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ADMINISTRATION OF CONNEXIN-43 siRNA ABOLISHES SECRETORY PULSE SYNCHRONIZATION IN GNRH CLONAL CELL POPULATIONS

Sudeep Bose¹, Gilles M. Leclerc², Rafael Vasquez-Martinez³, and Fredric R. Boockfor¹

¹Department of Cell Biology and Anatomy, Medical University of South Carolina, 173 Ashley Avenue, Charleston, South Carolina

²Department of Pediatrics, Hematology and Oncology, University of Miami, Miami, Florida

³Department of Cellular Biology, Physiology and Immunology, University of Cordoba, Cordoba, Spain

Abstract

GnRH is released from hypothalamic neurons in coordinated pulses, but the cellular basis for this process is poorly understood. Previously, we found that secretory pulses from GT1-7 cells became synchronized with time in culture. Using this culture model, we investigated whether the gap junction proteins connexin43 (Cx43) and/or connexin26 (Cx26) are involved in this synchronization. Our results reveal that cytoplasmic densities immunoreactive for Cx43, and mRNA or protein for Cx43 increase with time in culture. Also, microinjection of day 3 cultures with siRNA for Cx43 abolished synchronized activity at day 7. Interestingly, cytoplasmic plaques, mRNA, or protein for Cx26 remained stable with culture time and Cx26 siRNA administration did not alter secretory activity. Our findings demonstrate that Cx43, but not Cx26 is necessary for synchronized secretory 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.

Keywords

rhythmicity; single-cell imaging; GT1-7 cells; microinjection; Real-time RT-PCR; siRNA

Introduction

It is generally accepted that GnRH is secreted in pulses from the hypothalamus [1]. These pulses reach the pituitary through the portal vasculature and stimulate the release of LH and FSH, also in a pulsatile fashion. In fact, the intermittent stimulation of gonadal and accessory sex tissues by these gonadotropin pulses is believed to be required for proper reproductive function [2,3]. The cellular basis for this pulse activity is not completely understood. A growing

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Address all correspondence and requests for reprints to: Dr. Fredric R. Boockfor, Laboratory of Molecular Dynamics, Department of Cell Biology and Anatomy, Medical University of South Carolina, 173 Ashley Avenue, Charleston, SC 29425. Phone: (843) 792-3526 FAX: (843) 792-0664. Boockfor@musc.edu.

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body of evidence suggests that the release of GnRH in pulses is an intrinsic property of GnRH neurons. This was suggested when pulsatile release of GnRH was observed from isolated hypothalamic fragments and cultures obtained from hypothalamic tissue dispersions [1,4-6]. With each of these preparations, GnRH release occurred in pulses even though they were devoid of outside input. The confirmation that hypothalamic neurons possess intrinsic pulsatile activity occurred with the development of GT1 cultures [7]. These cultures consisted only of GnRH-expressing cells and were found to release GnRH in a rhythmic fashion [8-11]. Thus, on the basis of these observations, it appears that GnRH neurons have intrinsic pulsatility with regard to GnRH secretion.

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. Until recently, very little information was available concerning this process. This was due mainly to the difficulty of studying these GnRH cells that are few in number and scattered throughout the hypothalamus [12]. By using GT1-7 clonal cell cultures in our laboratory in conjunction with a method that enables measurement of individual cell secretory activity, we were able to demonstrate that pulsatile function among GT1-7 cells becomes synchronized with time in culture [13]. Moreover, our observations of LY-dye transfer between these synchronized cells and a loss of this synchrony following treatment with octanol, a gap junctional blocker, suggested that an important part of this communication involves gap junctions.

Gap junctions are very complex structures well-recognized for their role in cell-to-cell communication. Each gap junction is composed of a specific type or combination of protein subunits termed connexins (Cx)[14]. The type of Cx protein comprising a particular gap junction is very specific and in conjunction with other factors confers the functional parameters of that junction [15]. In fact, at least two types of Cx proteins have been associated with GnRH neurons, Cx43 and Cx26 [16,17], also termed Gja1 and Gjb2, respectively. Our goal in the following study was to determine whether one or both of these proteins may play a role in the synchronization of GnRH neurons in our GT1-7 cultures.

Materials and Methods

Cell Culture

GT1-7 cells (provided by Dr. Richard Wiener, University of California, San Francisco, CA) were maintained as reported [13]. In short, cells were cultured in high glucose Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc, Logan Utah) and antibiotics (100U/ml penicillin, 100 ug/ml streptomycin and 2.5 ug/ml Fungizone; Invitrogen, Carlsbad, CA) in saturated atmosphere of 5%CO₂/95% air at 37 C. For fluorescent measurement of secretion, the detached cells were plated onto Matrigelcoated (1:25: BD Biosciences, Bedford, MA) 25-mm coverslips (Bellco Glass, Inc, Vineland, NJ) containing alphanumeric grids for re-identification of specific locations on each coverslip. The cells were kept in culture for 2-7 days before experimental manipulation.

Measurement of fluorescent membrane densities or "plaques"

Cells grown on glass coverslips for 2 and 7 days were fixed with B5-buffered formalin for 45 min. The coverslips were rinsed and the cells subjected to immunofluorescent detection of Cx43 or Cx26. Fixed cells were incubated with rabbit anti-mouse Cx43 (anti-Cx43; Sigma, St Louis, MO) (1:3000) [18-20] or Cx-26 (anti-Cx26; Zymed, San Francisco, CA) [21,22] (1:2000) at 4 C overnight. The next day the coverslips were washed with 0.3% Triton X-100, incubated with Fluorescein (FITC)-goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch, 1:1000) for 2h, washed again, and mounted. Using a 10X objective (total magnification X100), a portion

of each coverslip containing cells that were attached, associated with surrounding cells, and free of debris was chosen. The magnification was changed and the cells imaged for fluorescence emissions. In our experiments, cells were selected under light and then viewed with fluorescence. Because of this, there was limited information available during the cell selection process occurring under light that would introduce bias relating to the type and degree of fluorescent emission. Fluorescence was visualized using an Axiovert fluorescent microscope (Carl Zeiss, Jena Germany) fitted with a Carl Zeiss Fluor 40 × oil immersion objective (numerical aperture+ 0.9). The cells were epi-illuminated at 490 nm. Image acquisition was controlled using Metafluor PC-software (Universal Imaging Corp., West Chester, PA) and fluorescence emissions (528nm) from the cells were captured using a CCD camera (C-4880-80), Hamamatsu, Hamamatsu City, Japan) running in 8-bit mode. The images acquired were transferred to off-line storage for analysis at a later time. Fluorescent plaques were quantified using Scion Image software (Scion Corporation, Fredrick, Maryland). During this process, images collected following immunostaining for Cx43 and Cx26 at both 2 and 7 days were analyzed using uniform thresholding indices. A particle size setting of 2-4 pixels was used to distinguish cytoplasmic densities. For preabsorption controls, anti Cx-43 and anti Cx-26 that were first incubated overnight at 4 C with mouse Cx-43 control peptide (#A6 678; Chemicon, Billerica, MA) and mouse CX-26 control peptide (#Cx26-P; Biotrend; Cologne, Germany), respectively, were then added to the fixed cells and processed the same way.

Real-time Reverse Transcription-PCR (RT-PCR)

Real-time RT-PCR was performed to measure the relative expression of Cx43 and Cx26 mRNA in GT1-7 cells for 2 to 7 days in culture. Approximately, 200,000 cells were seeded onto 35mm dishes in triplicate and grown for 2 to 7 days. Total RNA was extracted using an RNAeasy Microkit according the manufacturer's instruction (Qiagen, Inc. Valencia, CA). Then, RNA (0.5µg) was reverse transcribed in a 20µl reaction volume using a cDNA synthesis kit (Quanta Biosciences, Inc, Gaithersburg, MD) and then diluted to 100µl. Real-time PCR was conducted using SensiMixPlus SYBR & Fluorescein kit (Quantace, Inc, Taunton, MA) on an iCycler machine (BioRad) using 5µl of the cDNA in triplicate with mouse hypoxanthine-guanine phosphoribosyl transferase (hPRT) as the internal control. Briefly, amplification reactions were performed by a multiple step thermal cycling method that consisted of 3 min at 95C followed by 40 cycles at 95C each for 15 sec, 1 min at 58C, and finally 30 sec at 72C. Real-time RT-PCR analyses were conducted in triplicate for each sample. Relative changes in the levels of connexins were determined by the comparative C_T method [23] that enables the normalization of cDNA in each sample to an internal control (i.e. hPRT) and comparison of sample to sample. As a negative control, reactions devoid of cDNA template were also performed. The primer pairs used for Cx43 were 5'(F)-GTGCCGGCTTCACTTTCA and '(R)-GGAGTAGGCTTGGACCTTGTC and for hPRT 5'(F)-ATCATTATGCCGAGGATTTGGA and 5'(r) CACACAGAGGGGCACAATGT were designed through the Universal Probe Library Design Center (Roche Applied Science, Indianapolis, IN) and purchased from Fisher Scientific Corp (Pittsburgh, PA). C26 primers (cat # PPM28155A) were purchased from SA Biosciences (Frederick, MD).

Preparation of Total Cell Protein Extract and Western Analysis

For protein extracts, GT1-7 cell cultures were detached from the culture plates by exposure to 0.05% trypsin-EDTA (Sigma) for 5 min, and centrifuged at 1200 rpm for 10 min. The pellet was resuspended in ice-cold PBS containing 0.1% protease inhibitor cocktail (Sigma), and centrifuged at 1200 rpm for 5 min at 4 C. Cells were lysed in lysis buffer using mammalian lysis buffer kit (Sigma) containing 1 mM DTT and 0.1% protease inhibitor cocktail. Lysates were incubated for 15 min on ice followed by centrifugation at 13,000 rpm for 20 min at 4 C. Total protein was recovered in the supernatant and concentrations were estimated by Bio-Rad assay and stored in aliquots at -80 C. For Western analysis, 35 µg of protein sample was mixed

with 4x NuPAGE LDS buffer containing 1 mM β -mercaptoethanol, boiled for 5 min followed by separation on a 4-12% gradient NuPAGE gel (Invitrogen, Carlsbad, CA). Proteins were transferred from the gel to a PVDF membrane followed by blocking for 2-3 hours at room temperature in 5% non-fat dried milk in TBS-T buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20). Blots were then probed with anti-Cx43 (cat. # C6219, SIGMA) or anti-Cx26 (cat. # 71-0500, Zymed) antibody at 1:1000 dilutions. After washing, the membranes were incubated with anti-rabbit antibody conjugated to alkaline-phosphatase (AP) (dilution 1:5000; Thermo Fisher Scientific, Inc, Rockford, IL), and the signal was visualized using CDP-Star chemiluminescence reagents (Perkin Elmer, Waltham, MA). As a control, blots were stripped and re-probed with mouse anti- α -tubulin or β -actin primary antibodies (both at 1:1000; Sigma). The protein-specific signals were measured densitometrically using the Image J program (developed by NIH and available at <http://rsb.info.nih.gov/ij/>). With this program, the protein signal of a target band is determined and compared to the intensity of the band of the housekeeping protein from the same sample.

Analysis of siRNA administration on FM1-43 fluorescence in GT1-7 cultures

SiRNA reagents against Cx26 (sc-37051), Cx43 (sc-35091) and scrambled siRNA (4618G, Ambion) were obtained from Santa Cruz Biotechnology, Inc, (Santa Cruz, CA). First, we confirmed the effectiveness of these reagents in GT1-7 cultures. Cells were seeded at a density of 250,000 per 35 mm dish in triplicate in antibiotic-free medium and grown overnight at 37 C before transfection. The next day, cells were transfected with 50nM siRNA using siPORT amine (Applied Biosciences/Ambion, Austin, TX) transfection agent in optimum-plus reduced serum medium overnight at 37 C according to manufacturer's protocol and reports of others [24]. Briefly, 5ul of si-PORT-Amine or siRNA were diluted separately in 100ul of OPTI-MEMI medium per 35mm dishes at room temperature for 10 min under dark condition. Then diluted transfection agent was mixed with the diluted siRNAs and incubated for 10min at room temperature. The medium covering the adhering GT1-7 cells were discarded from the 35 mm dishes followed by addition of the siRNA/transfection agent complexes. Finally, the volume was adjusted up to 2 ml with OPTI-MEMI. The next day, medium was replaced by normal high glucose containing DMEM supplemented with 10% calf serum and antibiotics and the cells grown for an additional 48 h period at 37 C. At the end of the culture period, cells exposed to these siRNA reagents were washed with 1X PBS containing 0.1% PI cocktail, harvested and total cell extract prepared was subjected to Western analysis.

Cells plated onto gridded coverslips were microinjected with these siRNA reagents against scrambled control sequences, Cx-43, or Cx-26, on day 3 of culture. Each coverslip was visualized with a light microscope at 100X magnification. A grid area was chosen that contained an attached monolayer of cells (greater than 80% confluent) and was free of debris or obvious structural defects (such as scratches or disruptions that may have occurred during cell seeding or culture). The injection was performed at a 400X magnification and all cells in the grid were injected. Each coverslip was injected with a single reagent at 2 μ M using a semi-automated micromanipulator and injection system (Eppendorf Microinjector 5242, Beckman Instruments, Inc., Westbury, NY)[25]. Conditions for injections were maintained at constant time (0.4 sec) and pressure (50-70hPa) for consistent delivery to each cell. All cells in a chosen grid area were injected. The coverslips were placed back into culture and incubated for an additional 4 days. On the day of analysis, the same region of the coverslip that was previously injected was re-identified by the alphanumeric grid pattern and the cells evaluated for secretory pulse activity as described [13]. In short, the cells were exposed to the fluorescent dye FM1-43 [N-(3-triethylammoniopropyl)-4-(p-dibutylaminostyryl)piradium dibromide], 2 μ M, (Invitrogen, Inc.), for 30 min at 37 C and the coverslips containing the cells were mounted in Sykes-Moore chambers (Bellco Glass, Inc, Vineland, NJ). The assembled chambers were placed on a temperature-controlled stage of an Axiovert microscope (Carl Zeiss, Jena,

Germany). The cells were epi-illuminated at 490nm for 100-500 msec every 5 min for 4-8 h and images were recorded from the microinjected cells. Image acquisition was controlled using Metafluor PC-software and fluorescent images were captured using a CCD camera (Hamamatsu, Hamamatsu City, Japan). Regions of the same field devoid of cells were selected for continuous monitoring of the background throughout each experiment. In light of the fact that the illumination intensity was reduced to less than 5% by placing filters in the light path and the absorption spectrum of FM1-43 (490nm) approached visible light, the phototoxic effects with prolonged exposure were minimal. The FM1-43 results were analyzed as described previously [13]. Briefly, the fluorescent signal tended to increase continuously throughout an experiment because the FM1-43 was maintained in the imaging medium. Each secretory event results in the addition of the secretory granule membrane to the cell membrane, exposing it to the dye in the medium. This is followed by reabsorption of this excess membrane into the cell, increasing the intracellular fluorescent signal. As the dye is incorporated in this manner, the fluorescent profile exhibits a stair-step increase in fluorescent signal. These increases in FM1-43 uptake were resolved by transforming the data to a differential over time (dy/dt ; rate of dye uptake), which yielded profiles with identifiable upstrokes and downstrokes that could be analyzed by the PULSAR program (NICHHD, Bethesda, MD). Pulse amplitude was calculated from the original datasets as the difference between the areas under the curve defined by the three points following and preceding a pulse. Rises greater than 5% were considered as significant changes in FM1-43 incorporation. To avoid false positives, we did not consider single point peaks as pulses in any of the FM1-43 profiles analyzed. With this approach, the presence, intensity, and duration of pulse activity could be determined over time in individual cells.

In order to assess the degree of pulse synchronization among cells within groups of neurons, we compared the extent of related events observed with that expected if pulse coincidence were random. This was accomplished using an algorithm model that consisted of numeric channels corresponding to the number of cells in a given experiment. The dataset generated for each numeric channel was composed of a numeric series corresponding to the number of 5-min points in the related dataset and each time point in the model was assigned a value based on the probability that a pulse would occur at that moment. Thus the data set generated for the random model paralleled that observed for neurons, except that pulse occurrence was determined by random coincidence. This model provided a measure of the extent to which random coincidence could be expected to occur during an experiment. Using this approach, the predicted probability that more than 50% of the cells would display a pulse within two adjacent 5 min periods was low and approached zero. This 50% probability was set as a cutoff point for discrimination between random and nonrandom synchronization. The index of synchronization (IOS) is the ratio of pulse coincidence observed in more than 50% of cells to that predicted by the random model. Using this index, a value of 1 would indicate that true synchronization was low, because pulse synchronization in the experimental dataset was not different from that expected from random occurrence. As rise in this value would indicate that synchronized pulse activity has increased.

Statistical Analysis

Statistical differences in plaque number, levels of RNA, protein, or frequency, duration, and amplitude of peaks were assessed by one-way Anova followed by the Newman-Keuls multiple comparison test or followed by unpaired Student's *t* test. Differences in the index of synchronization between cells injected with siRNA or scrambled controls were analyzed by a Kruskal-Wallis test followed by a Dunn's multiple comparison test. Differences were considered statistically significant at $P < 0.05$, unless noted otherwise.

Results

Our initial experiments were to determine whether Cx-26 and/or Cx-43 could be identified in GT1-7 cells and if so to assess whether the levels of these gap junctional-associated proteins differ between non-synchronized (day 2) and synchronized (day 7) cultures. In Figure 1 (panel A), immunoreactive Cx-26 was identified by cytoplasmic localizations or “plaques” characteristic of gap junction-related connexin protein [26], in both 2-day and 7-day cultures. Quantification revealed a similar number of these immunoreactive densities in cells from 2-day and 7-day cultures (Figure 1, panel B). As shown, (Figure 1, panel A), plaques were virtually undetectable when Cx26 antisera was first preabsorbed with Cx26 peptide. Additional work revealed that immunoreactive Cx-43 was also expressed in GT1-7 cells and as with Cx-26 was localized in “plaques” (Figure 2, panel A). Interestingly, the relative abundance of C-43 immunoreactivity appeared to be more prominent in cells from day-7 rather than day-2 cultures. In fact, analysis revealed approximately a 4-fold increase in Cx-43 plaques in cells from day-2 to day-7 (Figure 2, panel B). As a control, our use of Cx43 antisera that was first preabsorbed with Cx43 peptide abolished plaque detection, revealing the specificity of the antisera for Cx43 (Figure 2, panel A).

In our next experiments, we explored whether or not these patterns of connexin plaque abundance were reflected in gene expression changes with time in culture. Real time RT-PCR analysis was conducted on cells cultured for 2, 3, 4, and 6 days. As shown in Figure 3, relative expression of Cx-26 RNA (panel A) remained relatively stable throughout the culture period. In contrast, the level of mRNA for Cx-43 increased markedly with more than a doubling from day 2 to day 4 of culture. (Figure 3, panel B).

In order to determine whether or not these patterns of connexin expression were reflected in cellular protein content, we performed Western analysis on cells cultured for 2, 4 and 6 days. As shown in a representative example of Western analysis for Cx26 (Figure 4, panel A), the relative levels of immunoreactive Cx-26 protein did not appear to change throughout the culture period. Further analysis revealed no differences in relative Cx26 protein levels in cultures for 2, 4, and 6 days (Figure 4, panel B). However, as shown in a representative blot for Cx43 (Figure 4, panel C), the level of this protein changed with time in culture. In fact, the relative Cx43 protein expression increased approximately 2-fold from 2 to 6 days of culture (Figure 4, panel D).

The change in Cx43 associated plaques, mRNA, and protein during a period when the culture is shifting from a non-synchronized to synchronized function suggested strongly that Cx43 may have an important role in this process. In order to address this possibility, we reasoned that a reduction or elimination of Cx43 RNA, but not Cx26, early in the culture period would also disrupt synchronized activity between these cells. Intending to use siRNA for this purpose, we tested the efficacy of siRNA reagents directed against Cx26 and Cx43 with these cells. In these experiments, we found that siRNA directed against Cx26 and Cx43, when transfected into GT1-7 cultures, reduced protein expression for Cx26 and Cx43, respectively compared to scrambled siRNA controls (Figure 5, panel A + B for Cx26 and Figure 5, panel C + D for Cx43). Interestingly, the specificity of these reagents were also confirmed, in part, by our findings that administration of siRNA for Cx43 did not alter Cx26 protein levels (Figure 5, panel E) nor did application of Cx26 siRNA affect Cx43 protein expression in these cells (Figure 5, panel F).

Having demonstrated the efficacy of these reagents with GT1-7 cells, we tested whether or not synchronized activity would be disrupted following injection of siRNA directed against either of these connexins. As shown in Figure 6, panel A, cells from these cultures that were injected with scrambled siRNA continued to exhibit pulses that overlapped in time. Following injection

of cells with siRNA against Cx43 on day 3, it appeared that less clustered pulse activity occurred (Figure 6, panel B). Interestingly, pulse profiles following injection of siRNA against Cx26 (Figure 6, panel C) still exhibited coordinated pulse activity on day 7 as shown. Analysis using the IOS to quantify synchronized activity is presented in Figure 7. As stated above, this index represents the ratio of coordinated pulse activity in a population of cells to that occurring randomly and provides a means to estimate the relative degree of synchronized activity occurring within a group of cells. Using this approach, we found that treatment with siRNA against Cx43 markedly reduced the IOS (approximately 5-fold) in cultured cells when compared to those injected with scrambled control siRNA (Figure 7). As suggested by the profile presented above, treatment with siRNA against Cx26 yielded an IOS that was not different than that measured from cells receiving injections of scrambled control siRNA. Interestingly, the average pulse intensity (3.3 ± 0.4 vs 4.3 ± 0.7 or $4.3 \pm 0.6 \times 10^4$ relative fluorescent units for control vs siCx43 or siCx26 treated, respectively,) or the average pulse frequency (1.0 ± 0.1 vs 1.2 ± 0.1 and 1.0 ± 0.1 pulse/h for control vs siCx43- or siCx26-treated, respectively; mean + SE, n=5, 5, and 4 for control, siCx43 and siCx26, $P>0.5$) did not differ between treatments.

Discussion

Our results reveal that Cx43 expression increased in GT1-7 cells from 2 days to 7 days of culture. This increase is supported by several lines of evidence. First, a greater number of Cx-43 immunoreactive cytoplasmic densities or “plaques” 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. Our 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. 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. Moreover, the lack of a corresponding mRNA increase at day 6 suggests that some type of cellular control is imposed to limit the levels of Cx43 present. Interestingly, we found that increases in connexin abundance in GT1-7 during culture did not occur for every connexin. Rather, immunoreactive densities, mRNA, and protein for Cx26 all remained relatively constant throughout an identical culture period. These findings, when viewed in light of our previous observations of enhanced pulse synchronization in secretory activity among GT1-7 cells during this period [13] raised the possibility that increases in Cx43 may be associated with this synchronization process. Indeed, our final experiments using Cx43 siRNA early in the culture period to interfere with Cx43 expression resulted in an extensive reduction of cell-to-cell synchronization within these cultures. When taken together, our findings strongly support a role for Cx43 in GT1-7 cell coordinated function.

It is clear from our studies that both Cx43 and Cx26 are expressed in GT1-7 cell cultures. These findings confirm the results of others with Cx26 [16,27], but to our knowledge are the first reports of Cx43 in GnRH neuronal cultures of any type. Each of these connexins have been localized to several different neurons early in development, but their expression appears to diminish as development progresses [28,29]. It must be emphasized that the ability to reliably identify specific neuronal cell connexins in the adult is complicated by several factors, especially with GnRH neurons in the hypothalamus. First as mentioned above, GnRH neurons in mammals are few in number and are scattered from the olfactory bulb to the posterior hypothalamic area [12]. Such a distribution may decrease the likelihood of identifying moderate or reduced connexin expression in neurons particularly if specific connexin-related gap junctions occur mainly at points of neuronal contact. Indeed, GnRH neurons have dendritic processes that extend long distances and become relatively thin [30-32]. The localization of

connexin expression within individual dendrites would require immunocytochemical techniques that enable membrane preservation without compromising immunogenicity. This is a problem, especially with electron immunocytochemical detection [33]. Finally, the expression of certain connexins within GnRH neurons may depend on the specific physiological state of the donor animal. Interestingly, in one study using confocal microscopy, Cx43 and GnRH were colocalized in neuronal cell bodies and immunoreactive Cx-43 puncta were identified between GnRH fibers in sections prepared from hypothalami of castrated male rats [17]. In another experiment performed with cycling female rats and using standard epifluorescence, the co-localization of Cx-43 and GnRH was not observed in the hypothalamus [22]. Of course, it is quite possible that these differences in detection between the two studies could be attributed to different reagents used or the type of microscopic analysis employed. However, another possible explanation could be that Cx43 expression differs between cycling females and castrated males, perhaps due to the steroid environment existing upon sacrifice or during development [34]. Regardless of the explanation, the identification of Cx43 in GT1-7 cultures in our laboratory and its colocalization with GnRH in neurons derived from animals in at least one physiological state suggests that Cx43 is expressed in GnRH neurons.

A role for Cx43 in the coordination of GnRH cell function would be consistent with the manner in which this connexin functions in various other cells and tissues. Cx43 is expressed in a variety of locations such as the reproductive immune, digestive, and cardiac systems [26]. In almost every tissue type, Cx43 contributes markedly to coordination of cellular activity. The most noteworthy in this regard is the heart. It is well recognized that the proper functioning of the normal heart depends on coordinated function of cardiomyocytes for efficient pulse propagation [35,36]. A recent study found that administration of a dominant negative inhibitor of Cx43 abolished effective propagation of Ca^{2+} transients between cultured rat myocytes demonstrating the need for Cx43-related gap junctions in myocyte communication [37]. The importance of Cx43 in myocyte interaction in the ventricular myocardium is further indicated by several studies revealing an altered abundance of this connexin in diseased tissue, especially adjacent to infarcts [38,39], or in hypertrophied or failing hearts [40,41], all conditions lacking synchronized cell activity. From this work, there appears to be a direct association between Cx43 expression and coordinated cell activity in cardiac tissue. Although the case in the hypothalamus is not as strong, the possibility that Cx43 may function in a similar manner in GnRH neural function is suggested indirectly by the study of Cx43 mutant mice. Guinello and Etgen [34] demonstrated clearly that heterogenous Cx43 mutants displayed abnormal estrous cycles and irregular estrous behavior. Although the reason for this abnormal cyclic activity was not delineated, it was clear from this work that Cx43 was important for proper reproductive function. Moreover, the cyclic disruptions observed would not be inconsistent with an alteration of neuroendocrine pulse generation perhaps arising from abnormal GnRH neuronal communication.

The interconnection of cells via Cx43, regardless of the tissue, involves the passage of information from one cell to another. Indeed, it is well recognized that gap junctions formed from almost every type of connexin appear to be permeable to monovalent cations, and to a lesser extent anions [42], as well as to factors including second messengers, polypeptides and siRNA [43-46]. Interestingly, recent studies demonstrate that gap junctions formed from Cx43 have selectivity for negatively charged larger solutes. In fact, these channels were able to effectively transfer cAMP from cell-to-cell raising the possibility that a portion of this coordinated function in Cx43-containing cells may be due to the passage of cAMP [47]. Interestingly, a growing body of evidence suggests that cAMP is involved in the regulation of GnRH pulse frequency. It was observed that a reduction in intracellular cAMP concentrations by constitutive expression of cAMP-dependent phosphodiesterase (cAPD) in GT1 cells reduced the frequency of both spontaneous Ca^{2+} oscillations and pulses of GnRH release [48]. A reduction in GnRH pulse frequency was also observed in castrated transgenic rats in

which cAPD was continuously expressed [49]. 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 Ca²⁺ and/or other ions. Such a process would be able to coordinate the two connected cells with respect to GnRH pulse activity and could form 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, at least the cell bodies, would be extremely small in light of the few and scattered neurons in the hypothalamus. However, a recent series of studies have revealed several functional and structural properties of GnRH neurons that may help to explain how a scattered network of GnRH neurons could effectively communicate. GnRH neurons are uni- or bipolar, with extremely long dendrites [30-32]. More importantly, it was observed that input to the dendrites is capable of initiating action potentials in the GnRH neuron [50]. In fact, these reports by Roberts et al [50] suggest that distal dendrites of GnRH neurons appear to be the primary site of spike initiation in these cells. A contact of dendrite to dendrite or dendrite to cell body would provide a point of communication that could span appreciable distances 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 [51], [52,53]. Thus, neuronal communication via gap junctional contact between dendrites or dendrites and cell bodies may form the basis for a GnRH neuronal synchronized network. Our findings of Cx43 associated synchrony between GT1-7 neurons suggest that Cx43-related gap junctions may play an important role in this network structure.

In summary, our results demonstrate clearly that Cx43 is expressed in GT1-7 cells and increases from 2 to 7 days of culture, a period during which secretory synchronization occurs. That Cx43 is central to this synchronization process is revealed by the loss of synchronization when Cx43 siRNA is administered. These findings raise the 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, coordinate, and release a “pulse” of GnRH from the hypothalamus.

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A. **B.** **Figure 1.**

Measurement of particles or “plaques” immunoreactive for Cx26 in GT1-7 cells from 2 day and 7 day cultures. Shown in **panel A** is a representative example of plaques identified in cells following Cx26 immunocytochemistry (400X). **Panel B** shows a comparison of Cx26 immunoreactive particles between cells cultured for 2 days and 7 days (Data represents means + SE for 8 different cells each for 2 day and 7 day cultures).

A. **B.** **Figure 2.**

Identification of “plaques” immunoreactive for Cx43 in GT1-7 cells cultured for 2 days or 7 days. **Panel A** contains a representative example of cells cultured for 2 days or 7 days and then subjected to immunocytochemistry for Cx43 (400X). **Panel B** shows a quantification of cytoplasmic particles or “plaques” immunoreactive for Cx43. The data is expressed as the mean + SE of determinations made on 8 separate cells for each time point. (* denotes a significant difference between cells from 2 day and 7 day cultures, $P < 0.01$).

Figure 3.

Real time RT-PCR determination of Cx26 or Cx43 mRNA expression in GT1-7 cells cultured for various lengths of time. The cells were extracted and the RNA subjected to real-time RT-PCR to determine the relative expression of Cx26 (**panel A**) or Cx43 RNA (**panel B**). Each value represents the mean and the SE of 3 separate experiments for each connexin type and are expressed relative to day 2 mRNA levels. (* denotes significant difference between 2 and 4 days).

Figure 4.

Analysis of relative Cx26 and Cx43 protein expression in GT1-7 cultures. Cells grown for 2, 4, or 6 days were extracted and the proteins subjected to Western analysis for Cx26 or Cx43. **Panel A and C** shows representative examples of western blots performed for Cx26 and Cx43, respectively. The top row of each blot contains protein immunoreactive for a specific connexin and the bottom row contains protein immunoreactive for α -tubulin. **Panels B and D** presents results obtained from 3 separate experiments each for Cx26 and Cx43, respectively. The values (mean + SE) were derived as a ratio between the respective connexin and corresponding α -tubulin expression from same sample and are expressed relative to 2 days. (* denotes significant difference, $P < 0.01$, when extracts from 6 days are compared to those from 2 days).

Figure 5.

Western analysis of relative Cx26 and Cx43 protein expression after siRNA treatment of GT1-7 cultures. Cells treated with 50 nM of scrambled, Cx26, or Cx43 siRNA for 72 hr were extracted and the proteins subjected to Western analysis for Cx26 or CX43. **Panel A and C** shows representative examples of Western blots performed for Cx26 and CX43, respectively, after siRNA treatment. The top row of each blot shows immunoreactivity for a specific connexin protein and the bottom row reveals immunoreactivity for either α -tubulin or β -actin as loading controls. **Panel B and D** represents relative Cx26 and Cx43 expression after treatment with Cx26 and Cx43 siRNA, respectively. **Panel E and F** shows results obtained from 3 separate determinations of Cx43 and Cx26 relative expression after treatment with Cx26 and Cx43 siRNA, respectively. The values (mean $\pm$ SE) were derived as a ratio between the specific connexin indicated and α -tubulin or β -actin expression within those same samples and are expressed relative to controls. The controls were exposed to all reagents and parts of the procedure except addition of siRNA. (* denotes significant differences when results from connexin siRNA treated cultures are compared to control or scrambled siRNA values).

Figure 6.

Effect of siRNA treatment on pulse activity in GT1-7 cultures. Cells grown for 3 days were microinjected with siRNA and the cells assessed on day 7 for pulse synchronization by FM1-43 fluorescence analysis. **Panel A, B, and C** show examples of cells that were injected with scrambled, Cx43 or Cx26, respectively. Each panel contains pulse profiles from eight cells. (*) indicates overlapping pulse activity from at least 4 cells).

Figure 7.

Effect of siRNA treatment on the 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. From the profiles that occurred, the IOS was determined. Details of this analysis and calculation of the index are presented in Materials and Methods. Using this index, a value approaching 1 would be indicative of random activity. 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) (* denotes significant difference when compared to scrambled control siRNA treated, $P < 0.01$).