. Estrogen presence has a negative impact on FSH, but a positive impact on LH. Luteal progesterone has a positive impact on FSH and a negative impact on LH (Evans *et al.*, 1975; Gastal *et al.*, 2000).

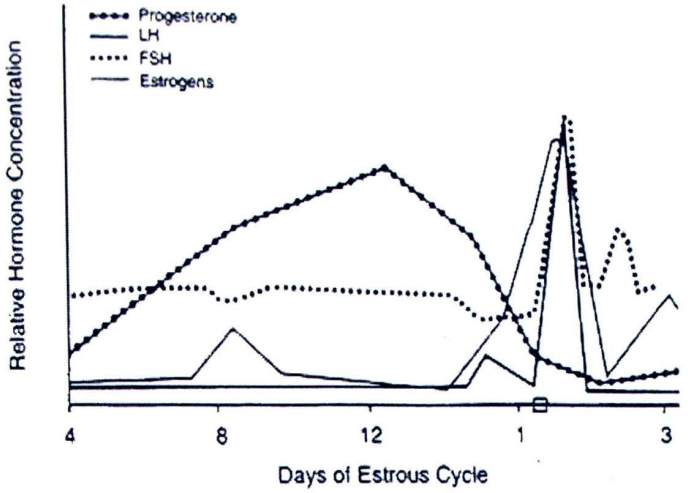

Figure 5. Hormonal changes in the plasma during the estrous cycle of the cow. The drop in progesterone on day 16, 17 or18 is followed by surges in estrogens, FSH and LH during late proestrus (Blowey, 1986).

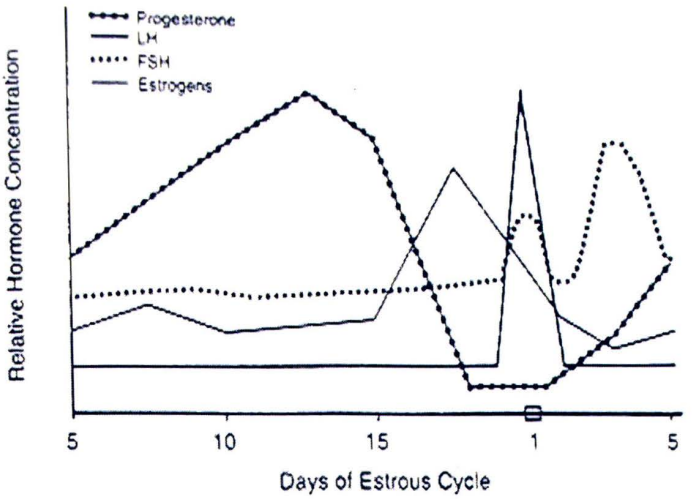

Figure 6. Hormonal changes in the plasma during the estrous cycle of the ewe. Pattenrns for the ewe are similar to that for other species. A reduction in FSH during proestrus is followed by a spike during estrus and another surge during metestrus (Blowey, 1986).

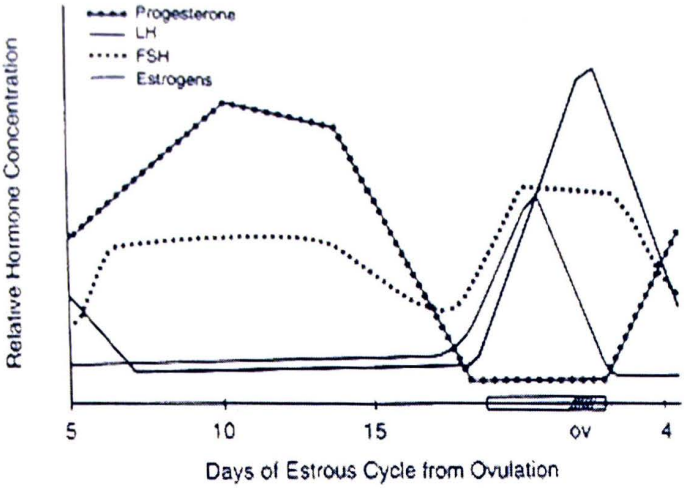

Figure 7. Hormonal changes during the mare estrous cycle. Patterns are similar to that of other species except that surges of FSH and LH during estrus last for several days (Blowey, 1986).

GT1 Cells: A model system for the study of GnRH secretion

GnRH analysis is difficult as it has a very short half-life of approximately 15 min in serum (Nett *et al.*, 1977), coupled with a small number of GnRH neurons that are scattered in the hypothalamus (Kuenzel *et al.*, 1991). To overcome these obstacles, a hypothalamic cell line was established with properties similar to native GnRH neurons (Mellon *et al.*, 1990). The cell line was developed by coupling the murine GnRH promoter to the SV40 T-antigen oncogene. This construct was used to create transgenic mice. Some of these transgenic mice developed tumors, from which immortalized cell culture system was established. Three different cell lines were obtained from the initial tumors: GT1-1, GT1-3 and GT1-7. Growth rates for these cell lines vary from doubling every 36 hr (GT1-3 and GT1-7) to every 3-4 days (GTI-1). These cell lines express GnRH mRNA and release GnRH in a pulsatile manner and in response to depolarization (Mellon *et al.*, 1990).

Several lines of evidence suggest that GT1 cells retain neuronal properties; consequently, they may be an excellent *in vitro* model for the study of GnRH release. GT1 cell bodies contain both forming and mature neurosecretory granules, golgi apparatus and rough endoplasmic reticulum (Mellon *et al.*, 1990; Liposits *et al.*, 1991). Thick processes contain neurosecretory granules that are immunoreactive for GnRH and GnRH-associated peptide (GAP), while thinner processes extending away from the cell bodies terminate in growth cones or bead like dilations (Mellon *et al.*, 1990; Liposits *et al.*, 1991). Transmission electron microscope visualization of GT1 cells confirms that they are rich in ribosomes and contain secretary vesicles and granules (Wetsel *et al.*, 1992). Nevertheless, one must always remember that these are transformed cells from immature mice that may not always act the same as mature GnRH neurons as GT1-7 cells lack the normal inputs from neurotransmitters, growth factors, and steroids, which are involved in the

maturation and maintenance of GnRH neurons in the brain. Since their creation, GT1 cells have been useful in the investigation of GnRH neurons. In particular, they have been used to help elucidate the intracellular mechanisms of GnRH pulse generation.

GnRH Pulse Generator: The Mechanism of GnRH release

An early question concerning the mechanism driving GnRH pulsatility was whether the pulse generator was intrinsic to individual GnRH neurons or was a property of neuronal networks. Suter *et al.* (2000) used transgenic mice in which green fluorescent protein was genetically targeted to GnRH neurons to determine whether GnRH neurons exhibit high frequency bursts of action potentials and are electrically coupled at or near the soma. They observed that GnRH neurons fired spontaneous action potentials, and in 15 of 21 GnRH neurons, the action potentials occurred in single bursts or episodes of repetitive bursts of high frequency spikes (9.77 ± 0.87 Hz) lasting 3–120 sec. Extended periods of quiescence of up to 30 min preceded and followed these periods of repetitive firing. Examination of 92 GnRH neurons (including 32 neurons that were located near another green fluorescent protein-positive neuron) revealed evidence for coupling in only 1 pair of GnRH neurons. The evidence for minimal coupling between these neuroendocrine cells suggested that direct soma to soma transfer of information, through either cytoplasmic bridges or gap junctions, has a minor role in synchronization of GnRH neurons. The pattern of electrical activity observed in single GnRH neurons within slices is temporally consistent with observations of GnRH release and multiple unit electrophysiological correlates of LH release (Suter *et al.*, 2000; Westel *et al.*,1992) demonstrated intrinsic pulsatile secretory activity from GT1 cells. Pulsatile GnRH release is intrinsic property of both GnRH neurons as well as immortalized GnRH neurons, meaning they do not require input from exogenous sources for patterned GnRH release (Charles and Hales, 1995; de la Escalera *et al.*, 1992; Kusano *et al.*,

1995; Vazquez-Martinez *et al.*, 2001). De La Escalera *et al.*, (1992) also used GT1-1 cells to investigate the endogenous pattern of GnRH release. ~~Cells attached to cover slips were superfused in Sykes-Moore chambers with Locke's medium, calcium free Locke's medium, or Opti-MEM (another defined medium) for 2 hr, and samples were collected at 4-min intervals. Release of GnRH in 17 of 18 superfusion chambers was seen to be pulsatile when data were analyzed by cluster analysis. Pulses exhibited a mean interpulse interval of 25.8 +/- 1.5 min, a mean duration of 18.8 +/- 1.4 min, and a mean amplitude of 150.5 +/- 6.0% above preceding nadir. The removal of Ca^{2+} from the Locke's medium resulted in the progressive reduction of the amplitude and eventually in the absence of identifiable pulses. Pulses reappeared after the return of Ca^{2+} to the medium which indicates that GT1-1 cell line secretes GnRH in a rhythmic pattern.~~

Initially, it was believed that the GnRH pulse generator might be composed of cycles of transcription and translation, similar to the mechanism used by circadian clocks. However, Pitts *et al.*, (2001) demonstrated that neither inhibitors of transcription nor translation were able to prevent the episodic secretion of GnRH, indicating that another mechanism must be involved. In more recent studies, much progress has been made towards identifying the mechanism involved in pulse generation. The pulsatile secretion of GnRH from normal and immortalized hypothalamic GnRH neurons is highly dependent on calcium and is stimulated by cAMP (Krsmanovic *et al.*, 2003; Hu *et al.*, 2006). Li *et al.*, (2012) showed that arcuate nucleus is the key site for kisspeptin modulation of LH pulse frequency, supporting the notion that kisspeptin-GPR54 signaling in this region of the mediobasal hypothalamus is a critical neural component of the hypothalamic GnRH pulse generator. Discovery of kisspeptin and, consequently, KNDy (kisspeptin/neurokinin B/dynorphin) neurons in the hypothalamus have provided a clue to the possible location of the GnRH pulse generator (Okamura *et al.*, 2013). KNDy cells may serve as

a central node in the control of GnRH secretion, acting as conduits for a variety of intrinsic and extrinsic regulatory signals (Lehman *et al.*, 2010). The characteristic pulsatile secretion of GnRH from hypothalamic neurons is dependent on an autocrine interaction between GnRH and its receptors expressed in GnRH-producing neurons. The ontogeny and function of this autoregulatory process were investigated in studies on the properties of GnRH neurons derived from the olfactory placode of the fetal rat. An analysis of immunocytochemically identified, laser-captured fetal rat hypothalamic GnRH neurons, and olfactory placode-derived GnRH neurons identified by differential interference contrast microscopy, demonstrated coexpression of mRNAs encoding GnRH and its type I receptor. Both placode-derived and immortalized GnRH neurons (GT1-7 cells) exhibited spontaneous electrical activity that was stimulated by GnRH agonist treatment. This evoked response, as well as basal neuronal firing, was abolished by treatment with a GnRH antagonist. GnRH stimulation elicited biphasic intracellular Ca^{+2} responses, and both basal and GnRH-stimulated Ca^{+2} levels were reduced by antagonist treatment. Perfused cultures released GnRH in a pulsatile manner that was highly dependent on extracellular ~~Ca^{2+}~~$^{+2}$Ca+2. The amplitude of GnRH pulses was increased by GnRH agonist stimulation and was diminished during GnRH antagonist treatment. These findings demonstrate that expression of GnRH receptor, GnRH-dependent activation of calcium signaling, and autocrine regulation of GnRH release are characteristics of early fetal GnRH neurons and could provide a mechanism for gene expression and regulated GnRH secretion during embryonic migration. This autocrine mechanism could serve as a timer to determine the frequency of pulsatile GnRH release by regulating $Ca^{2+}$$^{+2}$ and cAMP-dependent signaling and GnRH neuronal firing. The firing of individual and/or bursts of action potentials in spontaneously active GnRH neurons is followed after hyperpolarization that lasts from several milliseconds to several

seconds (Martinez-Fuetens *et al.*, 2004), and release GnRH in a pulsatile manner with frequency similar to that of GnRH secretion, *in vivo*.

GnRH Neuron Communication and Synchronization

The ability of a population of individually pulsing neurons to produce distinct episodes of GnRH secretion into the portal circulation requires a coordinating or synchronizing mechanism. Indeed, Vazquez-Martinez *et al.* (2001) demonstrated that GT1 cell cultures were able to resynchronize within three days of passaging. Three possible types of communication could be used by GnRH neurons: 1. a paracrine mechanism where one cell secretes a chemical messenger into the interstitial fluid which then diffuses to another GnRH neuron, 2. communication through a chemical synapse, and 3. communication via electrical synapses. In a study performed by Vazquez-Martinez *et al.*, (2001), it was shown that GT1-7 cells communicate through electrical synapses. They used FM1–43 to monitor the membrane turnover associated with exocytosis in single GT1–7 neurons and found an intrinsic pulsatility (frequency, 1.4 ± 0.1/h; pulse duration, 17.3 ± 0.6 min) that became progressively synchronized among neighboring cells as the length of culture increased. Voltage-gated Ca^{+2} channels and gap junctional communication each played a major role in synchronized pulsatility. An L-type Ca^{+2} channel inhibitor, nimodipine, abolished synchronized pulsatility (Choudhary *et al.*, 2006). In addition, functional gap junction communication among adjacent cells was detected, but only under conditions where pulsatile synchronization was also observed, and the gap junction inhibitor octanol abolished gap junction coupling by causing the acid shift in the cells without affecting pulse frequency or duration (Pappas *et al.*, 1996). These results provide strong evidence that the GnRH pulse generator in GT1–7 cells arises from a single cell oscillator mechanism that is synchronized through network

signaling involving voltage-gated calcium channels and gap junctions (Vazquez-Martinez *et al.*, 2001).

Gap Junction communication between GnRH neurons

Gap junctions are composed of 2 connexons, with each expressed by adjacent cells. The connexons are made up of 6 protein subunits (Figure 8), termed connexins (Evans *et al.*, 2002). The connexin gene family comprises 20 members in the mouse and 21 members in the human genome (Goran *et al.*, 2004). These gap junctions are specific to particular cells, the types of connexins expressed in different cells is listed in table 2a and 2b. Gap junctions are permeable to small compounds (up to a molecular mass of 1000 daltons) such as metabolites, ions, second messengers, water and electrical impulses to be exchanged between adjacent cells using diffusion (Kumar *et al.*, 1996). The conductance and permeability of hemichannels as well as gap junctions are regulated by the intracellular protons changes (Ek-Vitorin *et al.*, 1996; Morley *et al.*, 1997; Spray and Burt, 1990) and Ca^{+2} -concentrations (Spray *et al.*, 1985; Spray and Burt 1990) but also via phosphorylation of specific serine, threonine and tyrosine residues by kinases (Lampe and Lau, 2000) especially protein kinase C (PKC; Bowling *et al.*, 2001; Lampe *et al.*, 2000). In Cx43 gap junctions, the conductance and permeability of the unopposed connexin~~Cx~~ channels increased when Cx43 became dephosphorylated (Burt and Spray 1988, Lau *et al.*, 1991). The dephosphorylation of Cx43 is proposed to be the key mechanism to open connexin~~Cx~~ hemichannels in cardiac tissue during the metabolic inhibition in astrocytes culture (Contreras *et al.*, 2002). Figure 9 demonstrates the opening and closing of a gap junction channel, each hemichannel (green) can regulate its channel activity autonomously. The gap junction is open only when the plugs (red) in both hemichannels are displaced from the channel constriction

formed by the innermost helices C (yellow) toward the cytoplasmic side. The flexible connections of the plug with the channel are shown as red dashed lines (Oshima *et al.*, 2007).

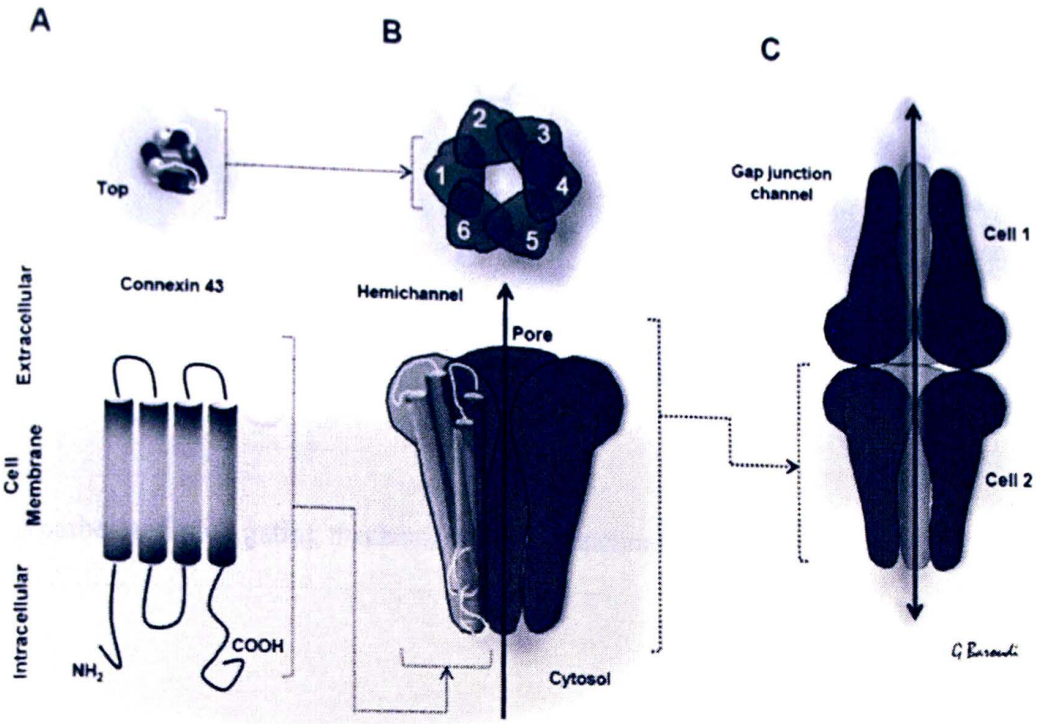

Figure 8. Connexin 43 structures. A, Secondary structure of Cx43 with insert showing a tridimensional view from the top; B, a single hemichannel formed by the association of six connexins to form central permeable pore; C, a gap junction channel formation with two hemichannels formed from the apposition of adjacent cells (Hawat *et al.*, 2012).

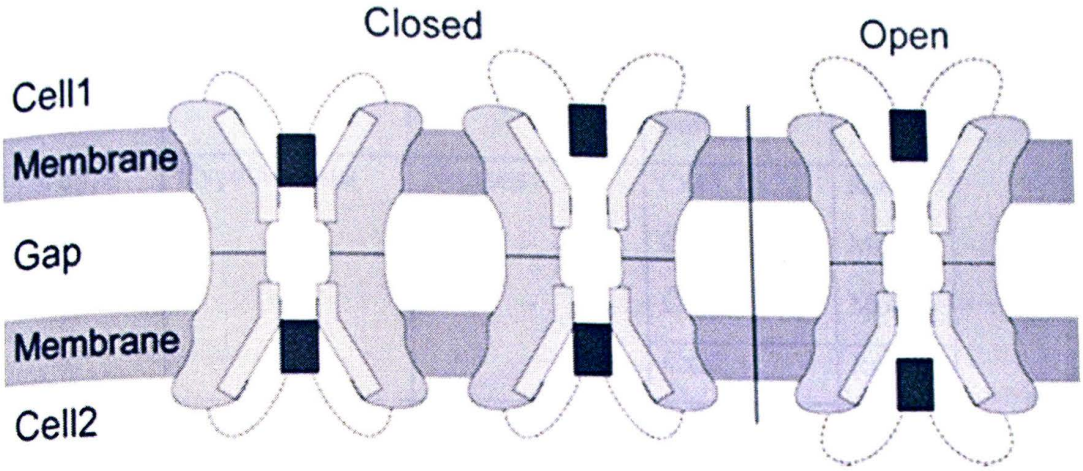

Figure 9. Hypothesized plug gating mechanism of gap junctions (Oshima A *et al.*, 2007).

Tables

Type of secretion	Gland	Cell	Connexin isoform	Species
Endocrine	Hypothalamus	Neurons	Cx43, Cx32	Rat
			Cx36	Mouse
			Cx43	Mouse
			Cx32	Rat
	Pineal	Pinealocytes	Cx26, Cx32	Rat
	Pituitary	Acidophil	Cx43, Cx26	Rat
	Thyroid	Follicular	Cx43, Cx32, Cx26	Rat
			Cx43	Rat
	Parathyroid	Principal	Cx43	Rat
	Pancreas	Beta	Cx36	Rat, mouse
			Cx36	Human
	Kidney	Myoepithelial	Cx40, Cx37, Cx45	Mouse, rat
			Cx40, Cx37	Human
	Adrenal	Medullary	Cx36, Cx43	Rat
Secretion			Cx36	Mouse
			Cx43, Cx50	Human
		Cortical	Cx43	Rat
			Cx43	Mouse

Table: 2a Connexins of vertebrate (neuro) endocrine glands (Firestone *et al.*, 2012)

	Testis	Leydig	Cx43	Rat
			Cx43, Cx30.2	Mouse
	Gland	Sertoli	Cx43	Rat
		Cell	Cx isoform	Species
Endocrine		Luteal	Cx43	Rat
			Cx43, Cx32, Cx26	Sheep
			Cx43	Human, baboon
	Ovary	Granulosa and theca	Cx43	Mouse, rat
			Cx43, Cx37, Cx26	Sheep
			Cx43, Cx26, Cx30.3, Cx32	Pork
			Cx43, Cx37, Cx26, Cx32	Beef
			Cx43	Human
	Placenta	Throphoblast		Rat
				Human
Pheromone	Skin	Sebaceous	Cx43	Human
	Preputial glands	Parenchymal	Cx43	Rat

Table: 2b Connexins of vertebrate (neuro) endocrine glands (Firestone *et al.*, 2012)

Three different types of connexins are associated with GnRH neurons namely, Cx32, Cx43, and Cx26 (Hosny *et al.*, 1998; Hu *et al.*, 1999; Matesic *et al.*, 1993;Tsukahara *et al.*,1999; Houang at al., 2002). There was no further research reported on Cx32 in gap junction communication between GnRH neurons. Initial studies focused on the role of Cx26 in synchronizing GnRH neurons. Matesic *et al.*, (1993) reported the expression of Cx26 in GT1-7 cells and also reported that 20% of the cells were coupled with Cx26. In this study, GT1-7 cells were found to express a connexin 26-like protein that co-migrated with mouse liver connexin 26 and reacted with connexin 26-specific antibodies on Western blots. Immunofluorescent staining revealed punctate staining in a fraction of the cells, often present at points of apparent contact with neighboring cell bodies or processes. Fluorescence recovery after photobleaching analysis of 5, 6-carboxyfluorescein loaded GT1-7 cells showed dye coupling among 20-30% of cells that made contact with other cells, suggesting the presence of functional gap junctions in this cell line (Matesic *et al.*, 1993). In contrast, Bose *et al.*, (2010) reported no significant amount of Cx26 produced during GnRH cell synchronization in GT1-7 cells. They also showed that application of siRNA targeting Cx43 abolished the pulsatile release of GnRH in cultures of immortalized GT1-7 neuronal cells cells, suggesting that Cx43 is involved in communication of GT1-7 cells. The same study showed that siRNA targeting Cx26 did not abolish the pulsatile release of GnRH in cultures indicating that Cx26 was not involved in GT1-7 cell communication. Finally, microinjection of day-3 cultures with siRNA for Cx43 abolished the synchronized release of GnRH at day 7, whereas siRNA for Cx26 administration did not alter secretary activity at day 7 (Figure 10, 11).

Conventional blockers of connexin channels act unselectively on both gap junction channels and unapposed hemichannels, independent of the connexin isoforms forming these channels (Scemes *et al.*, 2009). Interestingly, synthetic connexin-derived structural mimetic peptides (CxMPs) emerged as powerful and unique tools capable of blocking unapposed hemi–channels with little or no immediate effects on gap junction channel. These were initially developed with the intention to modulate the function of gap junction channels (Gomes P, *et al.*, 2005), subsequent studies unraveled their preferential action on unopposed hemichannels (Leybaert L, 2003). By mimicking short amino acid sequences on connexins extracellular loops, CxMPs bind to these structures and cause inhibition of unapposed hemichannels by a yet undetermined mechanism. Because each CxMP contains a conserved motif that is not consistently found in other connexins or cell surface proteins, it is generally admitted that CxMPs interact specifically with connexins in an isoform-selective manner (Ghayda Hawat, *et al.*, 2010; Nan wang, *et al.*, 2012). The specific type of connexin proteins comprising a particular gap junction and in conjunction with other factors such as cell type-independent (ubiquitous) and dependent transcription factors, transcription initiation site, binding sites for TATA box-binding protein, confers the functional parameters of that junction (Oyamada *et al.*, 2005). By means of electrophysiology, Desplantez *et al.*, (2012) compared the effects of Gap26, a mimetic peptide corresponding to a short linear sequence in the first extracellular loop of connexin43, on connexin channel function in HeLa cells expressing Cx43. They demonstrate that Gap26 inhibited electrical coupling in cell pairs mediated by gap junctions after exposure for 30 min.

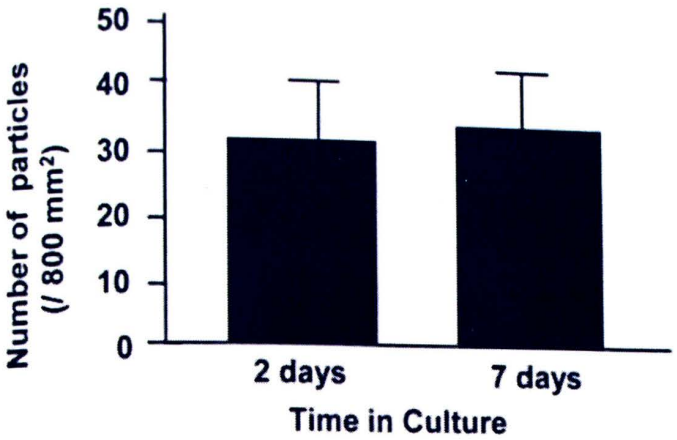

Figure 10. Immunoreactive Cx26 particles or "plaques" in GT1-7 cells from 2 day and 7 day cultures. The bar graph is showing a comparison of Cx26 immunoreactive particles between cells cultured for 2 days and 7 days with time in culture (Bose *et al.* 2011).

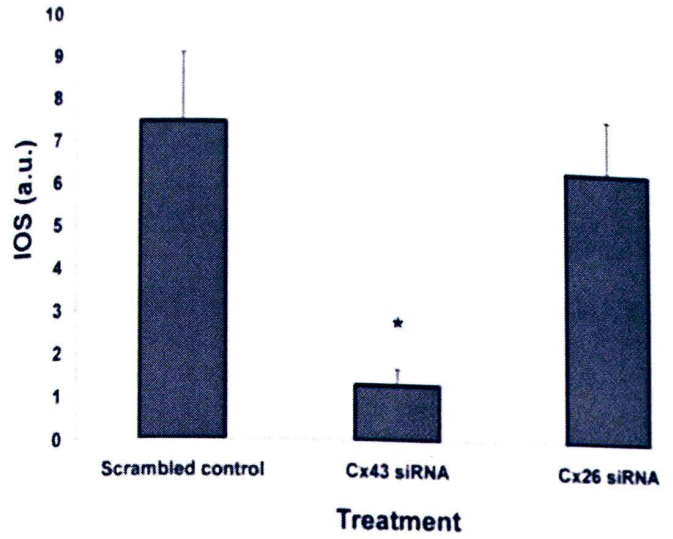

Figure 11. Effect of siRNA treatment on the index of syncronization (IOS) of populations of GT1-7 cells. As above, cells were grown for 3 days, microinjected with siRNA, and then on day 7 analyzed for pulse activity with FM1-43. Data are expressed as the mean + SE of 5 experiments for scrambled control and Cx43 injected (total of 40 cells for each treatment) and 4 experiments for Cx26 siRNA treated (total of 32 cells; Bose *et al.* 2011).

There is no direct mechanism to measure GnRH secretion from individual cells. Therefore, the amount of GnRH released is inferred based on events that lead to the release of GnRH. There are two main research methods that can be used to infer GnRH release, they are: 1. Measuring membrane potential using patch clamp (Eisthen *et al.*, 2000; Zhang *et al.*, 2007) and 2. Fluorescent microscopy using a dye for Ca^{+2} (Terasawa *et al.*, 1999; Kim *et al.*, 2006). In the present research fluorescent microscopy is used. During GnRH secretion intracellular Ca^{+2} levels increase (Krsmanovic LZ., *et al.*, 1992; Ei Terasawa *et al.*, 1999), triggering exocytosis (Kaftan *et al.*, 2000). Thus, by measuring the Ca^{+2} concentrations using calcium specific fluorescent dye one can infer the GnRH release.

Calcium imaging

Calcium imaging has been used to trace the intracellular free $Ca^{2+\pm2}$ concentration within GT1-7 cells (Kawahara *et al.*, 2000). GnRH release requires depolarization followed by $Ca^{2+\pm2}$ entry through voltage-sensitive $Ca^{2+\pm2}$ channels (Terasawa *et al.*, 1999). Individual GnRH cells exhibit $Ca^{2+\pm2}$ oscillations which are synchronized at intervals of nearly 50 min (Krsmanovic *et al.*, 1992; Terasawa *et al.*, 1999). Therefore, examination of $Ca^{2+\pm2}$ levels are able to infer the release of GnRH. Fura-2-acetoxymethyl (Fura-2 AM) (Figure 12) is a widely used UV-excitable fluorescent calcium indicator (Hyeon *et al.*, 2006). Using a xenon lamp and a shutter wheel, cells were excited successively with 340 and 380 nm UV light, and an emission fluorescence light of 510 nm was captured every 1 sec by a video camera attached to the microscope. The ratio of the emission from 340 nm excitation to 380 nm excitation is proportional to $Ca^{2+\pm2}$ concentrations (Terasawa *et al.*, 1999).

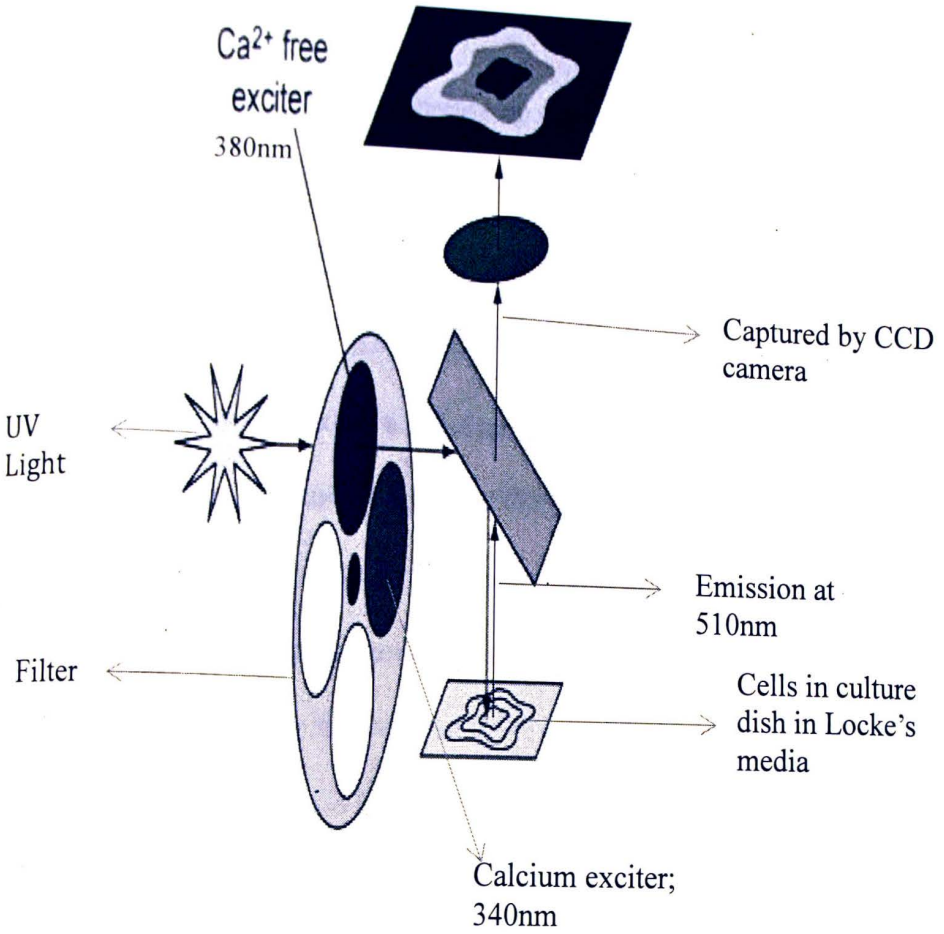

Figure 12. A diagram showing the process of fluorescent microscopy. The light source sends light rays to shutter attached with UV 340nm and UV 380nm filters, with the help of software the shutter shifts back and forth to 340nm and 380nm at a set autoexposure time. The transmitted light falls on sample that is arranged on stage and the emission light at 510 nm is recorded and captured by CCD camera. All data are sent to computer attached to the microscope.

Summary

GnRH is a neurohormone that is secreted by the hypothalamus and controls the release of LH and FSH from the anterior pituitary. The frequency of GnRH determines the ratio of LH and FSH release. Higher frequencies of GnRH favor the release of LH while lower frequencies favor the release of FSH. These hormones control the reproductive system in both males and females.

GnRH secretion is controlled by a pulse generator that is intrinsic to GnRH neurons. The pulses secreted by GnRH synchronize with time. GnRH neurons communicate through electrical synapses comprised of gap junctions. Gap junctions are made of hemi channel proteins known as connexins. Connexin 43 and connexin 26 are the predominant connexins found in GnRH neurons. A previous study showed that Cx43 may be involved in GnRH syncronization and not Cx26. In the present study, Cx43 specific blocker Gap 26 is applied to GT1-7 cells and the cells are treated with a calcium specific fluorescent dye fura-2AM, to study the calcium oscillations inside the cells.

Chapter 3 Materials and Methods

GT1-7 cell culture

GT1-7 cells were grown on 100mm poly-D lysine coated tissue culture dishes (BD Falcon, Franklin Lakes, NJ; Catalogue # P35GC-1.0-14-C) and maintained in Dulbecco's modified Eagle medium nutrient mixture (DMEM/F12; Invitrogen, Grand Island, NY; Catalogue # 12634-010) with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and Streptomycin (100 µg/ml). Cells were incubated at 37°C in a humidified 5% CO_2 environment and passaged when they reached approximately 70-90% confluence.

Calcium Imaging

Intracellular calcium measurement was done as previously described (Kim *et al.*, 2006). To prepare for imaging, approximately 20,000 cells were grown to 30% confluence on 35mm glass bottom dishes (Catalog # P35GC-1.0-14-C , MakTek, , Ashland, MA) and incubated for 24h-48hrs in the medium mentioned above. After incubation the cells were washed thrice in OptiMEM media (Catalog #11058-021 11320-033; Invitrogen, Grand Island, NY) and were incubated for another 24hrs.

The cells were washed twice with Locke's media containing (in mM): 154 NaCl, 5.6 KCl, 2.2 $CaCl_2$, 1.0 $MgCl_2$, 2.0 HEPES, 0.6 $NaHCO_3$, 10 D-glucose and 0.02 bacitracin. Control cells were imaged in 1 ml of Locke's medium, while test cells were imaged in 1ml of Locke's medium containing 30 µM Gap26 (VCYDKSFPISHVR;Catalog # 1950, TOCRIS Bioscience, Minneapolis, MN, diluted in locke's medium) which was added to the cultures and the cells ~~are~~ were incubated in the dark for 20hrs and 30 minutes at 37°C and 5% CO_2. After ~~the~~ incubation ~~the~~ cells were ~~are~~ treated with 10 µl (1mg/ml) of the fluorescent indicator, Fura-2 AM (Catalog#

F-1221, Molecular Probes, Junction City, OR) in dark and cells were ~~are~~ placed back into the incubator for about 30minutes.

Following incubation, cells were washed thrice with Locke's media in the dark and were placed on the Nikon FN1 upright microscope (Nikon Instruments, Tokyo, Japan) for calcium imaging. The microscope was prepared prior to placing the cells on the stage for imaging. First, the microscope and computer were turned on. Second, NIS elements, ANDOR program (NIS elements software, version: 3.2.1) was run on the computer to allow live imaging of the cells. The cells were set on the microscope stage, a drop of oil was added to the objective, and the cells were brought into focus using transmitted light. The cells were illuminated at 340 and ~~at~~ 380 nm and the fluorescence was examined using the eyepieces. Resting cells appeared dim at 340 nm and bright at 380 nm. Finally, the exposure time was set and was always maintained below 200 ms to prevent photo-oxidation. When the setup was complete, a test run was performed for 1min to determine if all the settings were correct. The actual runs were performed for 120 min and images were recorded. Stored images were analyzed using a CCD camera and software system controlled by a computer. Briefly, regions of interest (ROI's) were randomly selected and the fluorescence was recorded at the emission wave length of 510 nm from pixels of images obtained at the excitation wavelengths of 340 and 380 nm. As the Ca^{+2} concentration rises, the intensity of fluorescence at 340 nm rises and a fluorescence at 380 nm falls. The ratio of fluorescence seen in response to these two excitation wavelengths (340nm fluorescence/380 nm fluorescence) was used to determine the intracellular Ca^{+2} concentration. The recordings of ratio intensities of each ROI were collected for every 5sec interval, and stored as a MS Office Excel file. The graphs are constructed using the Excel data.

tatistical analysis

All data are presented as mean ± standard error. Statistical analysis was performed using the JMP statistical analysis software (JMP pro 10)._Data were analyzed using a two-tailed paired t-test. A P-value of 0.05 was considered to be statistically significant.

CHAPTER 4. Results

Optimizing the concentration Fura-2 AM

Before the effect of Cx43 could be examined, experiments were conducted to determine the minimum amount of Fura-2 AM to be used for the experiment. Fura-2 AM (1 mg) was dissolved in 1 ml DMSO, producing a 1 mM solution. Four volumes of this solution were tested 5 µl, 10 µl, 15 µl and 20 µl by adding them to GT1-7 cells bathed in 1 ml of Locke's medium (Figure13). These volumes correspond to total concentrations of 5, 10, 15, and 20 µM, respectively. The cells loaded with 5 µl of Fura-2 AM showed less background intensity and clearer cells, compared to the cells loaded with other concentrations of Fura-2 AM.

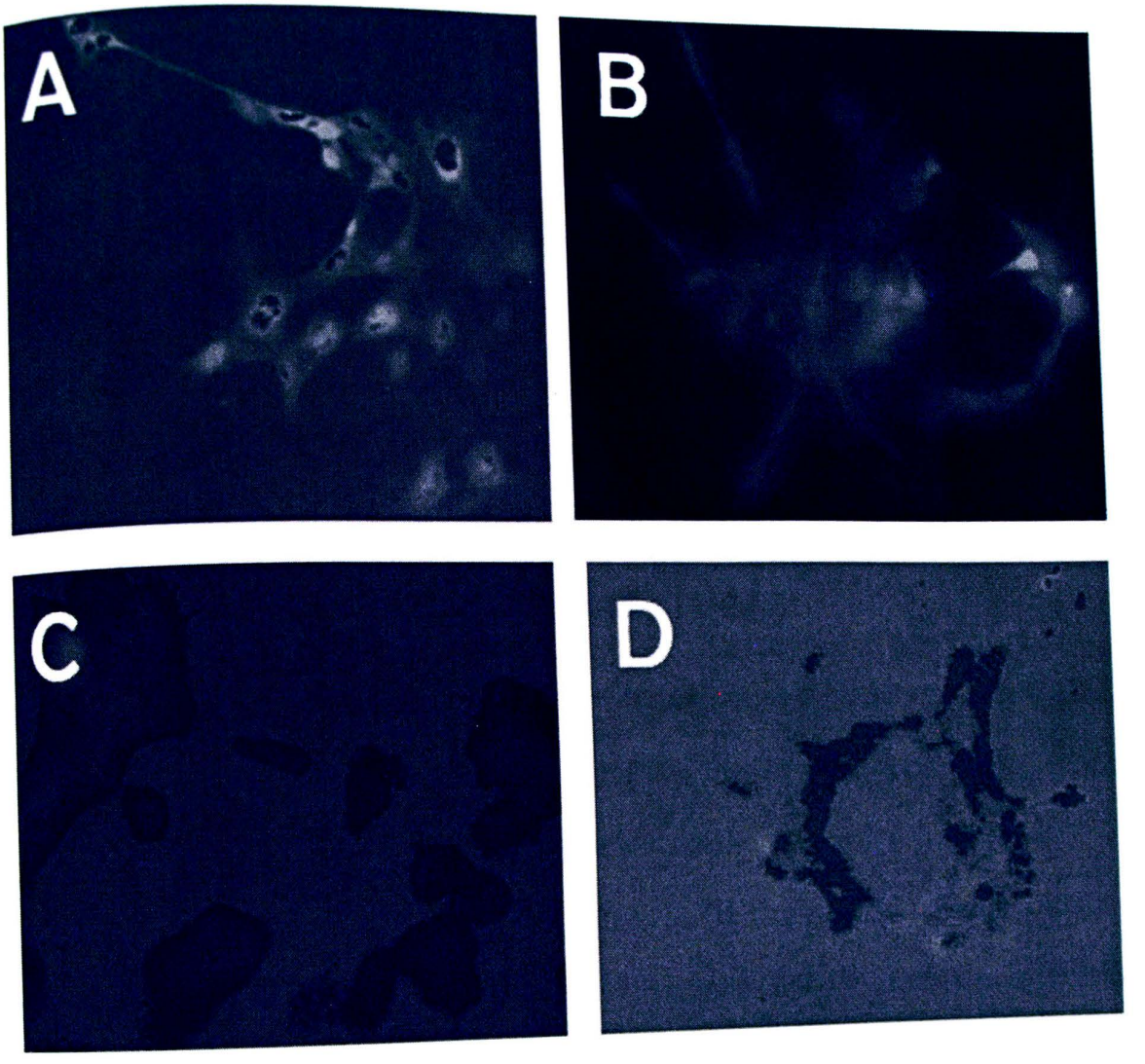

Figure 13. The effect of Fura-2 AM on GT1-7 cell image quality after excitation at 340 nm. A) Cells loaded with 5 µl of Fura-2 AM exhibited clear cytoplasms with less background intensity. B) Cells loaded with 10 µl of Fura-2 AM had clear cytoplasms and bright background intensity, which would interfere with the data analysis. C, D) Cells loaded with 15 µl and 20 µl of Fura-2 AM, respectively

Effect of Cx43 blockade on GnRH release

Intracellular Ca^{+2+2} rhythms were examined in adjacent GT1-7 cells to determine the involvement of Cx43 in intercellular communication. Three sets of experiments were conducted. Each set contained three control cells and three cells treated with the Cx43-specific blocker, Gap26. Control sample is the set of cells that are not treated with Gap 26, but are cultured under similar conditions as Gap 26 treated cells. In control samples instead of Gap26 we added Lockes medium, the medium in which Gap26 was dissolved. Figures 7-9 depict images of the control treatments and 10-12 depict the Gap26-treated cells. Panels A-C show the Fura-2 AM loaded cells while the pattern of Ca^{+2} oscillations is depicted in panel D.

The percentage of synchronized Ca^{2+2} peaks was calculated for each imaging run. 87.56 ± 0.52 % of Ca^{2+2} peaks were synchronized in control samples (Figure. 13, n=3, Run 1: 86.7%, Run 2: 88.5%, and Run 3: 87.5%). Ca^{2+2} peaks exhibited less synchronization in Gap 26-treated cultures ($p \leq 0.01$, Average: 9.17 ± 0.45%, n=3, Run 1: 13.3%, Run 2: 14.2%, and Run 3:0%). These results indicate that Gap 26 disrupted the synchronization of Ca^{+2} oscillations in the cells. This infers that the synchronization of GnRH secretion was also disrupted in GT1-7 cells. The frequency of Ca^{+2} oscillations in control sample ranged from 9-22 peaks/hr and that of Gap26-treated cultures ranged from 4-5 peaks/hr.

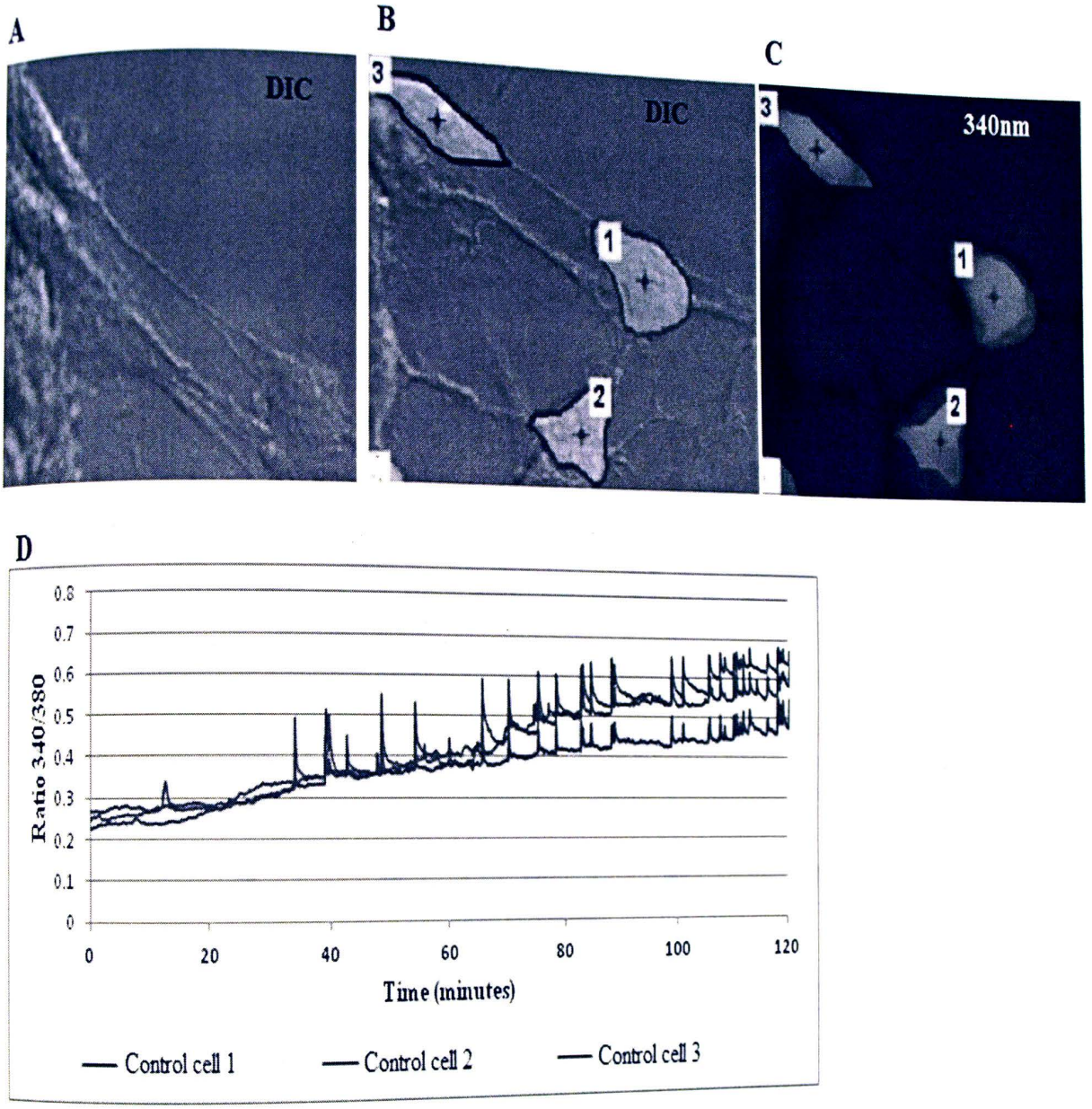

Figure 14. Synchronization of GT1-7 cells in control Run 1. A) Differential interference contrast (DIC) image of GT1-7 cells taken prior to the experiment using confocal microscope with 40X oil immersion objective lens. B) Three GT1-7 cells were selected using the imaging software. C) Fura-2 fluorescence resulting from excitation at 340 nm. D) Intracellular Ca^{+2} levels indicated by the fluorescence intensity ratio (340/380 nm) throughout the 120 minute experiment. Tracings 1, 2 and 3 depict cells 1, 2 and 3, respectively. An average of 86.7% of Ca^{2+2} peaks were synchronized

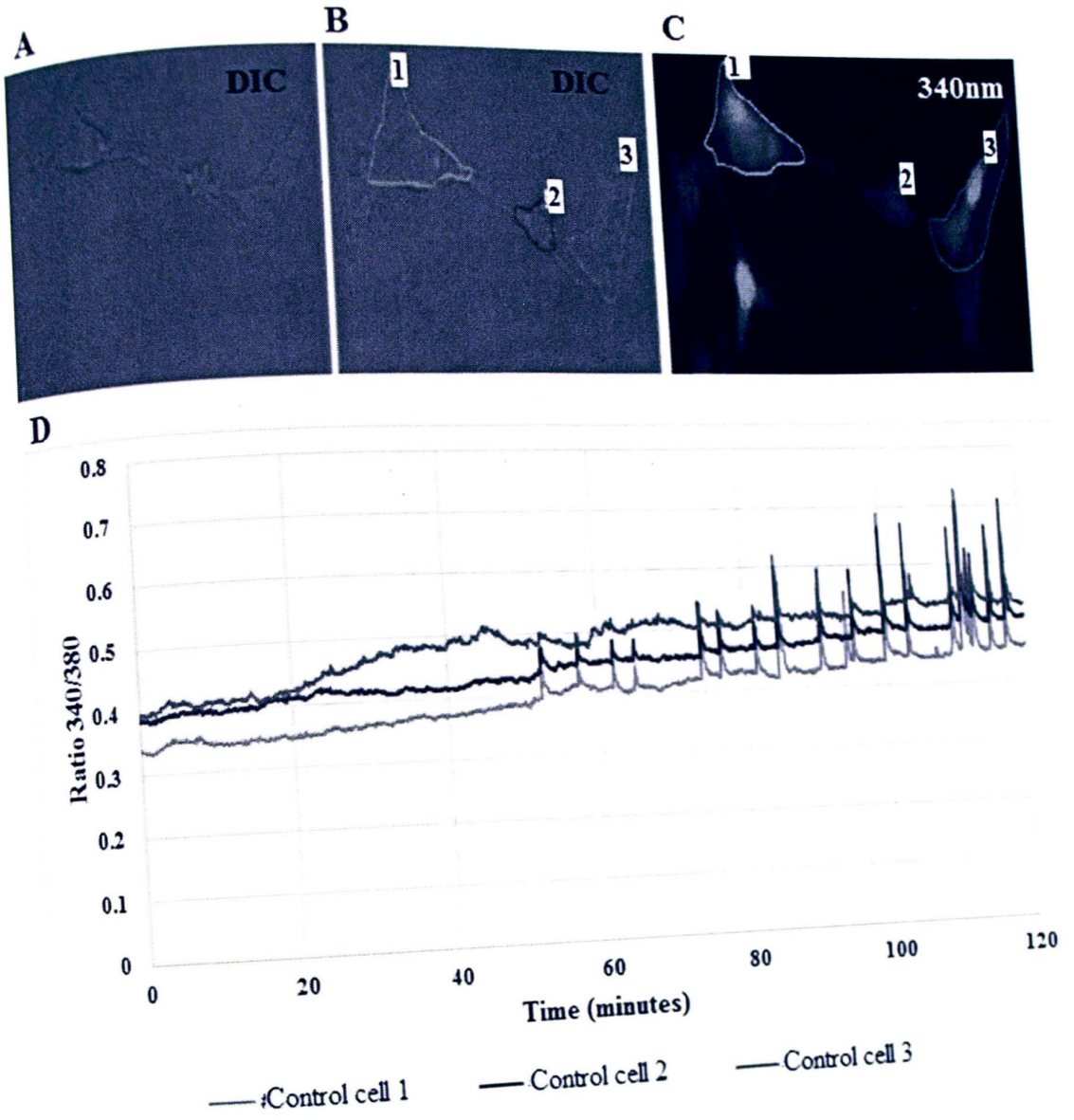

Figure 15.Synchronization of GT1-7 cells in control Run 2. A) Differential interference contrast (DIC) image of GT1-7 cells taken prior to the experiment using confocal microscope with 40X oil immersion objective lens. B) Three GT1-7 cells were selected using the imaging software. C) Fura-2 fluorescence resulting from excitation at 340 nm. D) Intracellular Ca^{+2} levels indicated by the fluorescence intensity ratio (340/380 nm) throughout the 120 minute experiment. Tracings 1, 2 and 3 depict cells 1, 2 and 3, respectively. An average of 88.5% of $Ca^{2+\pm2}$ peaks were synchronized.

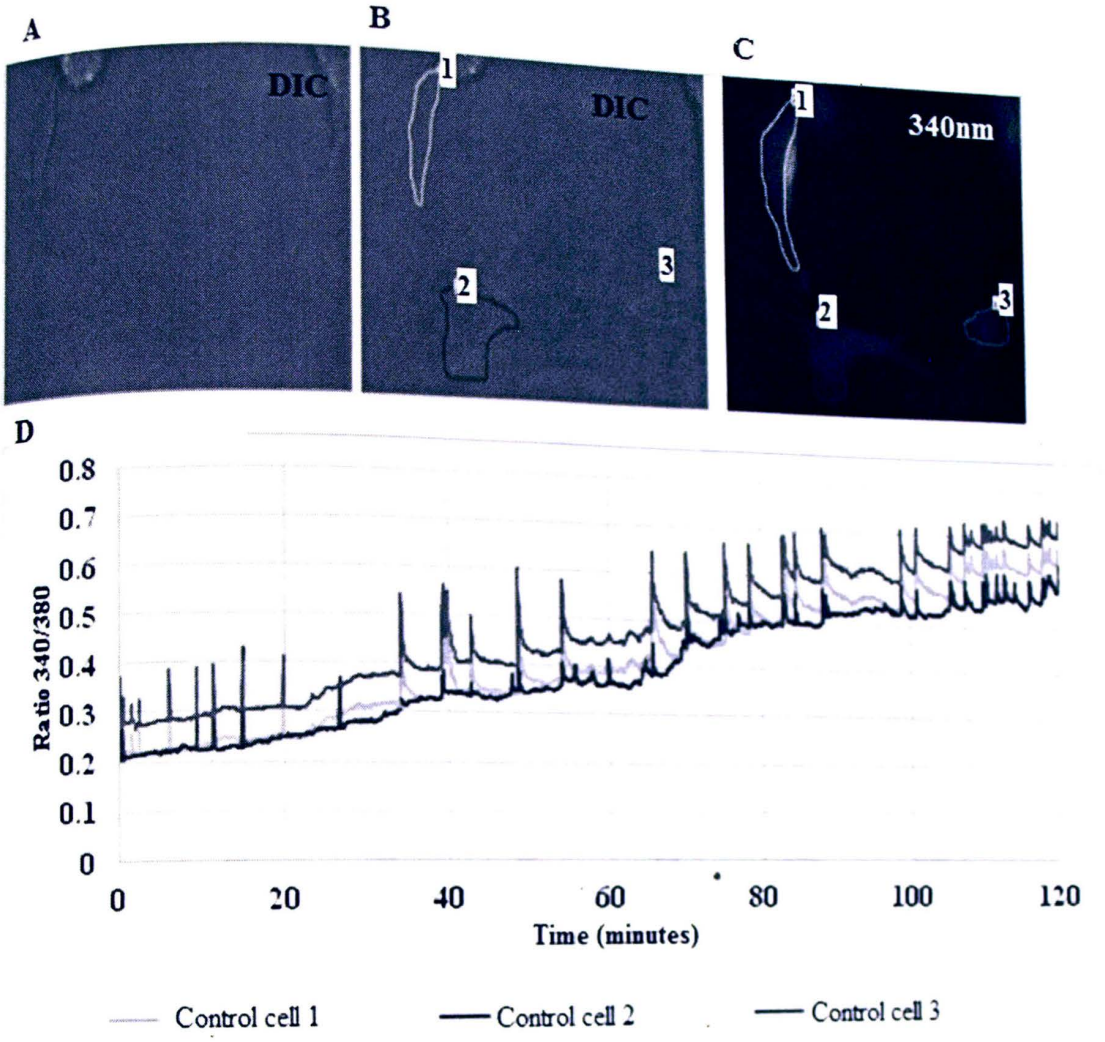

Figure 16. Synchronization of GT1-7 cells in control Run 3. A) Differential interference contrast (DIC) image of GT1-7 cells taken prior to the experiment using confocal microscope with 40X oil immersion objective lens. B) Three GT1-7 cells were selected using the imaging software. C) Fura-2 fluorescence resulting from excitation at 340 nm. D) Intracellular Ca^{+2} levels indicated by the fluorescence intensity ratio (340/380 nm) throughout the 120 minute experiment. Tracings 1, 2 and 3 depict cells 1, 2 and 3, respectively. An average of 87.5% of $Ca^{2+/+2}$ peaks were synchronized.

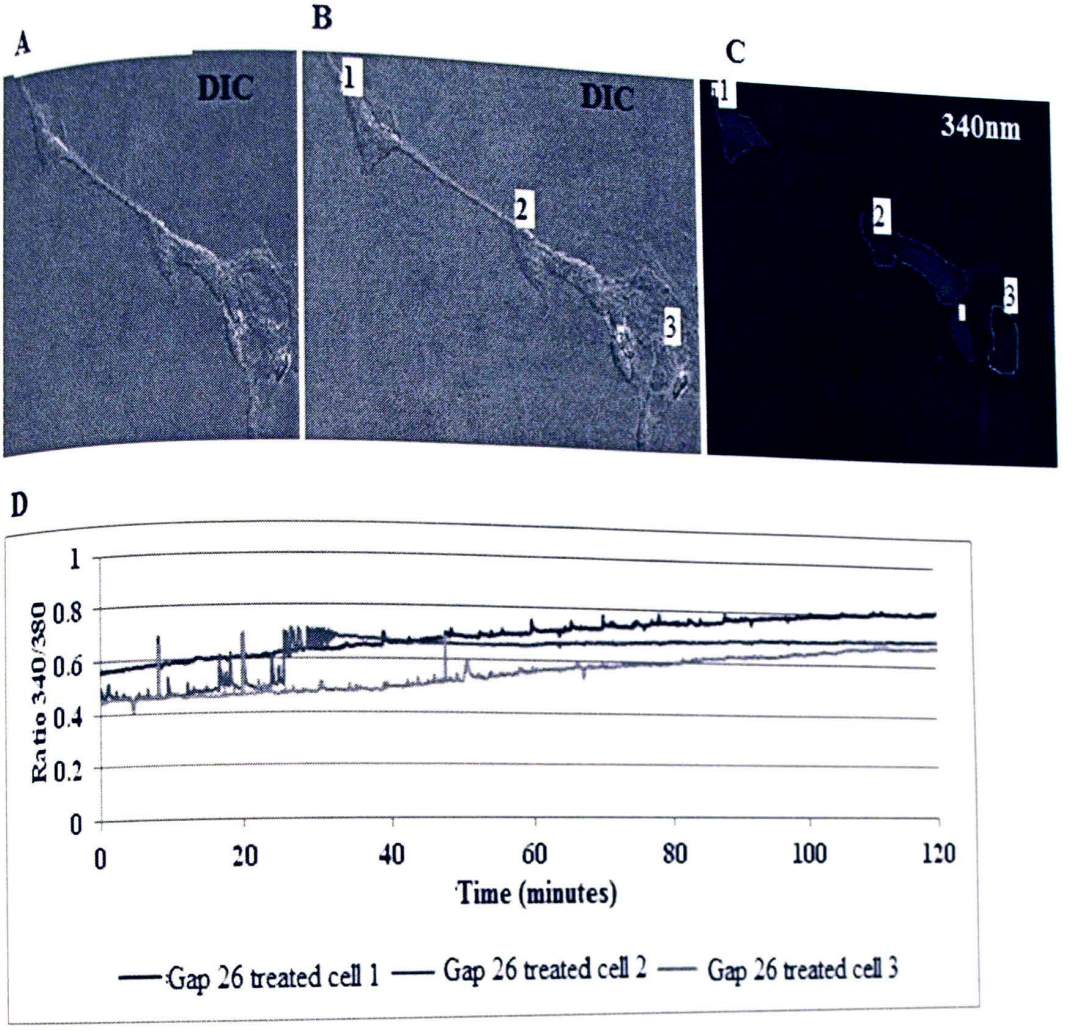

Figure 17. Synchronization of GT1-7 cells following GAP 26 treatment (Run 1). A) Differential interference contrast (DIC) image of GT1-7 cells taken prior to the experiment using confocal microscope with 40X oil immersion objective lens. B) Three GT1-7 cells were selected using the imaging software. C) Fura-2 fluorescence resulting from excitation at 340 nm. D) Intracellular Ca^{+2} levels indicated by the fluorescence intensity ratio (340/380 nm) throughout the 120 minute experiment. Tracings 1, 2 and 3 depict cells 1, 2 and 3, respectively. An average of 13.3% of $Ca^{2+\pm2}$ peaks were synchronized.

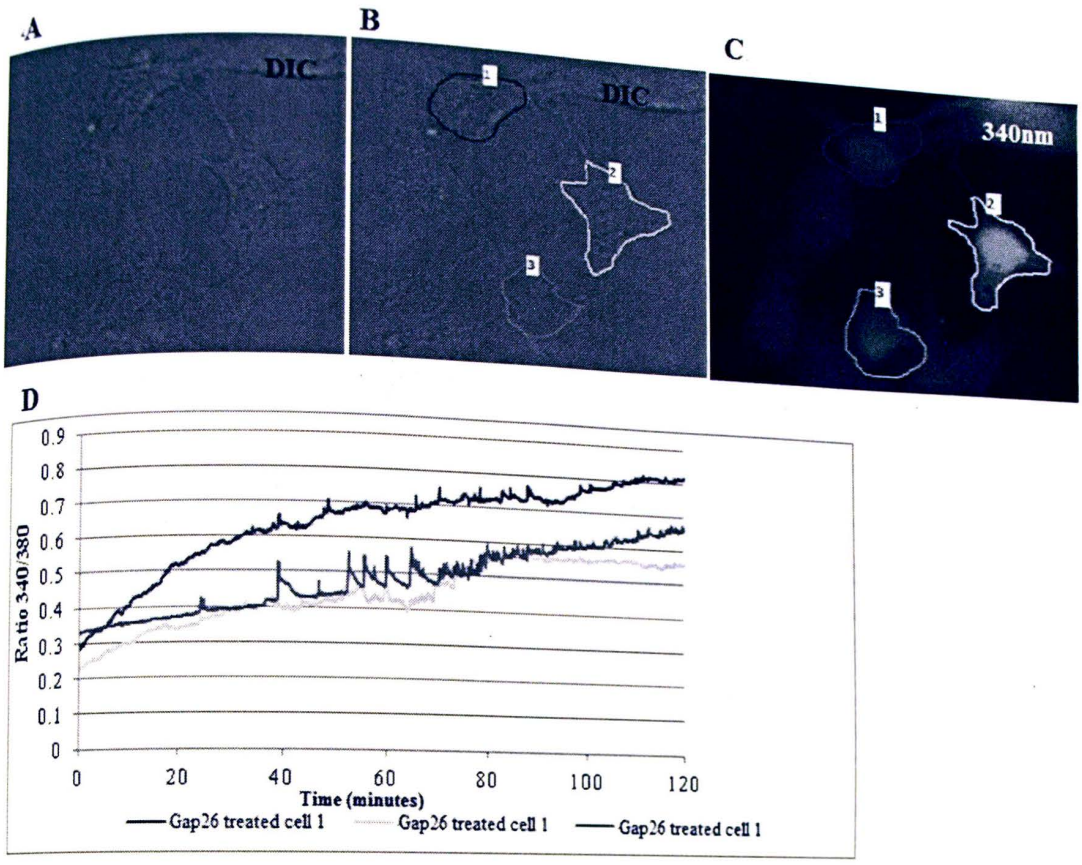

Figure 18. Synchronization of GT1-7 cells following GAP 26 treatment (Run 2). A) Differential interference contrast (DIC) image of GT1-7 cells taken prior to the experiment using confocal microscope with 40X oil immersion objective lens. B) Three GT1-7 cells were selected using the imaging software. C) Fura-2 fluorescence resulting from excitation at 340 nm. D) Intracellular Ca^{+2} levels indicated by the fluorescence intensity ratio (340/380 nm) throughout the 120 minute experiment. Tracings 1, 2 and 3 depict cells 1, 2 and 3, respectively. An average of 14.2% of $Ca^{2+\pm2}$ peaks were synchronized.

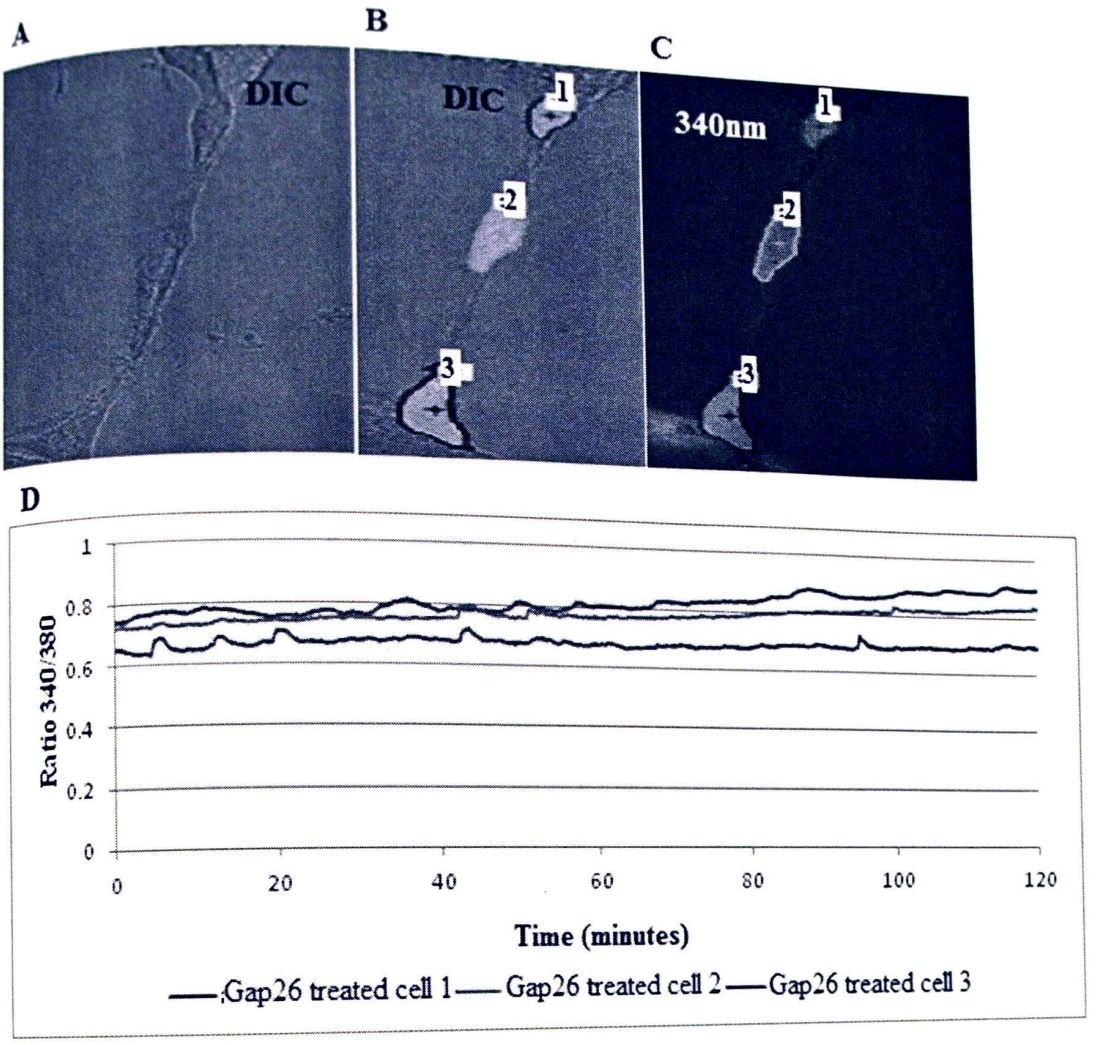

Figure 19. Synchronization of GT1-7 cells following GAP 26 treatment (Run 3). A) Differential interference contrast (DIC) image of GT1-7 cells taken prior to the experiment using confocal microscope with 40X oil immersion objective lens. B) Three GT1-7 cells were selected using the imaging software. C) Fura-2 fluorescence resulting from excitation at 340 nm. D) Intracellular Ca^{+2} levels indicated by the fluorescence intensity ratio (340/380 nm) throughout the 120 minute experiment. Tracings 1, 2 and 3 depict cells 1, 2 and 3, respectively. None of the Ca^{2+2} peaks were synchronized.

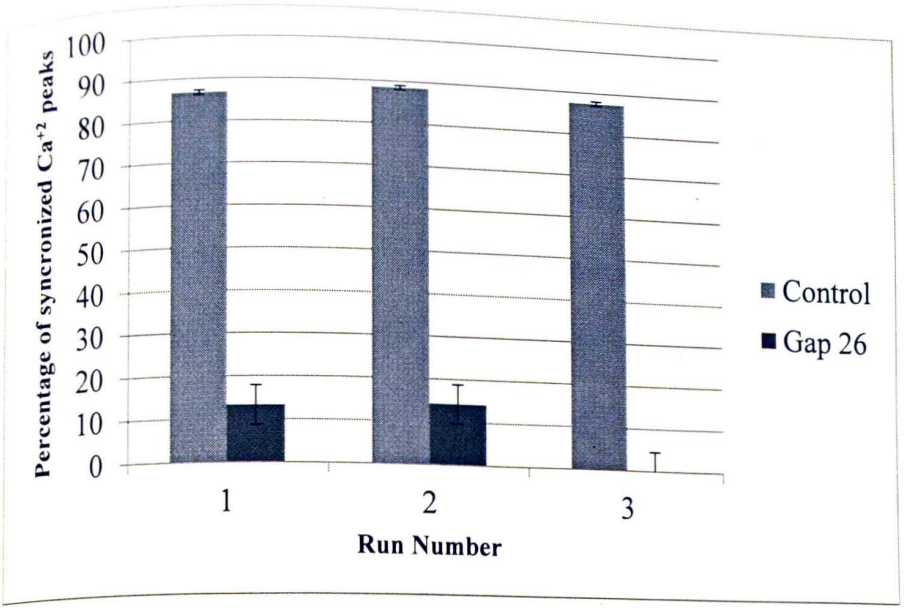

Figure 20: The percentage of synchronized Ca^{+2} oscillations in cells from three different runs (1, 2 and 3) of both control and 30μM Gap-26 treated samples. Data are presented as mean ± standard error. The percentage of synchronized $Ca^{2+\pm2}$ oscillations differed significantly between control and GAP 26-treated cells ($p \leq 0.0039$), inferring that the synchronization of GnRH release by GT1-7 cells is mediated by Cx43.

The goal of the research was to discover if gap junctions comprised of Cx43 have a role in the synchronization of Ca^{2+} oscillations within GT1-7 cultures. Since Ca^{2+} influx is associated with exocytosis (Kerboeuf *et al.*, 1990) these results may infer something about the pattern of GnRH secretion. The results show that Cx43 gap junction channels are involved in communication between the GT1-7 cells. Our results confirm the postulate of a previous study which stated that there is intriguing possibility that, Cx43 related gap junctional contacts between neurons may serve as a structural link that would enable a scattered GnRH nerve cell population to communicate during GnRH pulsatile release (Bose *et al.*, 2010). From the studies of Bose *et al.*,(2010) it is clear that both Cx43 and Cx26 are expressed in GT1-7 cell cultures. They measured the expression of Cx43 and Cx26 in GT1-7 cells and found that the expression of Cx43 increased in GT1-7 cells from 2 days to 7 days of culture through a series of experiments. First, a greater number of Cx43 immunoreactive cytoplasmic densities were found in cells from day 7 cultures when compared to those of day 2 cultures indicating that an increase in the number of Cx43 associated gap junctions occurred between these two culture periods, Cx26 immunoreactive cytoplasmic densities were found constant. The second series of experiments demonstrate a change in Cx43 mRNA occurring with time of culture. An increase was detectable at 4 days and appeared to return to beginning levels by day 6, there was no detectable increase in Cx26 mRNA. Finally, Cx43 protein was also found to change in lysates from cells cultured from 2 to 6 days of culture. This increase was not found to be significant until day 6. A rise in Cx43 protein levels following an earlier rise in Cx43 mRNA levels indicated that new synthesis was underlying these Cx43 changes but not Cx26 changes. From the following data it is evident that Cx43 but not Cx26 is necessary for synchronized secretary activity in these GT1-7 cultures and

raise the possibility that Cx43 related gap junctions may be important in GnRH neuronal coordination in the hypothalamus (Bose *et al.*, 2010).

Gulinello *et al.* (2005) reported increased Cx43 protein expression in the preoptic area of female rats following treatment with estrogen, progesterone, or a combination of the two steroids. The preoptic area is also where GnRH neurons are located (Leranth *et al.*, 1985). In contrast, Cx43 expression decreased in male rats treated with estrogen and progesterone (Gulinello *et al.*, 2005). These results indicate that steroid hormones differentially regulate Cx43 expression in specific regions of the brain in a sexually dimorphic manner. Since GnRH is important for the control of the estrous cycle important for proper reproductive function in both male and female rats. Bose *et al.* (2010) utilized Cx43 siRNA to show that GnRH neuron synchronization requires functional Cx43-related gap junctions.

Gap junctions function by letting signaling molecules move down their concentration gradient from one cell to another. It is well recognized that almost every type of gap junction is permeable to monovalent cations, and to a lesser extent, anions (Harris *et al.*, 2007), second messengers, polypeptides and siRNA (Niessen *et al.*, 2000; Goldberg *et al.*, 2004; Valiunas *et al.*, 2005; Bedner *et al.*, 2006). Recent studies demonstrated that gap junctions formed from Cx43 have selectivity for negatively charged larger solutes (Kanaporis *et al.*, 2011). Interestingly, these channels were able to effectively transfer cAMP between cells. This raises the possibility that the passage of cAMP through Cx43 may allow cells to coordinate their function (Kanaporis *et al.*, 2008).

A growing body of evidence suggests that cAMP is involved in the regulation of GnRH pulse frequency. Forskolin, a direct activator of adenyl cyclase, increases GnRH secretion two fold in GT1-7 cells (Wetsel, 1995; Roy *et al.*, 2002). Presumably, is does this by promoting cAMP formation and increasing intracellular Ca^{2+} concentration (Krsmanovic *et al.*, 2001; Nunnez *et al.*, 2000). The frequency of both spontaneous Ca^{2+} oscillations and pulses of GnRH release were reduced following the catabolism of cAMP through constitutive expression of cAMP-dependent phosphodiesterase (cAPD) in GT1 cells (Yoshida *et al.*, 2003). GnRH pulse frequency was also lowered in castrated transgenic rats in which cAPD was continuously expressed (Paruthiyil *et al.*, 2002). Thus, it appears to be quite possible that cAMP, transferred from one neuron to another via Cx43-related gap junctions, could stimulate GnRH pulse activity in some manner by inducing rapid ionic changes in Ca^{2+} and/or other ions. Such a process would be able to coordinate the GnRH secretory activity of connected cells, forming the basis for a functionally synchronized network of GnRH neurons.

The ability of neurons to initiate or maintain gap junctional communication with each other would require contact. At first glance, it appears that the likelihood of contact between GnRH neurons would be extremely low in light of the scarce scattering of GnRH neurons in the hypothalamus. GnRH neurons are uni- or bipolar, with extremely long dendrites (Campbell *et al.*, 2005; Ybarra *et al.*, 2011). In fact, Roberts *et al.* (2008) suggest that distal dendrites of GnRH neurons appear to be the primary site of spike initiation in these cells. A dendrite to dendrite or dendrite to cell body contact could theoretically provide a point of communication between these scattered neurons in the hypothalamus. The possibility that gap junctions may serve as this point of contact in the GnRH neuron is suggested by observations that other neuronal networks in the mammalian brain can be mediated by gap junctional contact between

neurons (Bennett *et al.*, 2004; Connors *et al.*, 2004; Hormuzdi *et al.*, 2004). Thus, neuronal communication via gap junctional contact between dendrites or dendrites and cell bodies may form the basis for a GnRH neuronal synchronized network.

The loss of synchronization of secretary activity of GnRH following the treatment of GT1-7 cells with Cx43 siRNA, clearly demonstrates the role of Cx43 in GT1-7 cell synchronization (Bose *et al.*, 2010). In the present study, the role of Cx43 in GnRH cell synchronization was studied. The loss of synchronization of pulsatile secretion of GnRH was observed, by measuring the intracellular Ca^{+2} oscillations in the cells, when the GT1-7 cells are treated with Cx43 specific blocker Gap26. The 87.56 ± 0.52 % of $Ca^{2+\pm2}$ peaks were synchronized in control samples, while only 9.17 ± 0.45 % were synchronized following treatment with GAP 26, indicating the loss of GnRH synchronization in GT1-7 cells. These results also confirm that Cx43 plays a key role in synchronization process of GT1-7 cells, as the cells treated with Cx43 blocker Gap26 showed the loss of synchronization.

In summary, our results clearly demonstrate that Cx43 is central to the synchronization of GT1-7 cells. These findings confirm that Cx43 related gap junctional contacts between hypothalamic neurons is important for GnRH pulse generation. Our study provides valuable insight into the mechanisms that underlie the GnRH pulse generator. Cx43 gap junctions serve as a structural link that would enable a scattered GnRH nerve cell population to communicate, coordinate, and release a "pulse" of GnRH from the hypothalamus.

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