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The Functional Significance of Neurokinin 3 Receptor Signaling in Gonadotropin-Releasing Hormone Neurons

A thesis submitted in partial satisfaction of
the requirements for the degree Master of Science

in

Biology

by

Paul Peng Lin Shao

Committee in charge:

Professor Pamela L. Mellon, Chair
Professor Michael David, Co-Chair
Professor Ella Tour

2012

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The thesis of Paul Peng Lin Shao is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2012

I dedicate this thesis to my loved ones:

To mom and dad for supporting and loving me always.

To my dear brother Richie for believing in me and filling my life with joy.

To Matthew, Narmon, Bing and Samuel, for their encouragement, love and care.

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LIST OF ABBREVIATIONS

7-TM-GPCR	7-transmembrane G-protein coupled receptor
AC	adenylate cyclase
AP-1	activation protein 1
ARC	arcuate nucleus
ATF	activating transcription factor
AVPV	anteroventral periventricular nucleus
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
CRE	cAMP-reponse element
CREB	cAMP-response element binding proteins
CSF	colony stimulating factor
DAG	diacylglycerol
ELK	ETS-like transcription factors
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
ETS	E-twenty six transcription factors
FGFR1	fibroblast growth factor 1
FSH	follicle-stimulating hormone
GMCF	granulocyte-macrophage colony stimulating factor
GnRH	gonadotropin-releasing hormone
GPR54	kisspeptin receptor
HPG	hypothalamic pituitary gonadal
ICV	intracerebroventricular

IHH	idiopathic hypogonadotropic hypogonadism
IP_3	inositol trisphosphate
JAK	janus kinase
JNK	jun N-terminal kinase
KAL	kallman syndrome gene
KNDy neurons	kisspeptin-neurokinin B-dynorphin neurons
LH	luteinizing hormone
MAPK	mitogen-activated protein kinase
ME	medial eminence
MEKK	MAP kinase kinase kinase
NELF	nasal embryonic LHRH factor
NK3R	neurokinin 3 receptor
NKB	neurokinin B
OVLT	organum vasculosum of the lamina terminalis
PDGF	platelet-derived growth factor
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKD	protein kinase D
PLC	phospholipase C
PROK2	prokineticin-2
PROK2R	prokineticin-2 receptor
PVN	paraventricular nucleus

shRNA	small hairpin RNA
SRF	serum response factor
STAT	signal transducer and activator of transcription
TPA	12-O-tetradecanoylphorbol-13-acetate
TCF	ternary complex factor

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ABSTRACT OF THE THESIS

The Functional Significance of Neurokinin 3 Receptor Signaling in Gonadotropin-Releasing Hormone Neurons

by

Paul Peng Lin Shao

Master of Science in Biology

University of California, San Diego, 2012

Professor Pamela L. Mellon, Chair

Professor Michael David, Co-Chair

Gonadotropin-releasing hormone (GnRH) is a decapeptide hormone secreted by hypothalamic neurons that is essential for puberty and reproduction. Human studies identified a critical role for the neurokinin 3 receptor (NK3R) and its ligand, neurokinin B (NKB), in puberty. NK3R is expressed in multiple cells within the GnRH neuron network, including GnRH neurons themselves. To date, *in vivo* studies have not been able to discriminate between the direct and indirect effects of NKB on GnRH neurons. The goal of this thesis is to determine the functional significance of NKB signaling

directly to NK3R-expressing GnRH neurons. We show that GT1-7 cells, an *in vitro* model of the mature differentiated GnRH neuron, express NK3R, and acute treatment with senktide, a NK3R agonist, induces GnRH peptide secretion. Moreover, senktide induces the repression of GnRH gene transcription by a mechanism that involves Fos induction, AP-1 complex formation, and direct binding of Fos protein to a novel AP-1 half-site in enhancer 1. We also find that senktide induces c-Fos gene transcription by a mechanism that involves STAT (-310 bp) and SRF (-345 bp) binding sites. Finally, we identify the PKC pathway acting downstream from the NK3R in GnRH neurons. In summary, NKB signaling directly to NK3R-expression GnRH neurons activates GnRH secretion and represses GnRH transcription by a mechanism that involves a PKC pathway-dependent induction of Fos gene transcription and enhanced Fos protein binding to a novel AP-1 site identified in enhancer 1.

I

Introduction

Hypothalamic-Pituitary-Gonadal Axis

Reproduction is essential to life on Earth. Without a mechanism of producing offspring, plant and animal populations would become extinct. In mammals, sexual reproduction is controlled by an intricate and complex neuroendocrine system known as the hypothalamic-pituitary-gonadal (HPG) axis. Gonadotropin-releasing hormone (GnRH) is at the apex of the HPG axis. GnRH is a peptide hormone that is synthesized in a specialized population of about 800 neurons in the hypothalamus. GnRH is released from axon terminals at the median eminence in a pulsatile manner and travels through the hypophyseal portal vascular system to the anterior pituitary gland, where it acts on GnRH receptor in gonadotrope cells to stimulate the synthesis and secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Circulating LH and FSH act on receptors in the gonads, testes in male and ovaries in female, to trigger gametogenesis and the synthesis of sex steroids (1). Sex steroids, mainly estrogen in females and testosterone in males, provide neuroendocrine feedback at the level of hypothalamus and pituitary to regulate the further release of GnRH and the gonadotropin hormones. In females, estrogen is able to suppress basal GnRH secretion and induce a GnRH surge that induces a LH surge and ovulation. GnRH neurons receive inputs from neural pathways that convey information about environmental factors that can modulate GnRH release,

such as the circulating level of sex steroids, stress, metabolic state and, in seasonal breeding species, length of day. GnRH neurons and all other neurons that regulate GnRH secretion are collectively referred to as the "GnRH neuron network".

GnRH Neuron Development and Puberty

The GnRH neurons originate from the olfactory epithelium outside of the brain (2-4). During development, GnRH neurons migrate, crossing the cribriform plate, into forebrain following the tracts of olfactory sensory neurons. GnRH neurons migrate until they reach the rostral hypothalamus and then extend their axons to the median eminence at the base of the hypothalamus (5, 6). Studies in human patients with idiopathic hypogonadotropic hypogonadism (IHH), a disorder characterized by delayed or absent puberty and low levels of circulating LH and FSH, have identified mutations in several genes whose products are involved in GnRH neuron migration such as Kal (7, 8), NELF (9), FGFR1 (10), and PROK2 and its receptor PROKR2 (11). Moreover, treatment of patients with hypogonadotropic hypogonadism due to GnRH insufficiency with exogenous GnRH has shown that a pulsatile pattern of GnRH stimulation is strictly required for pituitary sensitivity (12). These studies illustrate the importance of successful targeting of GnRH neuron axons to the ME and a pulsatile pattern of GnRH secretion for normal pubertal development and reproductive maturation.

The GnRH neuron network continues to mature after birth and this is reflected by changes in GnRH secretion. During the infantile period, GnRH secretion becomes activated for a short time (13). This is important to the establishment of sexually

dimorphic traits in the brain, such as the female-specific preovulatory GnRH surge response to estrogen. Afterwards, throughout the juvenile period, the GnRH neuron network becomes dormant. The onset of puberty is marked by a sustained increase in pulsatile GnRH (14). In female monkey, the administration of pulsatile GnRH was shown to be sufficient to drive puberty onset (15). Thus, the increased activity of the GnRH neuron network and increased pulsatile GnRH secretion is thought to be responsible for puberty. However, the stimulus that triggers the end of the juvenile period and the onset of puberty remains largely unknown.

Kisspeptin Peptide and Kisspeptin Receptor

Kisspeptin peptide, encoded by the Kiss1 gene, is a key regulator of pubertal development and a potent stimulant of GnRH neurons, which express kisspeptin receptor, GPR54 (16). Kisspeptin is produced in neural cell bodies located in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC) regions of the rodent hypothalamus within the brain. Kisspeptin neurons participate in the estradiol-mediated GnRH stimulation of the LH surge (17, 18). Genetic studies reveal that loss-of-function mutation in Kiss1 and GPCR54, which encodes kisspeptin receptor, result in hypogonadotropic hypogonadism in humans (19). Furthermore, Kiss1 and GPR54 knock-out mice are infertile and display developmental abnormalities and low FSH level consistent with hypogonadotropic hypogonadism (20-22). Multiple *in-vivo* studies have shown that kisspeptin administration strongly stimulates GnRH and LH secretion in

rodents (23-27), sheep (28), monkeys (29, 30), and humans (31). Therefore, kisspeptin has proven to be a likely candidate for an activator of puberty.

Neurokinin B Peptide and Neurokinin 3 Receptor

Neurokinin B (NKB) is a peptide protein that belongs to the tachykinin family and is widely conserved across vertebrates (32). It is synthesized from the *preprotachykinin-B* gene, which is currently designated as *TAC3* in humans and *Tac2* in rodents. Like kisspeptin, recent studies in humans have implicated NKB and its receptor, the neurokinin 3 receptor (NK3R), in puberty onset and regulation of gonadotropin secretion. Loss-of-function mutations in genes that encode NKB or NK3R were found in patients with IHH (33). Treatment of patients with NKB or NK3R mutations with pulsatile GnRH successfully restored serum LH levels, sex hormone secretion, and in a few cases, fertility (34), indicating hypogonadotropic hypogonadism is due to a disruption in GnRH secretion at the level of the hypothalamus.

In contrast to GPR54 knock-out mice, male and female Tacr3 knock-out mice display normal timing of puberty. Also, male Tacr3 knock-out mice demonstrate normal fertility despite being hypogonadal and having low circulating levels of FSH. Female Tacr3 knock-out mice, however, are subfertile, have smaller uteri, display disrupted estrous cycles and a reduced number of corpora lutea in the ovary, indicating disrupted ovulation (35). Since female Tacr3 knock-out mice were shown to ovulate in response to exogenous gonadotropins, anovulation is not due to an ovarian defect but is likely due to a neuroendocrine defect causing a disrupted LH surge at the level of the hypothalamus

and/or pituitary. Thus, although data in Tacr3 knock-out mice failed to confirm a central role for NKB signaling in the onset of puberty, studies continue to suggest a critical role for NKB and NK3R in the regulation of gonadotropin hormone secretion and reproduction (35).

In Vivo **Studies Investigating the Regulation of Gonadotropin Hormones by NKB**

Many *in vivo* studies have investigated the regulation of gonadotropin secretion by NKB but, in contrast to kisspeptin, the results have been somewhat inconsistent. Two studies suggest NKB might be repressive towards serum LH. First, the central injection of senktide, a NK3R agonist, in ovariectomized female rats treated with low estrogen was reported to inhibit LH secretion (36). Second, in ovariectomized female mice, senktide administration was shown to reduce serum LH levels (37). These results were unexpected since NKB acting to suppress GnRH secretion is inconsistent with the low gonadotropin hormone levels observed in human patients with mutations in NKB and NK3R. Moreover, many other studies reported NKB to be stimulatory towards LH secretion. First, intracerebroventricular (ICV) injection of senktide was found to increase serum LH levels in diestrus female rats (38). Likewise, senktide treatment was reported to stimulate LH in follicular phase ewes (39) and prepubertal rhesus monkeys (40). Interestingly, estrogen was shown to down-regulate NK3R levels in the ARC (37) and some species were shown to have different receptor expression patterns in the brain (41). As a result, it is thought that inconsistencies in the response to senktide may be attributable to differences in the *in-vivo* models employed, including species, sex,

gonadal status, and the circulating hormonal milieu. Despite difficulty in reaching a consensus as to whether NKB is an activator or repressor of GnRH, these *in vivo* data indicate that NKB and NK3R signaling is involved in the central regulation of gonadotropin secretion, presumably by regulating GnRH secretion.

NKB May Directly or Indirectly Regulate GnRH Neuron Secretion

NKB peptide has been localized to neurons in the arcuate nucleus of the hypothalamus. This population of neurons also expresses kisspeptin and dynorphin and is commonly referred to as KNDy neurons. KNDy neurons express NK3R and extend axons to the medial eminence (ME) that come into close proximity to the termini of GnRH neurons (36, 42, 43). Since human and animal studies have shown kisspeptin to be a consistent and potent stimulator of GnRH and LH secretion, and involved in puberty onset, NKB has been speculated act autosynaptically or transynaptically on KNDy neurons to induce kisspeptin release and stimulate GnRH secretion by acting on GPR54 in terminals of GnRH neurons at the ME. Alternatively, a subset of GnRH neurons has been shown to express NK3R by immunocytochemistry and gene expression profiling experiments in rodents (44, 45), indicating that NKB may also be capable of directly regulating GnRH release.

Recent research has focused on determining the role of NKB in regulating kisspeptin release from KNDy neurons. A recent study in GPR54 knock-out mice showed no increased LH response to senktide, suggesting NKB requires kisspeptin to elicit its effects (46). Another showed that desensitization of GPR54 blocked the

stimulation of LH by senktide but desensitization of NK3R did not block response to kisspeptin (47). While these studies support a potential hierarchy where the response to NKB requires kisspeptin but the response to kisspeptin does not require NKB, they fail to discriminate between direct and indirect effects since GnRH neurons express both GPR54 and NK3R. One of the goals of this thesis is to determine the functional significance of NKB signaling directly to NK3R-expressing GnRH neurons.

GT1-7 Cell Model

GnRH neurons are few in number, about 800 in the mouse, and sparsely distributed throughout a large area including the forebrain and hypothalamus. As a result, the investigation of GnRH neurons *in vivo* is technically challenging. Our lab generated the immortalized GT1-7 cell line to study GnRH neurons. GT1-7 cell line was developed through targeted tumorigenesis of the GnRH neurons by expressing the SV40 T-antigen oncogene from the 5' flanking region of rat GnRH (48, 49). This cell line has been extensively used in our studies, and those of others, to investigate the molecular and cell biology of GnRH neurons. It is a very well-characterized model that has been reported to faithfully represent mature, post-migratory GnRH neurons. Similar to GnRH neurons *in vivo*, GT1-7 cells exhibit neuronal morphology, form neuronal synapses, synthesize and process GnRH mRNA and peptide, and secrete GnRH in pulsatile fashion (49, 50). Previously, GT1-7 cells have been used to study the GnRH neuron response to kisspeptin and GPR54 signaling pathways (51). In this thesis, the GT1-7 cell model enables the investigation of the direct effects of NKB signaling on GnRH neurons in the absence of the confounding variables that are present when doing *in vivo* studies such as changes in

kisspeptin release or differences in the hormonal milieu. In addition, GT1-7 cells allow detailed molecular studies to investigate transcriptional regulation by NK3R that would not be possible *in vivo*.

II

Materials and Methods

Cell culture

GT1-7 cells (49) were cultured in Dulbecco's modified Eagle's medium with 4.5 g/L glucose, L-glutamine & sodium pyruvate (DMEM; Mediatech, Manassas, VA) supplemented with 10% Foundation B fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA) in 5% CO_2/95% O_2 at 37°C. Senktide (Tocris Bioscience, Ellisville, MO) was dissolved in Dulbecco's phosphate-buffered saline without calcium or magnesium (dPBS; Mediatech) supplemented with 0.1% Fraction V bovine serum albumin (BSA; Sigma, St. Louis, MO).

The generation of GT1-7 cells with stable transfection of shRNA directed against β-Arrestin-1 (line 712), β-Arrestin-2 (line 153) and a control scrambled shRNA (line 013) was previously described. Western blot was used to confirm that these cell lines have reduced expression of β-arrestin proteins (52). In this study, cells were kept under constant selection by culturing in media supplemented with 0.5µg/mL puromycin. Media was changed every 3 days to ensure antibiotic potency.

Static culture secretion experiments

For GT1-7 static culture secretion studies, 2.5 x 10^6 cells were seeded into each well of a 6-well plate and allowed to adhere for 16-18 hours. Two hours before treatment,

media was changed to DMEM without serum + 0.1% BSA. Cells were treated with 1 ml of either vehicle or 50 nM senktide for either three or 24 hours. Conditioned media was transferred to a 1.5 ml microcentrifuge tube and centrifuged for 10 min at 2,000 rpm at room temperature to pellet contaminating cells. 860 µl of supernatant was transferred to 2 ml round bottom microcentrifuge tubes and frozen at -80°C. The experiment was repeated at least three times and then media was lyophilized using a speedvac. Dried samples were stored at -80°C until the GnRH content could be assayed by radioimmunoassay (RIA). The evening before the RIA, 0.2 ml PBS-gel [0.14M NaCl, 0.1M $NaPO_4$, 0.1% gelatin, (pH 7.4)] was added to dried samples. Samples were left to resuspend on a rocking shaker overnight at 4°C. The next morning, samples were vortexed and pipetted to fully resuspend. 0.1 ml aliquots were prepared in duplicate. The 1:100 stock of EL-14 GnRH primary antibody (Martha Bosch, Oline Ronnekleiv, and Martin Kelly, University of Oregon Health Sciences Center, Portland, OR) was diluted a further 1:700 (1:70,000 final) in NRS-EDTA [0.14M NaCl, 0.01M $NaPO_4$, 0.1% gelatin, 0.5M EDTA (pH 7.4) supplemented with 1% normal rabbit serum] and 50 µl was added to each sample. After incubating 48 hours at 4°C, 50 µl containing ~10,000 cpm of [^{125}I]Tyr^5-Luteinizing Hormone-Releasing Hormone (PerkinElmer, Boston, Massachusetts) diluted in PBS-gel was added. After incubating for 48 hours at 4°C, samples were precipitated with 3 ml ice-cold ethanol and centrifuged (15 min at 2500 rpm at 4°C). Supernatant was decanted and dried pellets were counted with a gamma-counter (Micromedic, Huntsville, AL) to determine CPM for bound radioactivity. Standards were run in triplicate. Duplicate standard curves were counted at the beginning

and end of the assay. GnRH content for experimental samples was extrapolated from the standard curve. The intra-assay coefficient of variability was 21.2%.

RNA isolation, RT-PCR and quantitative RT-PCR

Cells were grown to approximately 90% confluency prior to harvest for RNA. Briefly, media was aspirated and cells were rinsed with cold dPBS. 1ml of TRIzol (Invitrogen, Carlsbad, CA) was used to isolate RNA from each 10 cm dish and RNA was isolated according to manufacturer's protocol. Five micrograms of total RNA was DNase I treated using DNA-free (Ambion) and reverse transcribed in a total volume of 20 µl using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) using oligo-$(dT)_{20}$ (NK3R) or random hexamer (c-Fos). PCR was performed on the resulting cDNA using a thermocycler [MJ Research, St. Bruno (Quebec) Canada] and primers specific for NK3R (Table 2-1). qPCR was performed using the iQ5 Real-Time PCR Detection System and Software and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) according to the manufacture's recommendations, including melt curve analysis, with primers for c-Fos and βActin (Table 2-1) and tm = 58.7°C (βActin) and 55°C (c-Fos). Dilutions of a mouse c-Fos expression plasmid were used to generate standard curves. c-Fos expression was normalized using βActin, an unresponsive housekeeping gene.

Plasmids, transient transfections and luciferase assays

Cloning of the rat NK3R (53), murine c-Jun and c-Fos (54) and A-FOS (55) expression constructs were previously described. The -5K rat GnRH (-4984 to +22 relative to the transcription start site), 5' truncations and GnRH-E1/GnRH-P luciferase reporters have been previously described (56, 57). For the GnRH-E1/TKp luciferase reporter, enhancer 1 was digested from GnRH-E1/GnRH-P using KpnI and NheI and subcloned into a pGL3 vector containing the -81-bp thymidine kinase minimal promoter (GnRH-E1/TKp). AP-1 luciferase reporter (Agilent PathDetect in Vivo Signal Transduction Pathway *cis*-Reporting Systems; Agilent, Santa Clara, CA) contains a multimer of the AP-1 consensus binding sites $(TGACTAA)_7$. 5' truncations of murine c-Fos luciferase reporter, c-Fos reporters containing individual mutated response elements (μSTAT, μETS, μSRF, and μAP-1), and the SRF multimer reporter have been previously described (54). For the STAT multimer reporter, four copies of the STAT inducing elements with 2-bp spacers (tgTTCccgtCAAtc) were cloned into the Nhe1 and Xhol cloning sites in front of the thymidine kinase minimal promoter in the pGL3 basic luciferase reporter. The sequence is confirmed with sequence analysis using RV3F primer.

For transient transfections used for luciferase assays, approximately 24 hours prior to transfection, GT1-7 cells were seeded into 24-well plates at a density of 150,000 cells per well. Cells were transfected with plasmid DNA using Fugene 6 Transfection Reagent (Roche Diagnostics GmbH, Mannheim Germany) according to the manufacturer's protocol with a 3:1 ratio of μl Fugene to μg DNA. Approximately 24 hours after transfection, cells were switched to serum free media (DMEM + 0.1% BSA).

Cells were treated with pharmacologic agents as indicated in figure legends. Approximately 48 hours post-transfection, cells were rinsed 1 x with dPBS and lysed with 55 µl 100 nM KPO_4 + 0.2% Triton X-100. 20 µl cell lysate was used to measure luciferase activity on a Veritas Microplate luminometer (Turner Biosystems, Sunnyvale, CA) by injection with 100 µl luciferase assay buffer (25 mM Tris pH 7.4, 15 mM $MgSO_4$, 10 mM ATP and 65 µM luciferin) into each well. Subsequently, β-galactosidase activity was measured using the Tropix Galacto-light β-galactosidase assay (Applied Biosystems, Foster City, CA). For each well, relative luminescence unit (RLU) for luciferase was normalized to β-galactosidase to control for transfection efficiency. Transfections were performed in triplicate and repeated at least three times.

For transient transfection used for Western blot analysis, 3.5×10^6 GT1-7 cells were plated in 10 cm tissue culture dishes. GT1-7 cells were transfected with 8.7 µg rat NK3R using Fugene (Roche Diagnostics GmbH) according to the manufacturer's protocol with a 3:1 Fugene:DNA ratio. Cells were serum starved for a total of 24 hours and treated with vehicle, 30 nM senktide, 100 nM TPA for times indicated in figure legends.

Treatments and Inhibitors

Senktide (Tocris Bioscience, Ellisville, MO) was dissolved in dPBS (Mediatech) supplemented with 0.1% BSA (Sigma). Aliquots of stock solutions were stored at -80°C until use. After overnight starvation in serum-free media (DMEM + 0.1% BSA), GT1-7

cells were treated with vehicle, 100 nM TPA or 30 nM senktide for the time indicated in figure legends.

NK3R inhibitor (SB 222200, Tocris Bioscience), JNK-1 and JNK-2 inhibitor (SP600125, Calbiochem, La Jolla, CA), JNK-3 inhibitor XII (SR-3576, Calbiochem), p38 MAPK inhibitor (SB202190, Calbiochem), ERK1/2 inhibitor (U0126, Calbiochem) PKC inhibitor Go6983 and TPA (Tocris Bioscience) were dissolved in dimethylsulfoxide (DMSO; Sigma). Aliquots of stock solutions were stored at -80°C until use. Compounds that were dissolved in DMSO were further diluted in DMSO as needed followed by a final dilution of 1:1000 in media (0.1% DMSO final). For studies that employed inhibitors and transient transfection with the SRF multimer luciferase reporter, cells in each well of a 24-well plate were pre-treated for 30 minutes with 0.5 ml vehicle +/- inhibitor or DMSO and then treated by addition of 55 µl of 4 nM (10X) senktide for a final concentration of 0.4 nM. This was determined to be necessary in preliminary studies in order to avoid problems with cell adhesion due to changing the media multiple times within a short (3.5 hour) period.

Harvesting whole-cell extracts, protein quantification and Western blotting

At harvest, GT1-7 cells were rinsed with 5 ml dPBS (Mediatech) and lysed with 500 µl of cell lysis buffer (20 mM Tris pH 7.4, 140 mM NaCl, protease inhibitors from Sigma, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1% Nonidet P-40, 0.5 mM EDTA, and 1 mM EGTA). Protein concentrations were determined using 1 µl lysate and Bradford assay reagent (Bio-Rad Laboratories). 50 µg total protein was loaded per

sample along with a size ladder stained with dye in Polyacrylamide gel electrophoresis (10% gel with 4% stacking gel) and run for 1.2 hours with 115 volts at 4℃ in 1X running buffer (25 mM Tris, 200 mM Glycine and 0.3% SDS). The samples were transferred to a polyvinylidene fluoride membrane (Millipore Corporation, Billerica, MA) using transfer apparatus (Biorad), transfer buffer and 105 volts for 1.5 hours at 4℃. Successful transfer was confirmed using PageRuler Plus Pre-stained Protein Ladder (Fermentas, Glen Burnie, Maryland). The membranes were washed 4 x 5 minutes in TBS-T (20 mM Tris, pH 7.4; 0.1% Tween; 150 mM NaCl; and 0.5% BSA) and blocked in TBS-T with 5% milk for 1 hour at room temperature or overnight at 4℃. Next, membranes were hybridized for 1 hour at 4°C with 1: 3000 α-total ERK1/2 antibody (sc-94 Lot#F2906, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in TBS-T with 5% milk. The membranes were then washed 4 x 5 minutes in TBS-T before being hybridized for 2 hours at room temperature with 1:3000 secondary goat α-rabbit IgG (sc2004, Lot# E710, Santa Cruz Biotechonology). Bands on the membranes were detected with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) by following the manufacturer's manual. The FluorChemQ (ProteinSimple, Santa Clara, California) imaging reader was used to quantify the bands. After stripping the membrane in Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) for 15 minutes, membranes were again washed in TBS-T, blocked and hybridized as described above with a 1:1000 dilution of α-phospho-ERK (sc7383, Lot#D0910, Santa Cruz Biotechnology) for 1 hour at 4℃ in TBS-T with 5% milk. After washing, membranes were incubated with a 1:3000 dilution of a secondary goat anti-mouse IgG (sc2005, Lot# E2410, Santa Cruz Biotechnology) for 2 hours at room temperature. Bands were detected and quantified as described above.

The amount of the phospho-ERK was normalized to total ERK1/2 protein. Experiments were repeated three independent times.

Electrophoretic mobility shift assays

Approximately 24 hours before harvest, cells were switched to serum free media (DMEM + 0.1% BSA). Cells were treated with 100 nM TPA for three hours and nuclear extracts were harvested as previously described (58). Briefly, GT1-7 cells were rinsed in 10 ml cold dPBS buffer and then lysed in cell lysis buffer [20 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1 mM $MgCl_2$, 1 mM PMSF, 10 mM NaF, 10 μg/ml protease inhibitors (Sigma)]. Cells were transferred to 1.0 ml tube and allowed to swell on ice for 15 min. Cell lysate was passed through a 25 gauge needle four times and centrifuged at 4000 rpm for 4 min at 4°C. Supernatant was aspirated and pellets containing cell nuclei were resuspended in 40 μl of nuclear lysis buffer [20 mM Hepes (pH 7.9), 20% glycerol, 420 mM KCl, 2 mM $MgCl_2$, 1 mM PMSF, 10 mM NaF, 0.1 mM EDTA, 0.1 mM EGTA, 10 μg/ml protease inhibitors (Sigma)]. Nuclei were allowed to swell on ice for 30 min and then centrifuged at 14,000 rpm for 10 min. Supernatant containing the nuclear lysate was aliquoted and flash frozen using a dry-ice ethanol bath and stored at -80°C until use. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Oligonucleotide probes are listed in Table 3-1. All synthetic oligonucleotides are from Integrated DNA Technologies, Inc. (San Diego, CA). Annealed double-stranded oligonucleotides (1 pmol/μl) were end-labeled with T4 Polynucleotide Kinase (New England Biolabs) and [γ^{32}P]ATP (7000 Ci/mmol; MP

Biomedicals, Solon, OH). Probes were purified using Micro Bio-Spin 6 Chromatography Columns (Bio-Rad). Binding reactions contained 2 µg nuclear protein and 100,000 CPM of labeled probe in 10 mM HEPES (pH 7.9), 25 mM KCl, 2.5 mM $MgCl_2$, 1% glycerol, 0.1% Nonidet P-40, 0.25 mM EDTA, 0.25% BSA, 1 mM dithiothreitol, and 0.0125 µg/µl poly dIdC. For cold competitor, 1000-fold molar excess unlabeled competitor probe was added to the reaction and incubated for 10 min on ice. For supershift experiments, 2 µg of an antibody that recognizes all Fos proteins, sc-253X, a c-Fos-specific antibody (K-25), or normal rabbit IgG (Santa Cruz Biotechnology) was added to the reaction and incubated for 20 min on ice before the addition of labeled probe. After addition of probe, samples were incubated for 10 min at room temperature before loading on a 5% nondenaturing polyacrylamide gel in 0.25X Tris-borate EDTA buffer. Gels were run for 2.5 h at 200-250 V, then dried under vacuum and exposed to film with L-Plus intensifying screens (Fisher Biotech) as indicated in figure legends.

Statistical analyses

Statistical analysis was performed using JMP version 8 (SAS Institute, Inc., Cary, NC). When necessary to achieve a normal distribution, data was optimally transformed by the method of Box and Cox. Data were analyzed by Student's *t* test or ANOVA followed by Tukey honestly significant difference (HSD), as indicated in figure legends. A *P* value of less than or equal to 0.05 was deemed significant.

Table 2-1: Primer sequences generated for PCR.

Name	Primer sequence	Application
NK3R	Fwd 5'-TGGCTGCCTTCAACACCTT-3' Rev 5'-ATGATGAGCAATGGGAAACAG-3'	PCR
c-Fos	Fwd 5'-GGCAAAGTAGAGCAGCTATCTCCT-3' Rev 5'-TCAGCTCCCTCCTCCGATTC-3'	qPCR
βActin	Fwd 5'-GCTGTGCTATGTTGCTCTAGA-3' Rev 5'-CATAGAGGTCTTTACGGATGT-3'	qPCR

III

Direct Effects of NKB on GnRH Synthesis and Secretion

INTRODUCTION

GnRH Peptide

GnRH is a peptide hormone produced by a specialized population of neurons in the hypothalamus. It is composed of 10 amino acids (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Nh_2). As the central regulator of reproduction, GnRH is at the apex of the HPG axis. Pulsatile GnRH secretion from the axons of hypothalamic GnRH neurons at the median eminence stimulates pituitary gonadotropin hormone synthesis and secretion. Pituitary gonadotropin hormones act on the gonads to stimulate the development of gametes and steroid hormone synthesis. During the juvenile period of development, pulsatile GnRH secretion is held quiescent by the GnRH neuron network. The GnRH neuron network becomes activated at puberty onset and a sustained increase in pulsatile GnRH secretion drives reproductive maturation (14, 15). Thus, the regulation of GnRH secretion is pivotal for the onset of puberty and reproduction.

GnRH Gene Regulation

GnRH synthesis is regulated at the level of transcription. Sequence upstream from the GnRH gene contains several regions that are highly homologous between human, rat and mouse (59). In rat, these elements include a minimal GnRH promoter,

GnRH-P (-173/+1), and three upstream enhancers, GnRH-E1 (-1863/-1571), GnRH-E2 (-3135/-2631), and GnRH-E3 (-4199/-3895). Of these elements, the -173 bp minimal promoter (P) and enhancer 1 (E1) have been shown to be most critical in regulating GnRH gene expression (56, 60). GnRH-E1 is a neuron-specific enhancer that binds multiple transcription factors that have been shown to be important for the regulation of GnRH transcription (61-63). Deletion of more than 30 bp from E1 significantly reduces the transcriptional activity of a -173 GnRH promoter luciferase reporter *in vitro* (64). Also, interestingly, deletion of a region that contains E1 *in vivo* results in disrupted puberty and infertility (65). Thus, the regulation of GnRH gene transcription by modulating enhancer 1 function may be critically important to the onset of puberty and reproduction.

NKB Regulates Gonadotropin Hormones by Regulating GnRH Secretion

Recent studies have identified mutations in TAC3 and TACR3, the genes which encode NKB and NK3R, in humans with IHH, a disorder characterized by a failure to undergo puberty and low circulating levels of gonadotropin hormones (33, 34). Since the administration of pulsatile GnRH was able to rescue circulating LH levels in these patients, it is thought that NKB regulates gonadotropin hormone secretion at the level of the hypothalamus by regulating GnRH release (34). Animal studies employing the intracerebroventricular (ICV) injection of senktide, a NK3R agonist, continue to support a role for NKB in the regulation of GnRH secretion but there have been some inconsistencies. A short time after senktide treatment, serum LH became decreased in

ovariectomized female rats (36) and mice (37) treated with low estrogen. In contrast, serum LH became increased in gonad-intact diestrus female rats (38), follicular phase ewes (39) and prepubertal rhesus monkeys (40). Surprisingly, a recent investigation of Tacr3 knock-out mice reported the normal timing for the onset of puberty and normal circulating LH levels. However, male mice were reported to have small testes and low circulating FSH levels. Moreover, female mice had decreased uterine weight, abnormal estrous cycles, reduced fertility, and decreased occurrence of corpora lutea, suggesting an ovulatory defect (35). Thus, although NKB has not yet been definitively classified as an activator or repressor of GnRH secretion, nor the precise role of NK3R in the onset of puberty identified, *in vivo* studies suggest a key role for NKB in the regulation of gonadotropin hormone secretion and reproduction.

<u>NKB May Directly and Indirectly Regulate GnRH Secretion</u>

Kisspeptin is a potent activator of GnRH neurons and inducer of GnRH secretion (16, 66-69). Kisspeptin, NKB and dynorphin are co-expressed in a population of neurons referred to as KNDy neurons within the ARC, a region of the hypothalamus implicated in the control of pulsatile GnRH secretion (70). Axons from KNDy neurons project to GnRH neuron terminals in the median eminence. In addition, KNDy neurons in the ARC were shown to have bilateral projections to other KNDy neurons (71), express NK3R (37) and exhibit c-Fos induction, a marker of neuronal activation, in response to the central administration of senktide (37). Thus, it is speculated that NKB may act either autosynaptically or transynaptically on NK3R within KNDy neurons to induce kisspeptin

secretion and indirectly stimulate GnRH neurons. In addition, NK3R immunoreactivity has been detected in a subset of GnRH somata, about 16% at the OVLT, and in GnRH neuron terminals at the median eminence in the female rat (44). NK3R expression has also been detected by RT-PCR in 50% of pools of GnRH neurons from GnRH-GFP mice (45). Therefore, we hypothesize that NKB may also directly regulate GnRH secretion by acting on NK3R-expressing GnRH neurons. However, due to the complexity of the GnRH neuron network and the multiple cell populations that express NK3R, *in vivo* studies are unable to distinguish the direct from indirect effects of NKB on GnRH neurons.

Our lab previously generated an *in vitro* model of GnRH neurons, the immortalized GT1-7 cell line. The GT1-7 cell line has been well characterized and shown to faithfully represent mature, post-migratory GnRH neurons in morphology, function and neuronal activity (49, 50). Importantly, GT1-7 cells enable the evaluation of the direct effects of NKB on GnRH neurons without confounding variables present in *in vivo* studies, including changes in kisspeptin release or the circulating hormonal milieu. In this chapter, we employ the GT1-7 cell model to investigate the direct effects of NKB on NK3R-expressing GnRH neuron function, including GnRH peptide secretion and GnRH gene transcription.

RESULTS

GT1-7 cells express NK3R and respond to senktide with a time-dependent change in GnRH secretion

RT-PCR was performed with total RNA to determine whether GT1-7 cells express NK3R. Using primers specific for Tacr3 cDNA, a band of the expected 387-bp size was observed (Table 2-1). The band was sequenced and the identity of the PCR product was confirmed by a Blast search to be mouse NK3R. Next, the functional significance of NK3R signaling on GnRH secretion was examined using radioimmunoassay (RIA) to measure the GnRH content of conditioned media collected from GT1-7 cells grown in static culture. GT1-7 cells responded to treatment with 50 nM senktide, a NK3R agonist, with a time-dependent change in GnRH secretion. Acute (3 hour) treatment significantly increased GnRH secretion by 66% (Fig. 3-2, left), suggesting that NKB could directly induce secretion from NK3R-expressing GnRH neurons *in vivo*. In contrast, chronic (24 hours) senktide treatment reduced GnRH secretion 26% (Fig. 3-2, right). These data indicate that the duration of exposure to NKB could also play a role in regulating the GnRH secretion response.

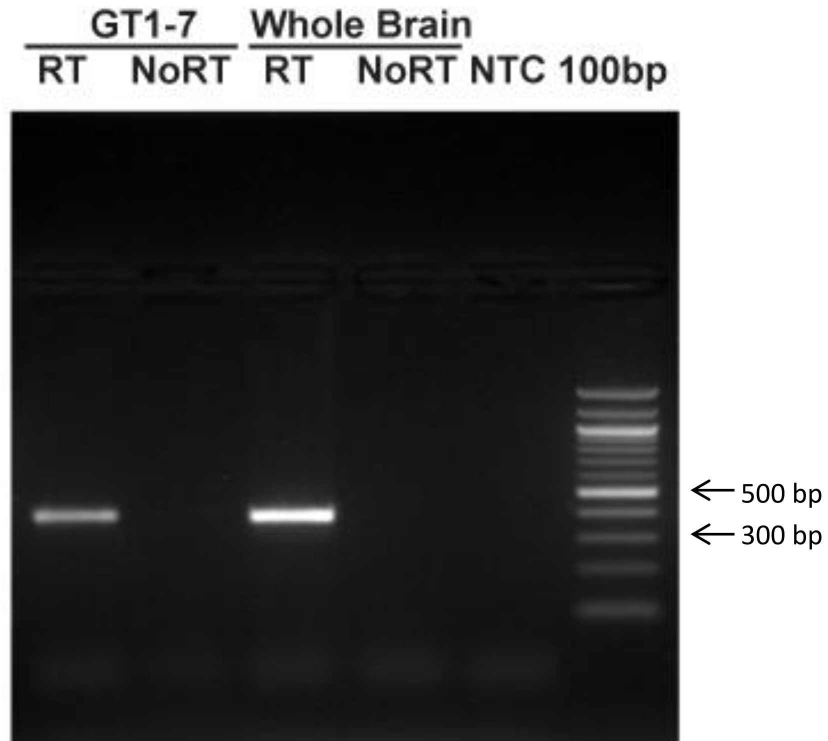

Figure 3-1: GT1-7 cells express mRNA for NK3R by RT-PCR.

RT-PCR using primers specific to NK3R, a single band of the expected 387 bp size was observed in cDNA from GT1-7 cells and mouse whole brain samples prepared with reverse transcriptase (RT), but not without enzyme (NoRT) or without template control (NTC). The band was excised and confirmed to be NK3R by sequencing.

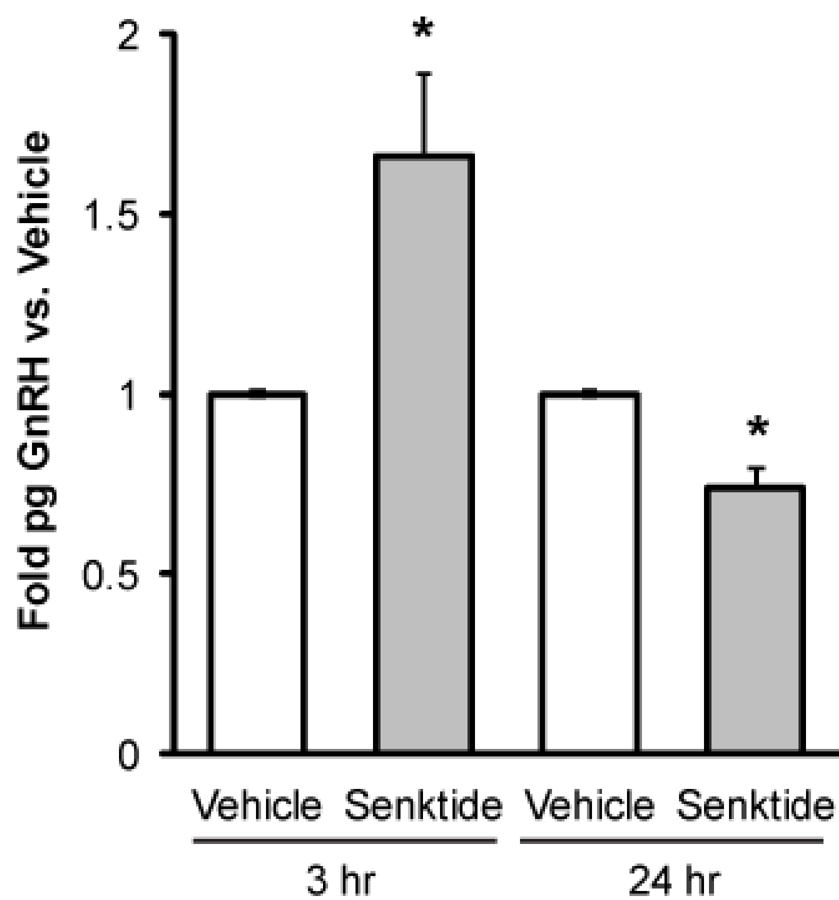

Figure 3-2: Senktide treatment induces a time-dependent change in GnRH secretion from GT1-7 cells.

Cells were treated with 50 nM senktide for either three or 24 hours and GnRH accumulation in conditioned media was measured by radioimmunoassay. Data shown are mean fold change in pg of GnRH versus vehicle controls ± SEM (n = 3 to 5). *, $P < 0.05$, senktide vs. vehicle control by Student's T-test.

Senktide treatment represses transcription of a rat -5Kb GnRH luciferase reporter

A decrease in GnRH secretion can result from reduced GnRH biosynthesis and a failure to replenish the releasable pool. Because GnRH biosynthesis is regulated at the level of transcription, long-term senktide treatment might act to repress GnRH gene transcription. To test whether long-term treatment with senktide represses GnRH gene transcription, GT1-7 cells were transiently transfected with a luciferase reporter containing five kilobase of upstream regulatory sequence of the rat GnRH gene (-5kb-GnRH-Luc) and treated for 10 hours with either 1 or 10 nM senktide treatment (Fig. 3-3). Senktide treatment significantly repressed GnRH reporter gene activity in a dose-dependent fashion. These data confirm that NK3R activation by senktide represses GnRH gene transcription. Interestingly, we observed a reduced sensitivity to the 1 nM dose of senktide in later trials and that expression of NK3R became reduced with passage or culture conditions by RT-PCR. To circumvent variability caused by differences in receptor expression levels, GT1-7 cells lacking endogenous NK3R were transiently transfected with a rat NK3R expression plasmid for the remainder of this study.

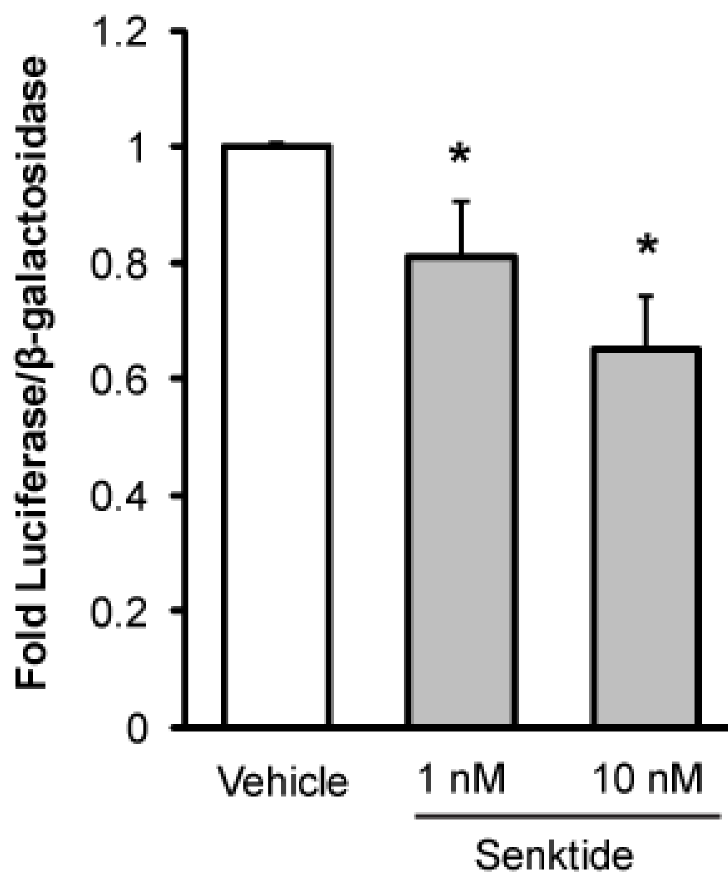

Figure 3-3: Senktide represses GnRH transcription.

GT1-7 cells were transiently transfected with a -5Kb GnRH luciferase reporter. Cells were treated with 1 or 10 nM Senktide for 10 hours and transcriptional activity was measured by luciferase assay. Luciferase data are normalized to β-galactosidase and represented as fold versus vehicle ± SEM (n = 3). *, $P < 0.05$, senktide versus vehicle control by Student's *t* test.

Enhancer 1 is sufficient to convey repression of -5 Kb GnRH luciferase reporter activity by NK3R activation

Truncation analysis was employed to map the elements in the -5 Kb regulatory region of the rat GnRH gene that are responsible for repression. Repression of GnRH gene transcription by senktide is maintained after truncation to -2168 bp. The remaining region includes a previously characterized enhancer, GnRH-E1, and promoter, GnRH-P. To determine the sufficiency of GnRH-E1 and GnRH-P for repression by NK3R activation in the absence of the flanking region, we used the GnRH-E1 and GnRH-P (E1/P) cloned into a luciferase reporter gene and tested reporter activity in response to senktide treatment. We found that senktide treatment represses -5 Kb GnRH and E1/P luciferase activity equivalently (Fig. 3-4). Therefore, GnRH-E1 and GnRH-P are sufficient for repression of GnRH gene transcription by NK3R activation.

To further test the sufficiency of enhancer 1 in mediating repression of GnRH gene by NK3R activation, GnRH-E1 was cloned upstream of a thymidine kinase promoter (TKp) that also contains a luciferase reporter. The thymidine kinase promoter alone does not respond to senktide treatment. However, senktide represses the luciferase reporter activity of GnRH-E1/TKp construct to a level similar to that observed previously with the -5Kb regulatory region (Fig. 3-4). These data suggest that enhancer 1 alone is sufficient to convey repression of GnRH transcription by NK3R activation.

We also sought to test the rat GnRH promoter for its sufficiency in mediating repression. However, without an enhancer, we have observed that the activity of the reporter containing only the promoter is generally too low to observe repression. We tried cloning it into vectors with the RSV enhancer or SV40 enhancer, but these vector

controls were stimulated by senktide. Thus, although our data identified GnRH-E1 as sufficient for NK3R to mediate its repression effect on GnRH transcription, this does not exclude the possibility that promoter region may also play a role in mediating repression.

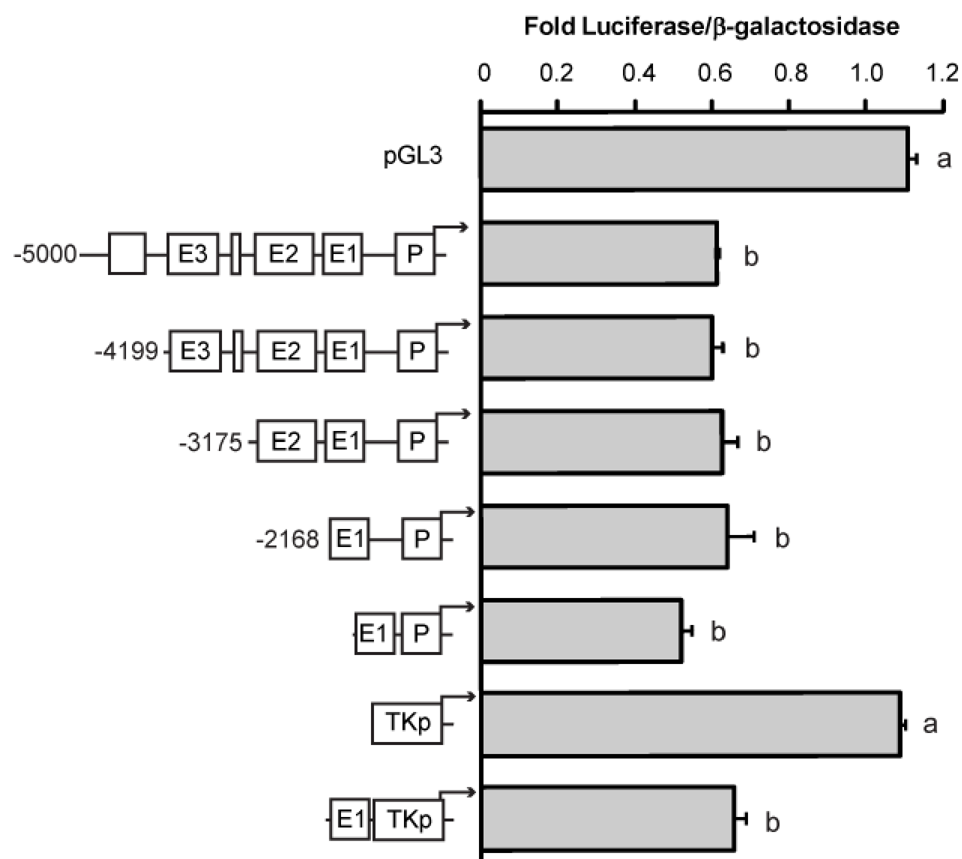

Figure 3-4: Enhancer 1 of the rat GnRH gene is sufficient for senktide-mediated repression.

GT1-7 cells were transiently transfected with NK3R and a luciferase reporter containing truncations of -5Kb GnRH regulatory region denoted as number of base-pairs upstream of the GnRH TSS or enhancer 1 (E1, -1863/-1571) and the promoter (P, −173/+112), or E1 with a heterologous -81-bp thymidine kinase minimal promoter (TKp). Cells were then treated with either vehicle or 30 nM senktide for 10 hours. PGL3 is the vector control. Luciferase data is normalized to β-galactosidase and are presented as fold vs. vehicle ± SEM. Bars not connected by same letter are significantly different by ANOVA, Tukey HSD.

Senktide treatment induce c-Fos expression and enhances AP-1 complex formation

c-Fos expression is often upregulated when neurons fire action potentials and can be used as an indirect marker for neuronal activity. c-Fos has been shown to be induced in a subset of GnRH neurons during the estrogen-induced GnRH/LH surge (72). Previous studies showed that the central administration of senktide induced c-Fos in NK3R-expressing neurons in the rat and mouse (36, 38, 73-75). Here, we show that one-hour treatment with 30 nM senktide in GT1-7 cells transiently transfected with NK3R causes a 79% increase in c-Fos RNA levels (Fig. 3-5). The induction of c-Fos, paired with an increase in secretion in response to acute senktide treatment, suggests that NKB would directly activate NK3R-expressing GnRH neurons.

Previous studies established a role for c-Fos in the repression of GnRH by TPA (76, 77). Therefore, we hypothesized that a similar mechanism of repression could be induced by senktide treatment. c-Fos protein regulates gene transcription by forming a heterodimer activation protein-1 (AP-1) complex with a variety of proteins, including JUN family protein such as c-Jun, JunB and JunD, as well as ATF2, ATF5 and MAF family protein (78). To determine whether the induction of c-Fos RNA by senktide results in increased AP-1 complex formation, we tested the activity of an AP-1 luciferase reporter, which contains 7 copies of AP-1 consensus binding sites (TGACTAA), in response to senktide treatment. Treatment with 30 nM senktide for 6 hours increased the activity of AP-1 luciferase reporter 5.2 +/- 0.139 fold in GT1-7 cells transiently transfected with NK3R (Fig. 3-6). These data suggest that NK3R activation not only induces c-Fos mRNA but also c-Fos protein and thus functional activity of the AP-1 transcription factor complex.

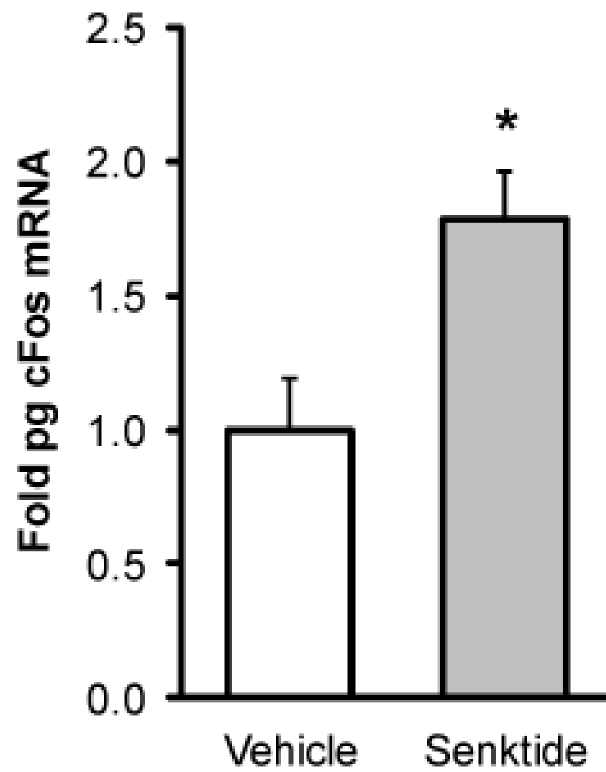

Figure 3-5: Senktide induces c-Fos RNA in GT1-7 cells.

Quantitative RT-PCR was performed on total RNA from GT1-7 cells transfected with NK3R and treated 1 hour with 30 nM senktide or vehicle control. The data is presented as fold vs. vehicle treated control ± SEM (n = 3). *, $P < 0.05$ by Student's *t* test.

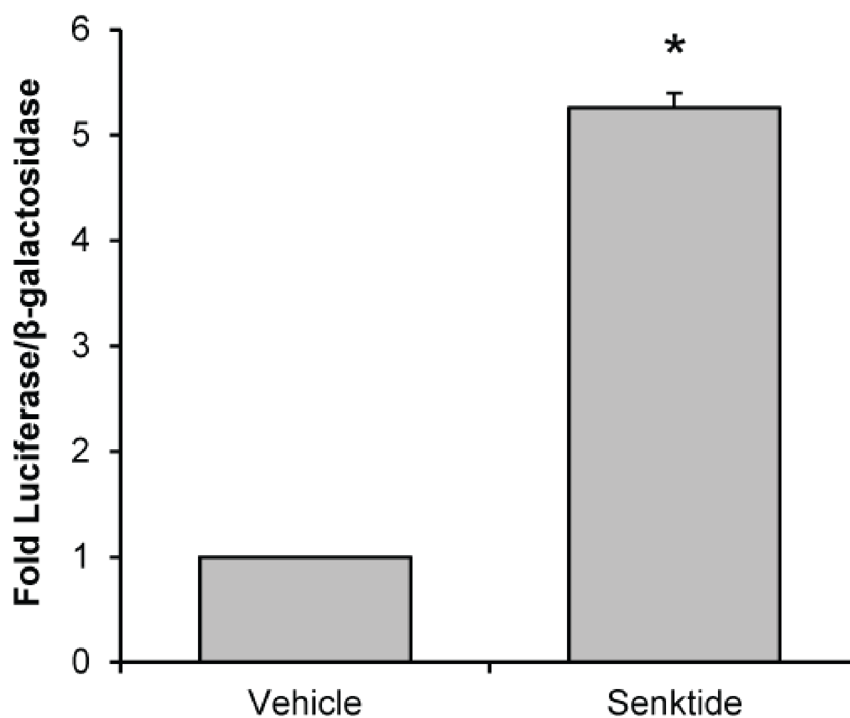

Figure 3-6: Senktide induces AP-1 luciferase reporter activity in GT1-7 cells.

GT1-7 cells were transiently transfected with NK3R and an AP-1 luciferase reporter that contains 7 copies of the AP-1 consensus binding sites (TGACTAA). Cells were serum starved 24 hours and treated for 6 hours with 30 nM senktide. Luciferase data is normalized to β-galactosidase and represented as fold vs vehicle ± SEM. *, $P<0.05$, senktide vs. control by Student's T-test.

AP-1 is involved in the NK3R-mediated repression of GnRH gene transcription

A previous study identified a region between -126 to -73 bp in the promoter of rat GnRH gene as responsible for the repression of GnRH transcription by Fos (76). However, our results map senktide-mediated repression to enhancer 1 region. It is possible that Fos-mediated regulation of GnRH transcription also occurs at enhancer 1 as well as the promoter. However, since our study mapped senktide-mediated repression to enhancer 1, we chose to further investigate whether Fos is able to regulate GnRH transcription through effects at this region. First, to elucidate c-Fos regulation of GnRH transcription at enhancer 1, we examined the effect of c-Fos and c-Jun (AP-1) over-expression on the activity of a GnRH-E1/TKp luciferase reporter. Over-expression of AP-1 significantly decreased GnRH-E1/TKp luciferase reporter activity by 0.57-fold verses a pcDNA3.1 control (Fig. 3-7). This result suggests a role for c-Fos and Jun in the regulation of enhancer 1 function.

To further confirm the role of AP-1 in regulating enhancer 1, we tested the effect of over-expressing a dominant negative to AP-1 complex formation, A-FOS. A-FOS consists of a dimerization domain and an extension of artificial acidic sequence at the N-terminus of the Fos leucine zipper, which replaces the normal basic region that is required for DNA binding (55). Over-expression of A-FOS alone increased GnRH-E1/TKp luciferase reporter activity (Fig. 3-7), suggesting that AP-1 complex may be involved in regulating basal expression of rat GnRH gene. In addition, repression of GnRH-E1/TKp transcription by AP-1 over-expression was significantly blocked by A-FOS (Fig. 3-7). These data indicate that c-Fos-mediated repression of E1 involves AP-1 complex formation.

Next, A-FOS was used to determine if senktide-mediated repression of enhancer 1 activity requires AP-1 complex formation. Over-expression of A-FOS blocked the repression of the GnRH-E1/TKp reporter by 0.4 nM senktide treatment in GT1-7 cells transiently transfected with NK3R (Fig. 3-8). Thus, AP-1 complex formation is involved in NK3R-mediated repression of GnRH transcription by effects mediated through enhancer 1.

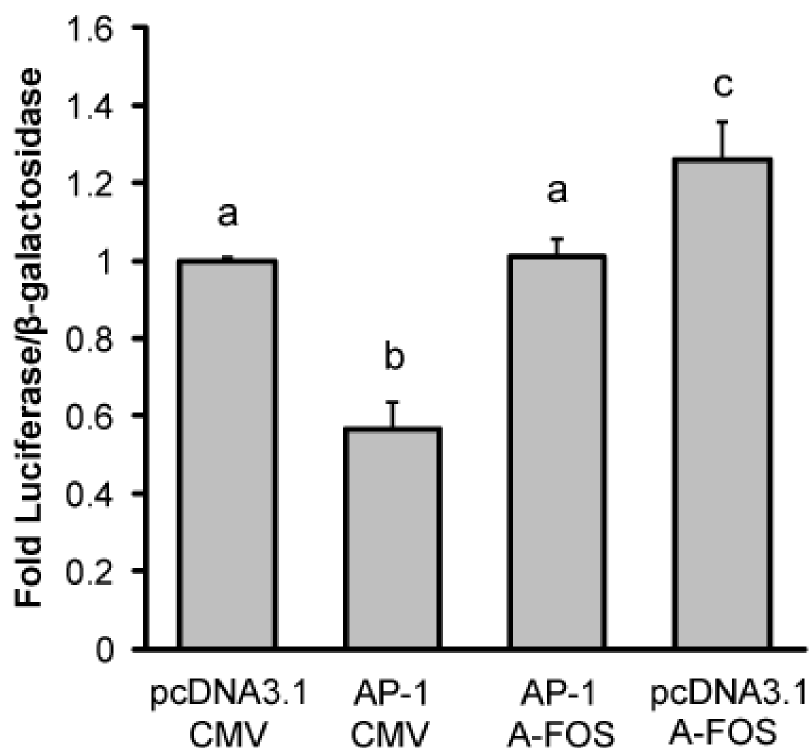

Figure 3-7: AP-1 is involved in repression of GnRH transcription by effects at enhancer 1.

Effect of over-expression of c-Jun and c-Fos (AP-1) is measured on activity of GnRH-E1/TKp luciferase reporter. Over-expression of a dominant negative to AP-1 complex formation, A-FOS, is examined for its ability to block effects of AP-1 over-expression. pcDNA3.1 is the vector control for c-Fos and c-Jun; CMV is the vector control for A-FOS. Luciferase data are normalized to β-galactosidase and represented as fold vs. pcDNA3.1 + CMV control ± SEM (n = 3). Bars not connected by same letter are significantly different by ANOVA, Tukey HSD.

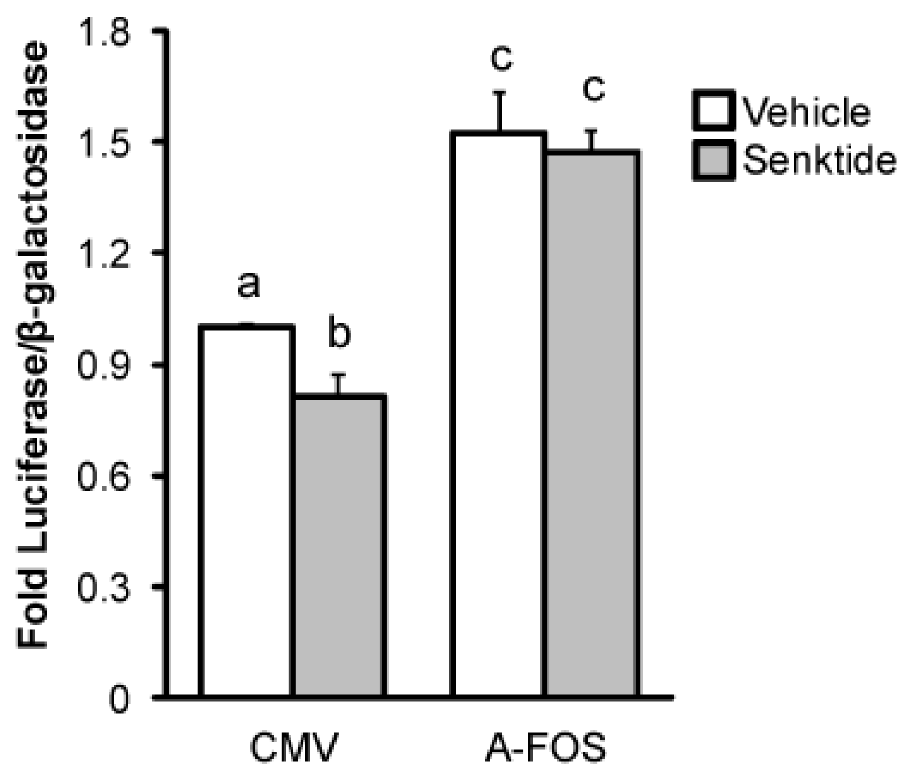

Figure 3-8: Senktide-mediated repression of enhancer 1 activity requires AP-1 complex formation.

GT1-7 cells are transfected with GnRH-E1/TKp luciferase reporter, NK3R and either A-FOS or CMV vector control. Cells are treated for 8 hours with 0.4 nM senktide after serum starved for 24 hours. Luciferase data is normalized to β-galactosidase and represented as fold vs. CMV vehicle-treated control ± SEM (n = 3). Bars not connected by the same letters are significantly different by ANOVA, Tukey HSD.

Fos protein directly associates with AP-1 half-sites in enhancer 1 of GnRH

Next, we sought to determine whether Fos proteins bind directly to enhancer 1 of the GnRH gene. Sequence analysis identified several putative AP-1 half-sites (E1A-D), each containing 5 of the 7 bases of the consensus AP-1 site (TGA$^{C}/_{G}$TCA), in enhancer 1 of the rat GnRH gene. To test if Fos proteins are directly bound to DNA, electromobility shift assay (EMSA) was performed. TPA was used to induce c-Fos in GT1-7 cells rather than senktide treatment to induce c-Fos in the whole population of cells avoiding the problems with NK3R transfection efficiency. Using western blot, our preliminary studies showed that 3-hour treatment with 100 nM TPA maximally induced c-Fos protein levels. As anticipated, TPA treatment induced formation of a new protein complex (C1) on a positive control AP-1 oligonucleotide probe (Fig. 3-9, lanes 1 and. 2) and the preincubation with cold competitor probe resulted in the loss of bands (Fig. 3-9, lanes 2 and 3). Preincubation with Fos but not IgG control antibody supershifted C1, confirming the direct binding of Fos protein to this sequence (Fig. 3-9, SS lanes 4 and 5) while preincubation with a c-Fos-specific antibody supershifted a portion of the TPA-responsive complex (Fig. 3-9, compare amount of C1 converted to SS in lane 5 vs. 6). These results indicate, as expected, that TPA treatment of GT1-7 cells induces binding of a new protein complex to a consensus AP-1 oligonucleotide probe that contains Fos proteins, including c-Fos.

Next, EMSA was performed to determine if Fos binds to oligonucleotide probes with sequences corresponding to putative AP-1 half-sites in enhancer 1 regions (Table 3-1). Similar to the AP-1 consensus probe, TPA treatment induced a new protein complex on E1A oligonucleotide probes (Fig. 3-9, compare amount of C1 in lanes 7 vs. 8) that

was lost upon preincubation with unlabeled probe (Fig. 3-9, compare amount of C1 in lanes 8 vs. 9) and supershifted by preincubation with Fos but not IgG control antibody (Fig. 3-9, compare amount of C1 converted to SS in lanes 11 vs. 10). Moreover, preincubation with a c-Fos-specific antibody induced supershift of a portion of the TPA-responsive complex for E1A (Fig. 3-9, compare amount of C1 converted to SS in lanes 11 vs. 12), indicating the presence of c-Fos protein in complex bound to E1A. In contrast, TPA treatment failed to induce any changes in protein complexes bound to E1B or E1D oligonucleotide probes (Fig. 3-10, C1 lanes 4 vs. 5 and 10 vs. 11). Also, despite observing a small amount of TPA-responsive complex, we were unable to confirm the presence of Fos protein bound to E1C oligonucleotide probe by supershift (Fig. 3-10, C1 lanes 7 vs. 8 and Fig. 3-11, C1 lanes 2 vs. 5). Therefore, the putative binding sites identified with E1B, (-1714/-1692), E1C (-1691/-1669) and E1D (-1616/-1594) were not confirmed to bind Fos. Thus, of the several putative AP-1 half-sites identified by sequence analysis, only the sites located within enhancer 1 (-1727/-1715) were confirmed to bind Fos proteins and only the site within -1727/-1715 was confirmed to bind to c-Fos, specifically.

Table 3-1: Oligonucleotide probes generated for EMSA.

Probe Name	**Oligonucleotide Sequences for EMSA (sense strands)**
AP-1 Consensus	5'-GATCCGGC**TGACTCA**TCAAGCTA-3'
E1A (-1737/-1715)	5'-AAGGTTACAA**AGTCA**TCACTGCT-3'
E1B (-1714/-1692)	5'-ATCATTT**TGAG**CT**A**TTTTAATGT-3'
E1C (-1691/-1669)	5'-TGAAAA**TGA**AG**CA**ATTTTCCTAT-3'
E1D (-1616/-1594)	5'-TTAAACCCAA**TTTGTCA**ATTTCA-3'

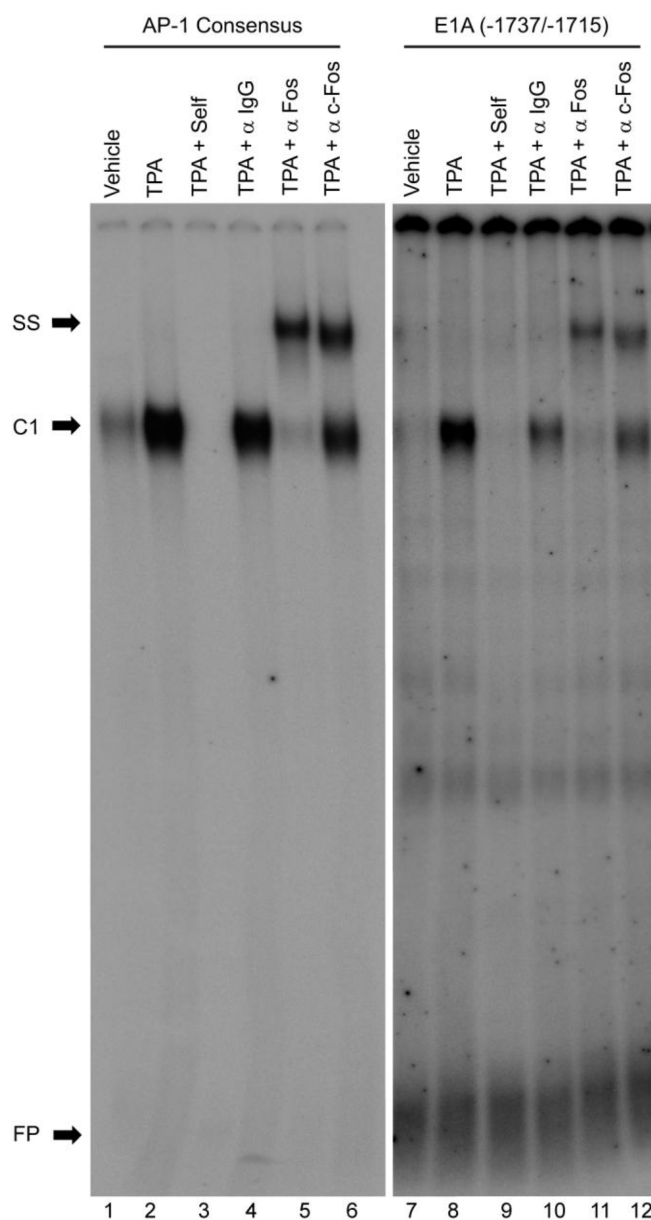

Figure 3-9: Fos protein is directly associated with the novel AP-1 half-site, E1A, in enhancer 1 of the rat GnRH gene.

Nuclear extracts were prepared from GT1-7 cells treated with 3 hours 100 nM TPA or vehicle control. EMSA was performed using an oligonucleotide probe corresponding to the AP-1 consensus sequence or AP-1 half-site in enhancer 1 rat GnRH gene (-1737/-1715). Supershift was performed using antibodies against Fos, c-Fos or IgG control. Exposure for AP-1 probe was 4.5 hours at room temperature. Exposure for E1A was 4.5 days at room temperature. TPA responsive complex (C1), antibody supershift (SS), Free-probe (FP).

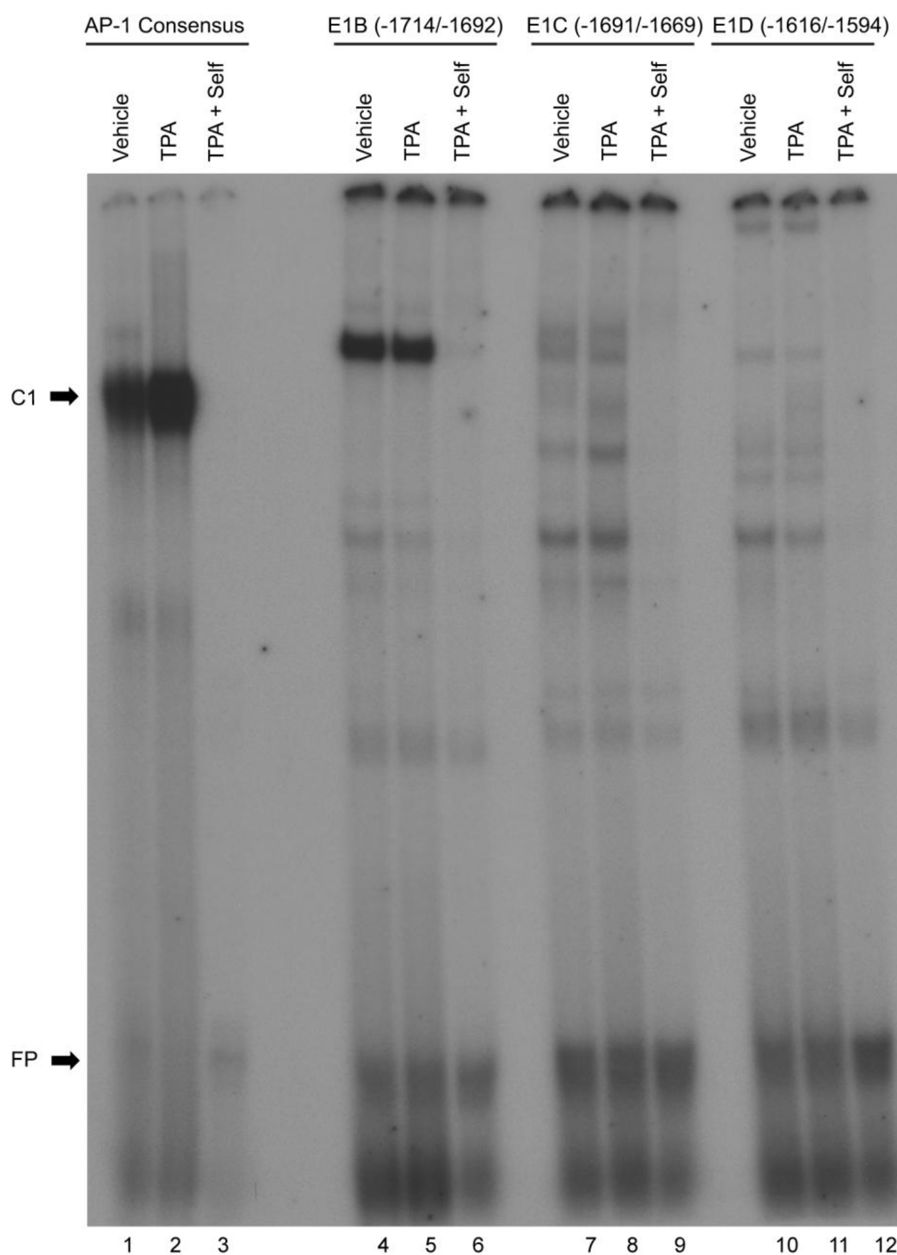

Figure 3-10: TPA-responsive complexes were not observed with E1B and E1D probes.

Nuclear extracts were prepared from GT1-7 cells treated with 3 hours 100 nM TPA or vehicle control. EMSA is employed with oligonucleotide probe corresponding to AP-1 half-site in enhancer 1 rat GnRH gene, E1B, E1C, and E1D, or AP-1 consensus sequence. 1/5 reaction volume was loaded for gel for AP-1 control. Films were exposed for 4 days at room temperature. TPA responsive complex (C1), antibody supershift (SS), Free-probe (FP).

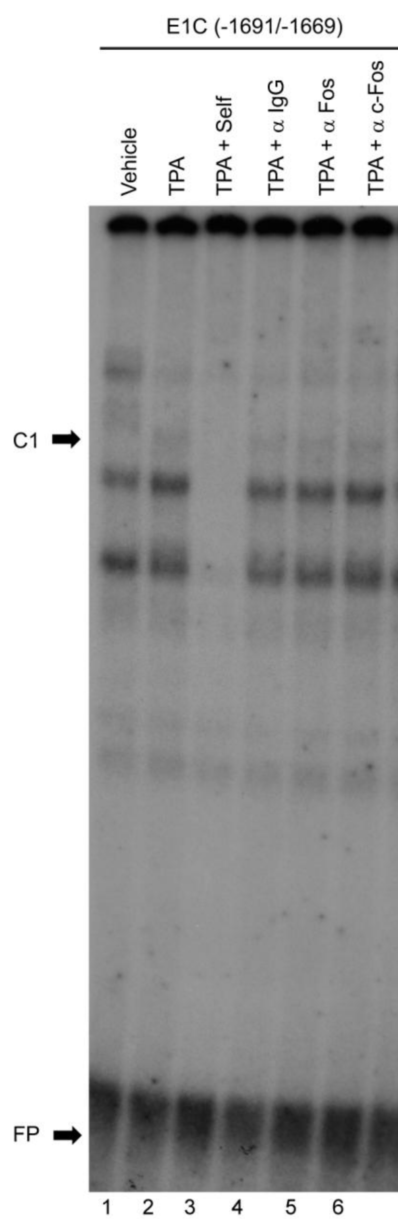

Figure 3-11: TPA-responsive complexes observed with E1C probe did not supershift with Fos antibodies

Nuclear extracts were prepared from GT1-7 cells treated with 3 hours 100 nM TPA or vehicle control. EMSA is employed with oligonucleotide probe corresponding to AP-1 half-site in enhancer 1 rat GnRH gene, E1C (-1691/-1669). Films were exposed for 4 days at room temperature. TPA responsive complex (C1), Free-probe (FP).

DISCUSSION

NK3R and its ligand, NKB, were recently implicated in human puberty and reproduction by genetic studies in patients with idiopathic hypogonadotropic hypogonadism (33, 34). Treatment of these patients with pulsatile GnRH increased serum LH [34], indicating NKB stimulates gonadotropin hormone levels by stimulating GnRH secretion. Since these initial studies, *in vivo* studies in various animal models have tried to identify the precise mechanism in the hypothalamus whereby NKB stimulates gonadotropin hormone secretion. Within the GnRH neuron network, NK3R expression has been shown in the majority of KNDy neurons and in a subset of GnRH neurons (37, 41, 44). Since senktide was shown to induce c-Fos in KNDy neurons, it has been suggested that NKB may stimulate kisspeptin secretion and indirectly drive GnRH secretion. However, the functional significance of NKB signaling directly to NK3R-expressing GnRH neurons has not been determined.

In this thesis, we employ the immortalized GT1-7 cell line model of the GnRH neuron to test the hypothesis that NKB can directly regulate GnRH neuron function. We find that GT1-7 cells express NK3R and respond to acute senktide treatment with increased GnRH secretion and c-Fos gene expression, suggesting that NKB would directly activate NK3R-expressing GnRH neurons *in vivo*. In addition, senktide represses GnRH gene expression by a mechanism that includes AP-1 complex formation binding to a novel AP-1 half-site identified in enhancer 1.

<u>NKB could directly activate NK3R-expresssing GnRH neurons</u>

Similar to GnRH neurons *in vivo* (44, 45), we find that GT1-7 cells express NK3R. Also, similar to *in vivo* studies which have reported an acute increase in serum LH in response to senktide (38-40, 46, 79, 80), GT1-7 cells respond to acute senktide treatment with an increase in GnRH secretion. Moreover, similar to *in vivo* studies that have reported c-Fos to be induced in NK3R-expressing neurons in response senktide treatment, senktide treatment induced c-Fos expression in GT1-7 cells. Importantly, c-Fos induction in GnRH neurons has been correlated with increased LH secretion and is thought to indicate activation of GnRH neurons and increased GnRH secretion (72). Taken together, these results indicate that NKB would directly activate NK3R-expressing GnRH neurons. It should be noted that it was recently reported that GnRH neurons did not exhibit c-Fos induction or increased electrical firing in response to senktide in male mice (46, 80), however, NK3R expression was not confirmed in the GnRH neurons in these studies. Thus, ours is the first report of the functional significance of NKB signaling directly to NK3R-expressing GnRH neurons.

The duration of NKB exposure may be a determining factor in the GnRH secretion response to NKB. When treated for three hours, senktide increased GnRH secretion from GT1-7 cells, while 24 hours treatment caused a modest decrease. Since decreased GnRH secretion can be the result of reduced GnRH biosynthesis and a failure to replenish the releasable pool, we employed transient transfection assays to examine the effect of senktide on GnRH gene transcription. We found that eight hours of senktide treatment repressed the transcriptional activity of a rat GnRH luciferase reporter. Although this result supports suggests reduced GnRH biosynthesis could underlie the

decrease in GnRH secretion that occurs in response to long-term senktide treatment, other mechanisms may be involved.

NK3R is a G protein-coupled 7-transmembrane receptor and studies have shown NK3R becomes desensitized in response to ligand by a β-arrestin-dependent mechanism in enteric neurons (53). Moreover, desensitization of NK3R was observed in monkey when, after 48 hours of continuous intravenous infusion of senktide, a bolus of senktide was unable to elicit GnRH release (40). However, if receptor desensitization is responsible for decreased secretion in GT1-7 cells when compared to vehicle controls, this would suggest that ligand is present in vehicle controls or ligand-independent signaling from NK3R may be involved in maintaining basal GnRH secretion. Interestingly, we were able to detect RNA encoding NKB in GT1-7 cells by RT-PCR, suggesting the potential for a NK3R/NKB autoregulatory system. Alternatively, cellular pathways activated by NK3R may initiate a negative feedback loop. Tachykinin receptors are thought to cause an increase in cAMP production (81), a factor strongly implicated in the pulsatile release of GnRH (82). Interestingly, cAMP was previously shown to induce a cAMP-dependent protein kinase (PKA)-dependent inhibition of adeylylate cyclase (AC) activity that results in decreased cAMP production (83). Thus, in addition to reduced GnRH biosynthesis, mechanisms such as receptor desensitization or a negative feedback loop may contribute to a decrease in GnRH secretion response to long-term senktide treatment.

Although we report that NKB directly regulates GnRH secretion from NK3R-expressing GnRH neurons *in vitro*, it remains unclear if NKB modulates GnRH secretion mainly through direct or indirect effects on GnRH neurons *in vivo*. In addition to a

subset of GnRH neurons, NK3R has been shown to be expressed in multiple populations of neurons in the GnRH neuron network, including the majority of KNDy neurons in the ARC. Recent studies have emerged suggesting that the response to NKB might require signaling by the kisspeptin receptor, GPR54. For example, in transgenic male mice, the increased serum LH response to senktide required the presence of the kisspeptin receptor, GPR54 (46). Also, in male monkeys, desensitization of the kisspeptin receptor blocked the response to senktide (79). However, while these studies suggest a potential signaling hierarchy where the response to NKB requires GPCR54, they do not distinguish direct from indirect signaling to GnRH neurons since the majority of GnRH neurons express GPR54. It could be reasoned that since only a subset of GnRH neurons, as compared to the majority of KNDy neurons in the ARC, express NK3R, then NKB is more likely to influence GnRH secretion by effects on KNDy neurons. However, it has been suggested that a subset of GnRH neurons could profoundly influence gonadotropin secretion, possibly by synchronizing GnRH neuron firing. For example, only a about 40% of GnRH neurons show Fos induction in response to estrogen during the GnRH and LH surge (72). Thus, NKB may directly regulate GnRH secretion by signaling to even a subset of NK3R-expressing GnRH neurons, possibly acting to synchronize firing to time the pulses of GnRH secretion.

NKB represses GnRH transcription by a mechanism that involves Fos binding to E1

GnRH transcription is regulated *in vivo* during development, aging, across the estrous cycle and in response to hormones and neurotransmitters (51, 84-89). We show

that NK3R activation in response to senktide treatment represses activity of a -5 kb rat GnRH luciferase reporter. Previous *in vitro* studies in GT1-7 cells identified a 300 bp region termed enhancer 1 (E1) and a -173 bp promoter (P) in the rat -5 kb GnRH regulatory region (60). Together, these two regions are sufficient to target GnRH expression to GnRH neurons *in vivo* (63, 64). Importantly, enhancer 1 is required for neuron-specific expression of GnRH (64) and for maintaining GnRH expression levels *in vivo* (90). Here, truncations and deletion analysis of the -5 kb rat GnRH promoter identified enhancer 1 as sufficient to confer senktide-mediated repression.

The induction of c-Fos with TPA and c-Fos over-expression were shown to repress the activity of a GnRH luciferase reporter (76, 77). Since we observed that senktide induced c-Fos mRNA levels and repressed GnRH, we hypothesized that the mechanism of repression might involve Fos. Moreover, senktide treatment induced the activity of an AP-1 luciferase reporter. In addition, over-expression of the dominant negative to AP-1 complex formation, A-FOS, blocks repression of GnRH by c-Fos and cJun (AP-1) over-expression as well as senktide treatment. These results indicate that the repression of GnRH transcription by senktide involves AP-1 complex formation. Next, we identified a putative AP-1 half-site at -1729/-1723 bp in enhancer 1 and showed it is able to directly bind to Fos proteins and specifically to c-Fos by EMSA. Interestingly, this novel AP-1 half-site is located near a region (-1736/-1728) previously shown to be involved in the repression of GnRH transcription by melatonin in GT1-7 cells by a mechanism that involved changes in binding of COUP-TFI and C/EBP β (91). Also, this AP-1 half-site is located between two GATA sites (-1743/-1748 and -1710/-1715) known to be essential for E1 function in GT1-7 cells and involved in the regulation of GnRH

(92-94). Furthermore, the deletion of a region containing enhancer 1 *in vivo* caused disruptions in the timing of pubertal onset and estrous cycling in mice (65). Thus, in addition to regulating GnRH secretion, perhaps NKB regulates puberty and reproduction by modulating GnRH gene transcription by altering the activity of enhancer 1.

In summary, in Chapter 3, we report that GT1-7 cells express NK3R and respond to acute senktide treatment with increased GnRH secretion. Additionally, long-term senktide treatment reduces GnRH secretion and represses GnRH transcription by a mechanism that includes the induction of Fos protein and binding to a novel AP-1 half-site located within enhancer 1. Based on these results, we propose a model whereby NKB acting directly on NK3R-expressing GnRH neurons would stimulate GnRH secretion and regulate GnRH transcription.

IV

NKB Induces c-Fos Expression in GnRH Neurons Via PKC

INTRODUCTION

Reproductive function and development is controlled by the pulsatile release of GnRH. Human genetic studies revealed that NKB signaling is necessary for normal reproductive development, including the initiation of puberty and maintenance of pulsatile LH (95, 96). Disruption of NKB/NK3R signaling due to mutations in either NKB or NK3R leads to gonadotropin deficiency and results in IHH (33, 34). Since treatment of patients with NKB or NK3R mutations with pulsatile GnRH was shown to rescue serum gonadotropin levels, it was concluded that NKB functions at the level of hypothalamus to regulate the release of GnRH [34]. NKB is co-expressed with kisspeptin and dynorphin in KNDy neurons in the arcuate nucleus (ARC) in the hypothalamus (40, 70, 97). In addition, KNDy neurons express NK3R. Thus, it has been proposed that NKB acts in an autocrine and/or paracrine way on KNDy neurons to induce the release of kisspeptin from KNDy neurons and indirectly stimulate secretion from GnRH neurons (37). In addition, a subset of GnRH neurons has been shown to express NK3R, suggesting NKB may also directly regulate GnRH neurons (44). However, the functional significance of NKB acting directly on GnRH neurons is not known. In the chapter 3, we demonstrated that acute senktide treatment increased GnRH secretion and

induced c-Fos expression, a marker for neuronal activation, in an *in vitro* model of the matured and differentiated GnRH neuron. These data suggest that NKB would directly activate NK3R-expressing GnRH neurons *in vivo*.

Different types of NK3R-expressing neurons respond to NKB with c-Fos induction and activation. Activation of NK3R increases the expression of c-Fos in vasopressin and oxytocin neurons in the paraventricular nucleus (PVN) in rats (98, 99). Similarly in female rats, c-Fos expression in kisspeptin neurons is induced in response to ICV injection of senktide (38). Fos induction in GnRH neurons has been correlated with increased GnRH secretion *in vivo* (72). Due to technical limitations, however, it has not been determined if senktide induces c-Fos in GnRH neurons *in vivo*. In the last chapter, acute senktide treatment was shown to induce GnRH secretion from GT1-7 cells, indicating that NKB would directly activate NK3R-expressing GnRH neurons *in vivo*. Also, senktide treatment increased c-Fos mRNA levels in GT1-7 cells. Thus, the induction of c-Fos is likely a general response to NKB in NK3R-expressing neurons.

The goal of chapter 4 is to elucidate the mechanism whereby NK3R activation causes c-Fos induction in GT1-7 cells in order to provide important insight to the cellular pathways activated downstream from NK3R in GnRH neurons. As an immediate early-gene, c-Fos is activated rapidly and transiently in most cell types in response to growth factors, cytokines, hormones and other extracellular stimuli. As shown in the chapter 3, c-Fos mRNA levels were induced significantly after just 1 hour of senktide treatment. In contrast, the repression of GnRH transcription required 8 hours of senktide treatment. Because of its more acute regulation, the c-Fos promoter is preferred to that of GnRH as

an end point for use in investigating the cellular pathways acting directly downstream from NK3R in GnRH neurons.

c-Fos regulates a wide array of cellular processes, including cell proliferation, apoptosis and differentiation. Much is known about the transcriptional regulation of the c-Fos gene (100, 101). Depending on the stimuli and cellular model, various signaling pathways have been shown altered binding at different *cis*-elements in the c-Fos promoter. For example, growth factor stimuli such as colony stimulating factor (CSF-1) and platelet-derived growth factor (PDGF) signal through the JAK-STAT pathway to activate transcription of c-Fos gene through a STAT-binding site at -345 bp upstream from the transcription start site. Alternatively, ERK activation by activation of MAPK-dependent kinases leads to c-Fos induction by Elk phosphorylation and enhanced binding to a serum response element (SRE) located at -310 bp. Ca^{2+} influx through voltage-gated calcium channels can also activate c-Fos gene transcription by Ca^{2+}/calmodulin-dependent protein kinase (CaMK) phosphorylation of cAMP-response element binding (CREB) transcription factors and increased binding at a cAMP-response element (CRE) at -303 bp. Alternatively, increased CREB binding to this CRE site may be induced by ligand-activated G protein-coupled receptors through activation of adenylyl cyclase (AC) and cAMP-dependent kinase (PKA). Finally, c-Fos transcription was also shown to be regulated by binding of AP-1 transcription factors, including autologous regulation by c-Fos, at an AP-1 site at -295 bp (102). This study will utilize the well-characterized c-Fos promoter to elucidate the cellular pathways activated downstream from NK3R in GnRH neurons. These pathways represent potential therapeutic targets for the treatment of IHH, infertility and disorders of puberty.

RESULTS

NK3R activation leads to increased c-Fos gene transcription

In Chapter 3, it was shown that 1 hour of senktide treatment increased c-Fos mRNA significantly in GT1-7 cells (Fig. 3-5). c-Fos mRNA could have been increased due to enhanced RNA stability or increases in RNA stability or gene transcription. Here, we test the hypothesis that NK3R activation leads to increased c-Fos gene transcription. GT1-7 cells were transiently transfected with a -1 Kb mouse c-Fos promoter luciferase reporter and either NK3R or pcDNA3.1 control vector. Treatment with 30 nM senktide significantly increased the activity of the -1 Kb c-Fos promoter luciferase reporter in cells transiently transfected with NK3R (Fig. 4-1). These data indicate that the increase in c-Fos RNA in response to NK3R activation involves an increase in gene transcription.

GT1-7 cells were previously shown to respond to the NK1R agonist, substance P, by an increase of IP_3 formation (103). Our lab confirmed NK1R expression in GT1-7 cells. NK3R, similar to all tachykinin receptors, exhibits ligand preference rather than ligand specificity (104) and although NKB has a greater affinity to NK3R, it is able to activate NK1R as well. Thus, we thought it important to verify that the induction of c-Fos by senktide treatment is specific to activation of NK3R. In contrast to cells transfected with NK3R, senktide treatment had no effect on the activity of the mouse -1Kb c-Fos promoter luciferase reporter in GT1-7 cells transiently transfected with pcDNA3.1 backbone vector (Fig. 4-1). Therefore, we confirm that the induction of

c-Fos gene transcription by senktide treatment in GT1-7 cells occurs specifically in response to activation of NK3R.

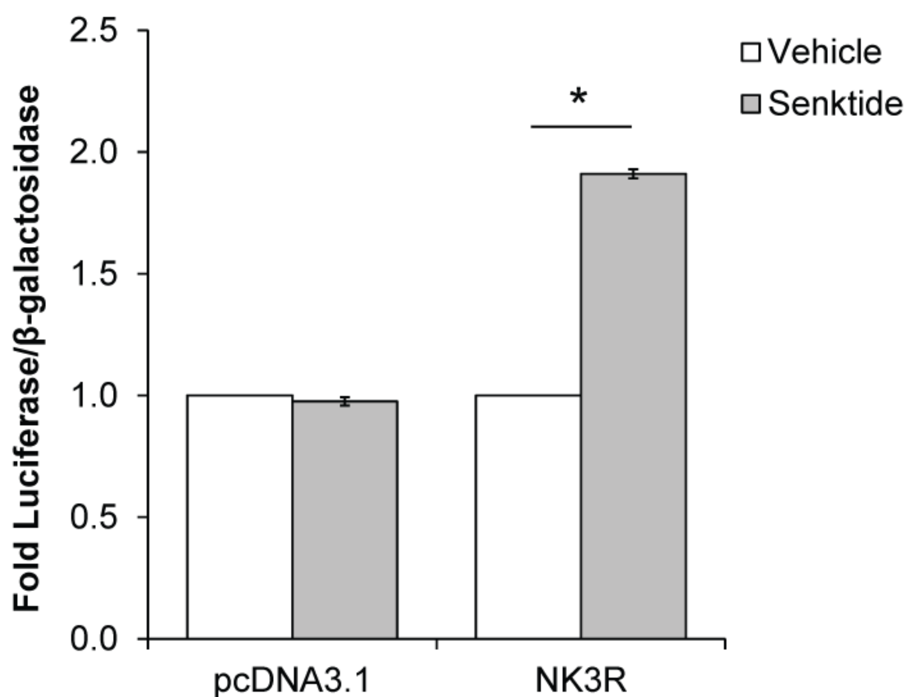

Figure 4-1: Senktide-mediated induction of c-Fos requires NK3R.

GT1-7 cells were transiently transfected with -1 Kb c-Fos luciferase reporter, and control vector, pcDNA3.1, or rat NK3R and treated with vehicle or 30 nM senktide. Luciferase data are normalized to β-galactosidase and represented as fold versus vehicle-treated pcDNA3.1 ± SEM. Bars not connected by same letter are significantly different by ANOVA, Tukey HSD.

The induction of c-Fos gene transcription by senktide precedes in time the repression of GnRH transcription and is dose-dependent

A time-course experiment with 2, 3, 4, 5, and 8 hours was performed to determine the optimal treatment time needed to achieve maximal induction of the -1Kb c-Fos luciferase reporter by 30 nM senktide. A 2-hour senktide treatment failed to change the activity of a -1 Kb c-Fos luciferase reporter significantly. However, a 3-hour senktide treatment maximally induced activity of the -1 Kb c-Fos promoter by 1.52 +/- 0.087 fold when compared to vehicle (Fig. 4-2). A similar induction of c-Fos promoter activity was also observed with 4, 5, and 8 hours of senktide treatment. Of note, induction of the c-Fos promoter reporter at 3 hours precedes the 8 hours of senktide treatment that was required to observe repression of the GnRH promoter. Thus, c-Fos induction occurs prior to the repression of GnRH transcription in response to NK3R activation in GT1-7 cells. Importantly, this result continues to support our model that the repression of GnRH transcription in response to NK3R activation occurs by a Fos-dependent mechanism. In addition, as it is more acutely regulated, the c-Fos promoter is preferred over the GnRH promoter for use in studies to identify the cellular pathways acting directly downstream from NK3R in GT1-7 cells.

Next, a dose-response experiment was performed to determine the dose of senktide required to obtain a maximal and sub-maximal induction of the -1 Kb c-Fos promoter. 3 hours of senktide treatment induced the activity of the -1Kb c-Fos luciferase reporter with an EC_{50} value of 0.037 +/- 0.010 nM (Fig. 4-3). In addition, a 1.6 nM dose of senktide was found to be sufficient to achieve maximal induction (Fig. 4-3). Thus, the 3-hour treatment with 30 nM senktide used previously should have been sufficient to

achieve maximal induction of c-Fos and will be utilized for remaining experiments. Alternatively, when a sub-maximal induction is required, the 3 hour treatment with 0.4 nM will be employed.

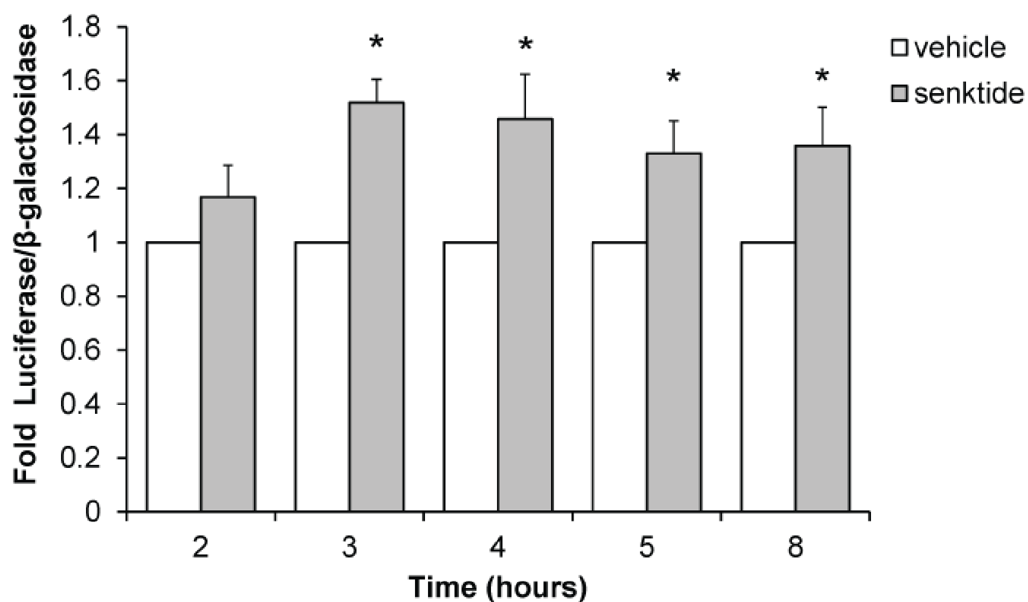

Figure 4-2: c-Fos is induced 3 hours after senktide treatment and maintained for 8 hours.

GT1-7 cells were transiently transfected with -1 Kb c-Fos luciferase reporter and with NK3R then treated with 30 nM senktide. Luciferase data are normalized to β-galactosidase and represented as fold versus vehicle controls ± SEM. *, $P < 0.05$, senktide versus vehicle control by Student's *t* test

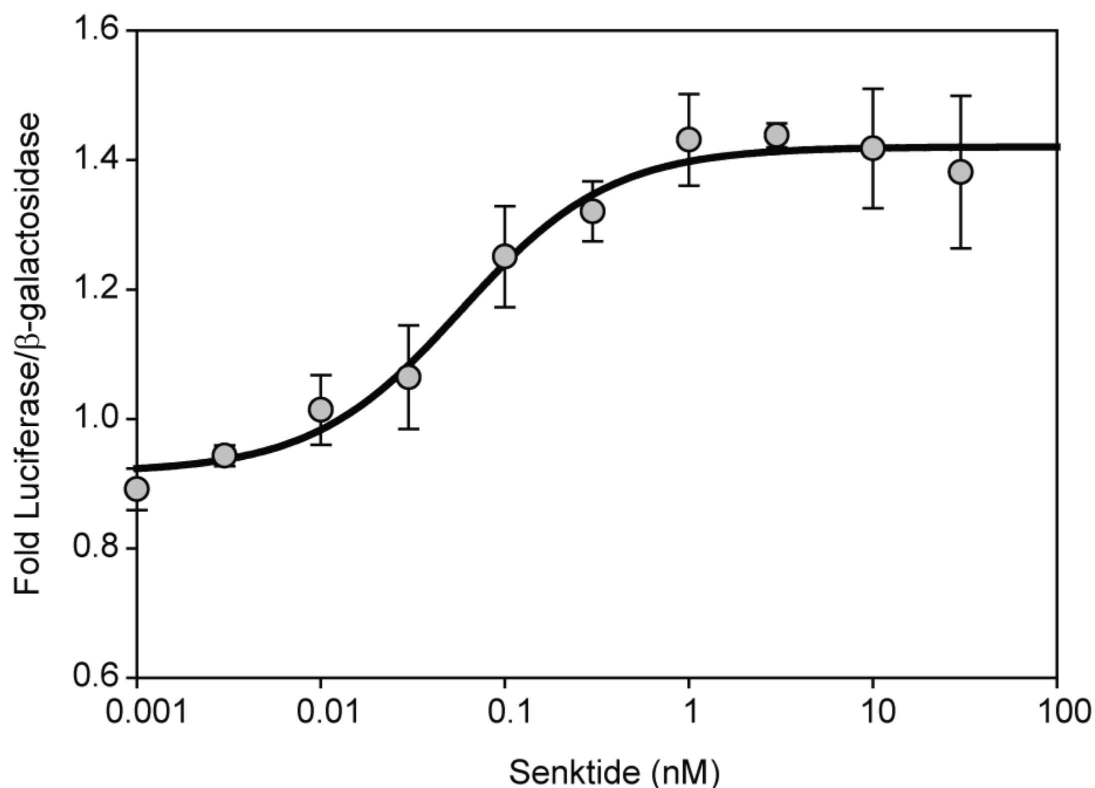

Figure 4-3: Senktide induces c-Fos transcription in a dose-dependent manner with EC_{50} = 0.04 nM and maximal dose = 1.6 nM.

GT1-7 cells were transiently transfected with rat NK3R and a -1 Kb c-Fos luciferase reporter and treated for 3 hours with vehicle or senktide at the indicated doses. Data shown are a representative dose-response curve fitted by nonlinear regression using a four-parameter logistic equation. Mean EC_{50} was determined to be 0.037 +/- 0.010 nM (n = 3) and maximal induction was observed with a dose of >1.6 nM.

<u>Senktide-mediated induction maps to between -400 and -200 bp in the mouse c-Fos promoter</u>

Transient transfection with truncations of the mouse -1 Kb c-Fos luciferase reporter was used to map the region responsible for senktide-mediated induction. 30 nM treatment of senktide significantly increases the activity of the wild-type -1 Kb c-Fos reporter by 1.52 +/- 0.072 fold. This induction is maintained when the -1 Kb c-Fos promoter is truncated to -600 and -400 bp. However, when the c-Fos promoter is truncated to -200 bp, induction of c-Fos reporter by senktide treatment is no longer observed (Fig. 4-4). Thus, the region that mediates repression by NK3R activation maps to between -200 and -400 bp upstream from the transcription start site in the mouse c-Fos promoter.

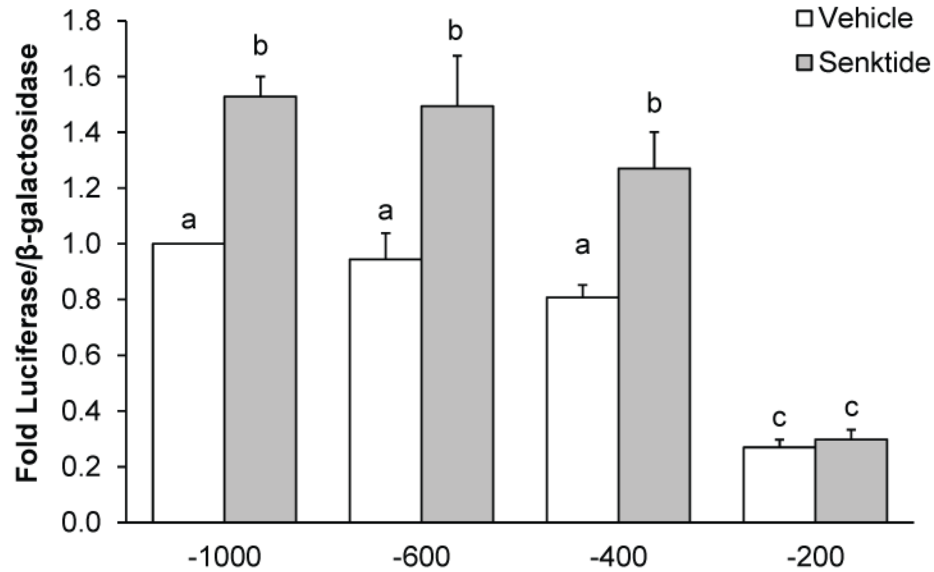

Figure 4-4: Senktide-mediated induction maps to between -200 and -400 bp in the mouse c-Fos promoter.

GT1-7 cells were transiently transfected with NK3R and truncations of a -1 Kb c-Fos gene, as indicated and treated with 30 nM senktide. Luciferase data are normalized to β-galactosidase and represented as fold versus vehicle-treated -1K c-Fos ± SEM. Bars not labeled with same letters are significantly different by ANOVA, Tukey HSD.

Senktide-mediated induction of mouse c-Fos requires the SRF and STAT binding sites

The region between -200 and -400 bp of c-Fos promoter contains binding sites for STAT, ETS, SRF, and AP-1 transcription factors, all of which have been previously reported to play a role in induction of c-Fos (105). The -1 Kb c-Fos luciferase reporter containing individual cis-mutations in these sites was used to determine if any of these elements are necessary for induction by NK3R activation. Mutations of these sites induced significant changes in vehicle treated controls, suggesting these *cis*-elements have a role in regulating the basal expression of c-Fos (Fig. 4-5). While mutations in STAT, ETS and SRF sites each decreased basal transcription of c-Fos, mutation of the AP-1 site leads to an increased reporter activity of c-Fos promoter. Next, the effect of *cis*-mutations on the response to senktide was determined. c-Fos reporters containing mutations in either the ETS or AP-1 binding sites were significantly induced by senktide treatment, indicating that these sites are not required for response (Fig. 4-5). However, reporters with mutations in either the STAT or SRF binding site were no longer induced by senktide. Therefore, we identified the STAT and SRF binding sites as required for the induction of c-Fos by NK3R activation.

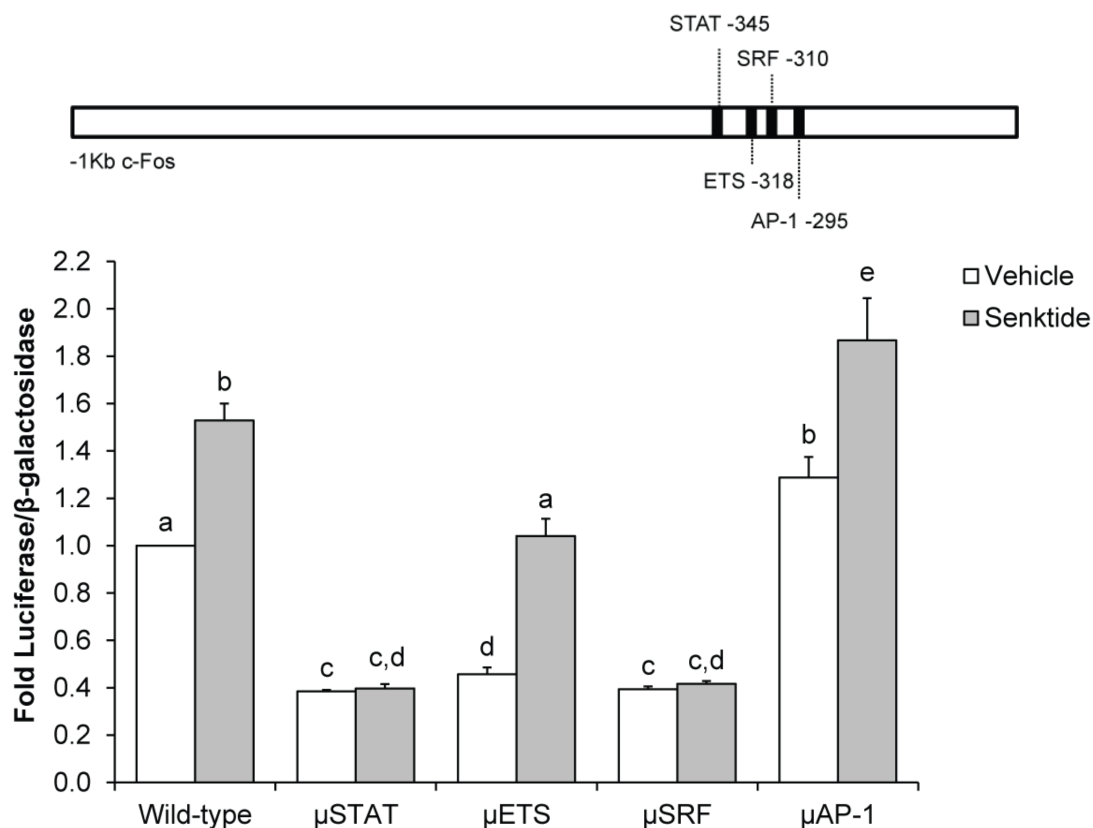

Figure 4-5: STAT and SRF sites are required for senktide-mediated induction of -1Kb c-Fos reporter activity.

GT1-7 cells were transiently transfected with NK3R and -1Kb c-Fos luciferase reporters containing cis-mutations in binding sites located between -200 and -400bbp of the c-Fos promoter. Luciferase data are normalized to β-galactosidase and represented as fold versus vehicle-treated wild-type -1 K c-Fos controls ± SEM. Bars not connected by same letters are significantly different by ANOVA, Tukey HSD.

The SRE but not STAT site is sufficient for senktide-mediated c-Fos induction

Multimers containing either four copies of the STAT or SRF binding site upstream from a thymidine kinase (TK) promoter were examined by luciferase assay to test the sufficiency of these sites for senktide-mediated induction. The multimer of the STAT sites was not induced by senktide treatment (Fig. 4-6). Thus, although required for induction of c-Fos by senktide, the STAT binding site is not sufficient for NK3R-mediated induction. This may suggest cooperation between the STAT-binding element and another site is necessary for the senktide-mediated induction of c-Fos promoter activity. In contrast, the activity of the luciferase reporter containing four copies of the serum response element (SRE) upstream from the TK promoter was significantly induced, by 1.69 fold, in response to 30 nM senktide (Fig. 4-6). Furthermore, ANOVA analysis shows that there is no significant difference between the level of senktide-mediated induction of the SRE multimer and the full-length -1 Kb c-Fos reporter. Thus, the SRF site is both necessary and sufficient for the induction by NK3R activation.

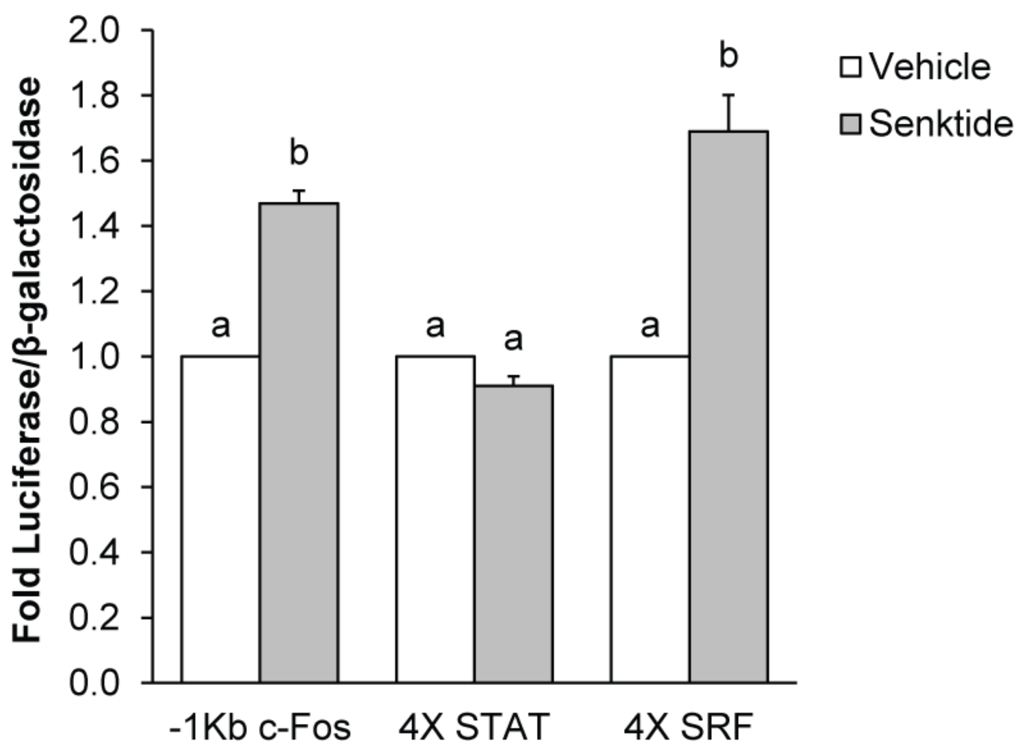

Figure 4-6: The SRF but not the STAT site is sufficient for senktide-mediated induction.

GT1-7 cells were transiently transfected with NK3R and luciferase reporters containing either -1 Kb c-Fos or 4 x multimers of STAT or SRF binding sites cloned upstream from a thymidine kinase promoter. Luciferase data are normalized to β-galactosidase and represented as fold versus vehicle-treated controls ± SEM. *, $P > 0.05\%$, senktide vs. vehicle control by Student's T-test.

Induction of SRF multimer activity by NK3R is independent of β-Arrestin

NK3R is a 7-transmembrane G-protein-coupled receptor (7-TM-GPCR). Upon ligand-stimulation, many GPCRs associate with β-arrestins to undergo internalization and desensitization. SRF activation is known to involve Elk phosphorylation and activation of ERK1/2. In addition to its role in receptor desensitization, β-arrestin has been shown to act as a scaffolding protein that facilitates ERK phosphorylation by Raf and MEKK (106). Previously, GT1-7 cells were shown to express β-Arrestin-1 and β-Arrestin-2. Also, in enteric neurons, NK3R was shown to associate primarily with β-arrestins 2 by a specificity that is determined by its cytoplasmic domain (53, 107). Therefore, we hypothesized that the senktide-mediated activation of the SRF activity may require ERK activation by a β-Arrestin-dependent pathway.

Previously, our collaborators created GT1-7 cell lines stably express small hair-pin RNAs directed against either β-Arrestin-1 or β-Arrestin-2. These cells have very low levels of β-arrestin protein when compared to GT1-7 cells with stable expression of a scrambled shRNA control. These cells were previously used to characterize the role of β-arrestins 1 and 2 in signaling by GPR54 in GT1-7 cells (52). Here, we use these cell lines to determine whether the induction SRF multimer activity by NK3R activation requires ERK activation by signaling via β-arrestins. Knock-down of either β-Arrestin-1 or β-Arrestin-2 failed to block the induction of luciferase activity from -1Kb or -400 bp c-Fos promoter or the SRF multimer by 30 nM senktide (Fig. 4-7). Furthermore, no significant difference in the induction level was observed between cells with β-Arrestin-1 or 2 knock-down and the scrambled control. These data suggest that neither β-Arrestin-1 nor β-Arrestin-2 is required for c-Fos induction by senktide.

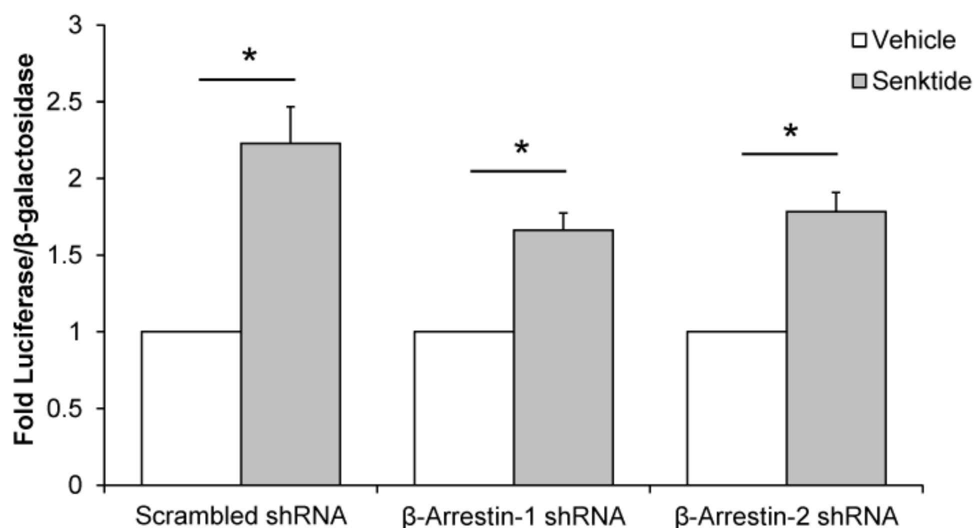

Figure 4-7: Knock-down of β-Arrestins does not block senktide-mediated induction of the SRF multimer reporter.

Stable shRNA knock-down β-Arrestin-1 and 2 GT1-7 cell lines were transiently transfected with NK3R and a SRF multimer luciferase reporter, and treated with 0.4 nM senktide. Luciferase data are normalized to β-galactosidase and represented as fold versus vehicle-treated controls ± SEM. *, $P < 0.05\%$, senktide vs. vehicle controls by Student's T-test.

ERK1/2 protein phosphorylation

To examine whether NK3R-mediated c-Fos induction involves ERK1/2 activation, whole cell lysates of GT1-7 cells transiently transfected with NK3R and treated with senktide at various time points were collected and assayed for phosphorylated ERK1/2 by Western blotting. Since TPA is known to increase ERK1/2 phosphorylation (108, 109) and induce c-Fos (110), TPA-treated GT1-7 cells were used as a positive control. TPA treatment significantly induced ERK1/2 phosphorylation by 5.5 fold versus vehicle control (Fig. 4-8). However, no significant change ERK1/2 phosphorylation was observed in response to senktide treatment regardless the length of the treatment time was used, either 5, 15, 30, 60, or 180 minutes (Fig. 4-8). These data may indicate that NK3R-mediated induction of the c-Fos promoter is independent of ERK1/2 protein phosphorylation. Alternatively, since the response to senktide depends upon the transfection of NK3R, the inability to detect changes in ERK1/2 phosphorylation may be due to insufficient transfection efficiency in GT1-7 cells. Transfect efficiency in GT1-7 cells is sufficient for luciferase assays since the luciferase reporter is co-transfected into the same subset of cells as the receptor expression vector. However, in the case of Western Blot, whole cell lysate is collected from all cells and the effect of senktide on ERK phosphorylation in cells successfully transfected with NK3R, only around 10% of the population, is diluted by protein derived from the majority of the cells that do not express NK3R and so do not respond to senktide treatment. Unfortunately, the GT1-7 cell isolates with endogenous NK3R expression used in previous studies were frozen down and, upon thawing, no longer show receptor expression. Efforts are currently underway to generate GT1-7 cells with stable NK3R expression or identify alternative

cell isolates with endogenous NK3R expression to further examine the phosphorylation of ERK1/2 in response to senktide treatment.

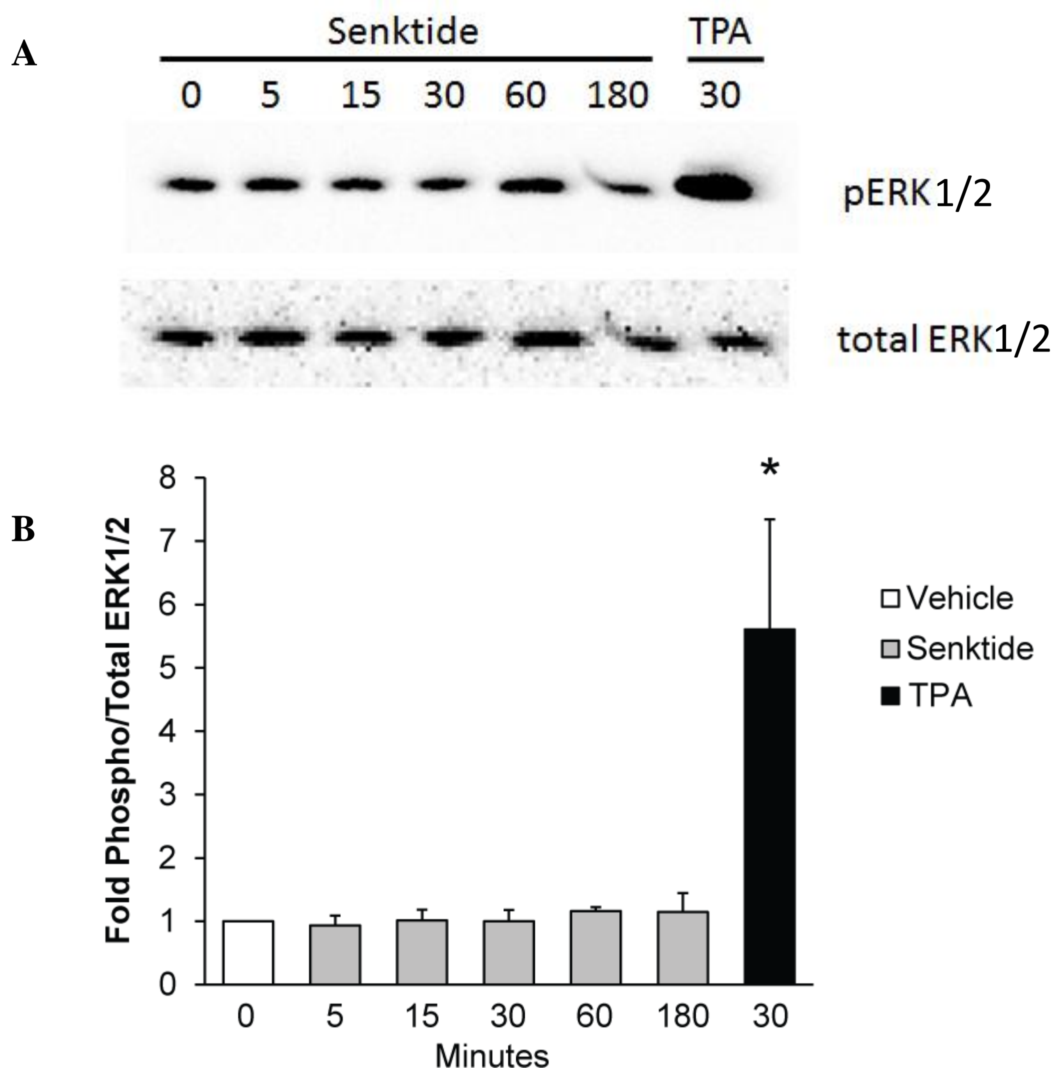

Figure 4-8: Senktide treatment does not induce ERK1/2 phosphorylation.

GT1-7 cells were transiently transected with NK3R and treated for indicated times (in minutes) with 30 nM senktide. Whole cell lysates were collected and assayed by Western blotting with antibodies against phosphorylated-ERK1/2 and total ERK. TPA is a positive control for ERK phosphorylation. Protein levels were quantified by chemiluminescence. In the graph, the levels of phosphorylated ERK1/2 have been normalized to total ERK. Data are represented as fold vs. 0 nM senktide-treated controls ± SEM (n=3). *, P < 0.05%, senktide or TPA vs. 0 nM senktide-treated controls by Student's T test.

PKC but not MAPK, p38 or JNK pathways may be activated downstream from NK3R in GT1-7 cells

The SRF site was determined to be sufficient for c-Fos induction by senktide. Therefore, we employed transient transfections with the SRF multimer treated with the ERK1/2 inhibitor, U0126, to test whether ERK1/2 activation is involved c-Fos induction by senktide. GT1-7 cells were pre-treated for 30 minutes with inhibitors at dosages ranging from 0.1 µM to 10 µM prior to treatment with 0.4 nM senktide treatment, a dose previously determined to be sufficient for a sub-maximal induction of c-Fos. Pretreatment with U0126 did not block induction of the -1 Kb c-Fos promoter or the SRF multimer in response to senktide (Fig. 4-9), indicating that ERK1/2 activation is likely not involved. These data confirm our previous results obtained by Western blotting. Therefore, NK3R activation appears to activate SRF by a mechanism other than MAPK activation of ERK1/2.

Other pathways that are known to activate SRF include the activation of p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal kinase (JNK). Using a similar approach, we employed inhibitors to examine these pathways. Pretreatment with SB202190, failed to block induction of the SRF multimer by senktide (Fig. 4-10), suggesting that p38 MAPK is not involved in the mechanism of Fos induction by NK3R. Next, a JNK-1 and JNK-2 inhibitor, SP600125 was employed. However, SP600125 also failed to block senktide-mediated SRF multimer induction (Fig. 4-11A). Similarly, a JNK-3 specific inhibitor, SR-3576 failed to block induction (Fig. 4-11B). These data suggest that p38 MAPK and JNK1/2/3 activation are not required for induction of SRF by NK3R activation in GnRH neurons.

c-Fos is known to be induced by TPA, by activation of PKC signaling. Moreover, neurokinin receptors have been described to activate PKC signaling in response to stimulation with ligand. Therefore, we hypothesized that NK3R might induce c-Fos gene transcription by activating PKC. Pretreatment with 10 μM PKC inhibitor, Go 6983, significantly blocked induction of the SRF multimer by 0.4 nM senktide treatment (Fig. 4-12). Therefore, PKC signaling may be activated downstream from NK3R in GnRH neurons and be involved in the induction of c-Fos.

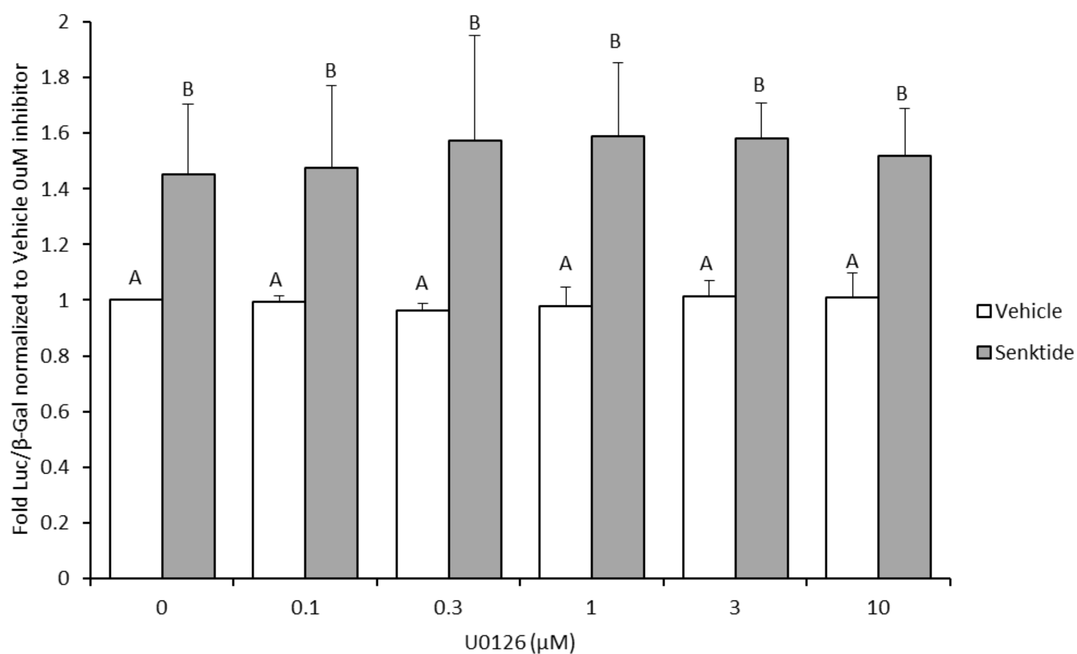

Figure 4-9: ERK1/2 inhibitor U0126 failed to block SRF induction

GT1-7 cells were transiently transfected with NK3R and SRF reporter and pre-treated with U0126 of doses indicated for 30 minutes followed by co-treatment with 0.4 nM senktide for 3 hours. Luciferase data are normalized to β-galactosidase and represented as fold versus vehicle -treated controls with 0 μM inhibitor ± SEM. Bars not connected by same letters are significantly different by ANOVA, Tukey HSD.

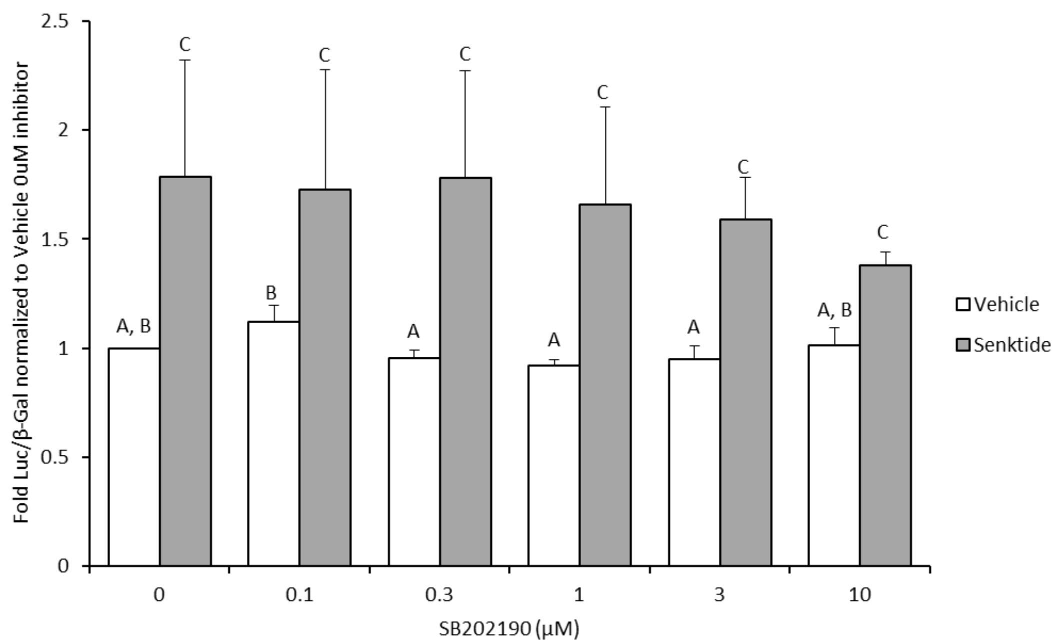

Figure 4-10: p38 MAPK inhibitor SB202190 failed to block SRF induction

GT1-7 cells were transiently transfected with NK3R and SRF reporter and pre-treated with SB202190 of doses indicated for 30 minutes followed by co-treatment with 0.4 nM senktide for 3 hours. Luciferase data are normalized to β-galactosidase and represented as fold versus vehicle -treated controls with 0 µM inhibitor ± SEM. Bars not connected by same letters are significantly different by ANOVA, Tukey HSD.

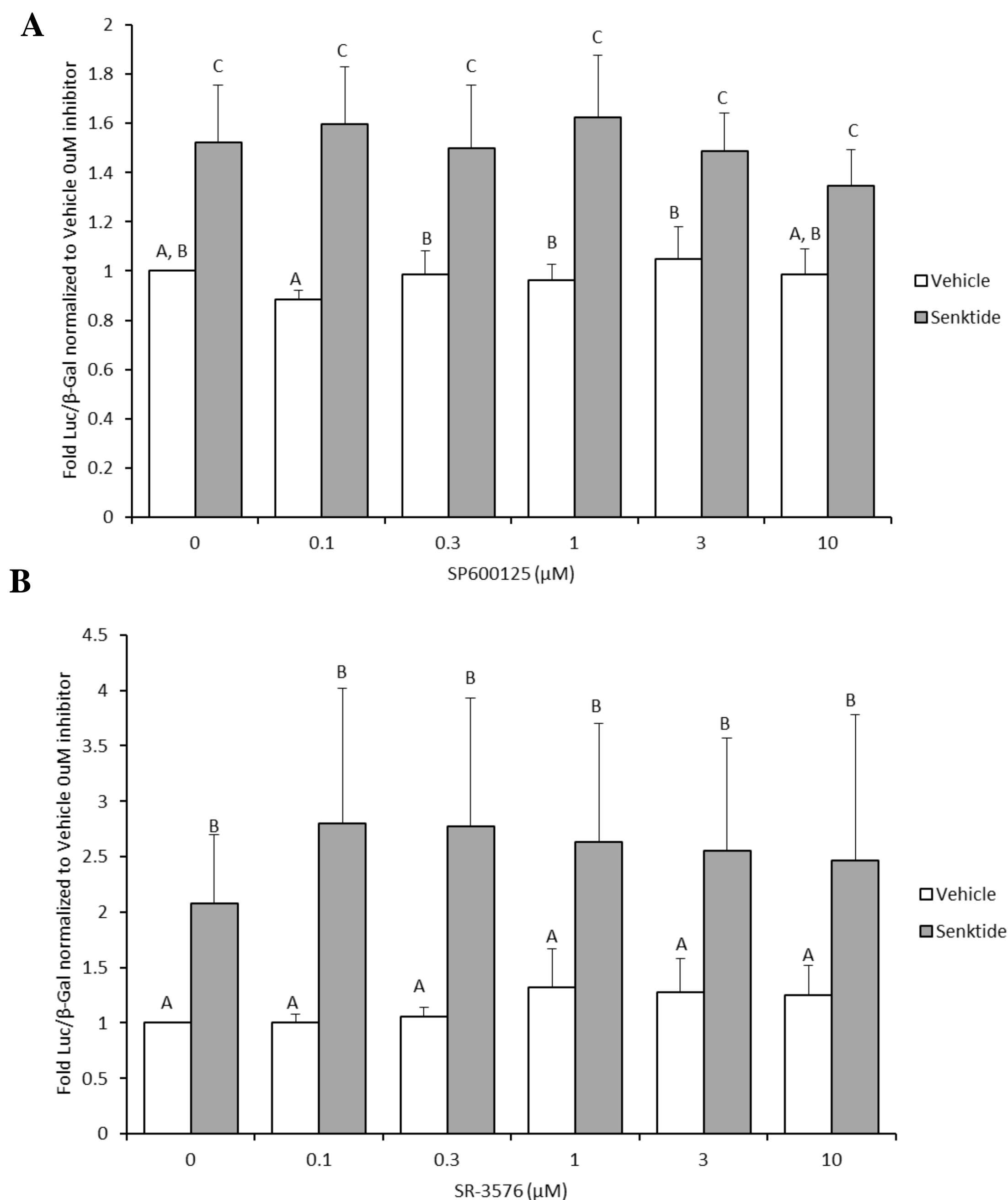

Figure 4-11: JNK1/2/3 inhibitors failed to block SRF induction by senktide

GT1-7 cells were transiently transfected with NK3R and SRF reporter and pre-treated with A) JNK1/2 inhibitor, SP600125, or B) JNK3 inhibitor, SR-3576 of doses indicated for 30 minutes followed by co-treatment with 0.4 nM senktide for 3 hours. Luciferase data are normalized to β-galactosidase and represented as fold versus vehicle -treated controls with 0 μM inhibitor ± SEM. Bars not connected by same letters are significantly different by ANOVA, Tukey HSD.

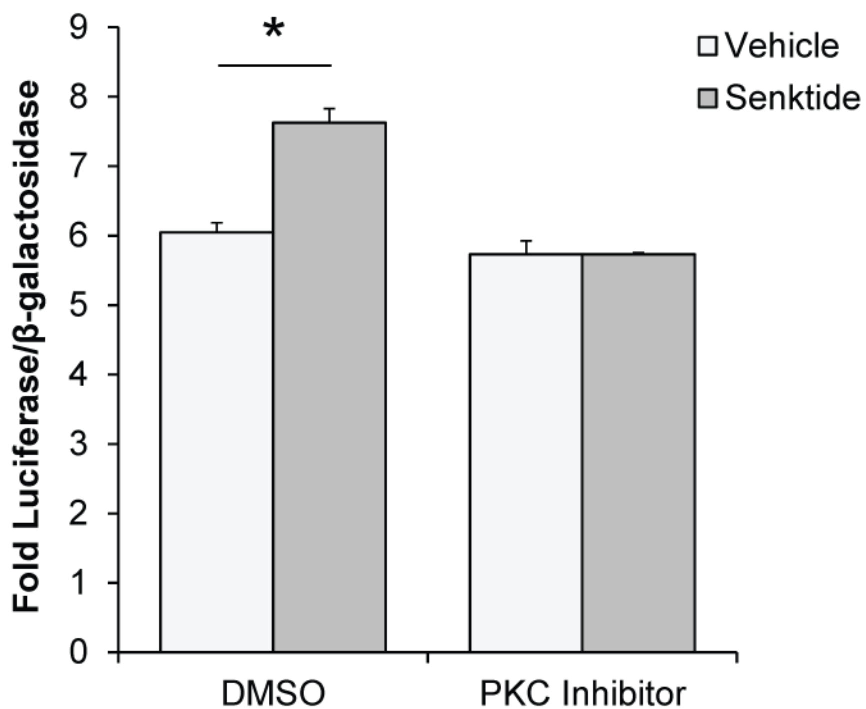

Figure 4-12: NK3R signals through activation of PKC to induce SRF in GnRH neurons

GT1-7 cells were transiently transfected with NK3R and pre-treated 30 minutes with the 10 μM PKC inhibitor, Go 6983, followed by co-treatment for 3 hours with 0.4 nM senktide. Luciferase data are normalized to β-galactosidase and represented as fold versus vehicle-treated DMSO controls ± SEM. *, $P < 0.05$ %, senktide vs. vehicle by Student's T-test.

DISCUSSION

In vivo studies have shown NK3R signaling to be essential for reproduction and the regulation of gonadotropin hormones and strongly suggest a mechanism that includes the regulation of GnRH neuron activity. NK3R has been shown to be expressed in neurons within the GnRH neuron network, including kisspeptin neurons and GnRH neurons themselves (38, 44, 45). The central administration of senktide was shown to induce c-Fos, a marker of neuronal activation, in NK3R-expressing kisspeptin neurons (37). As a result, NKB has been suggested to regulate GnRH neuron secretion indirectly through regulation of kisspeptin. However, it has not yet been determined whether NKB can directly regulate the activity of NK3R-expressing GnRH neurons. Two *in vivo* studies tried to examine whether NKB could directly activate GnRH neurons. One looked at whether c-Fos was induced in GnRH neurons in response to the central administration of senktide (44) and the other examined whether there was an increased firing response of GnRH neurons in response to senktide by electrophysiology (80). However due to technical issues, these studies did not confirm NK3R expression and, since only a subset of GnRH neurons express NK3R [44, 45], they likely measured response in GnRH neurons without NK3R. Thus, *in vivo* studies have not been successful in determining whether NKB can directly regulate the activity of NK3R-expressing GnRH neurons.

In chapter 3, we showed that NK3R-expressing GT1-7 cells respond to acute senktide treatment with increased GnRH secretion and an induction of c-Fos mRNA. These data indicate that NKB would directly activate NK3R-expressing GnRH neurons.

Moreover, we show that activation of NK3R also causes a Fos-dependent repression of GnRH transcription in GT1-7 cells. Also, senktide treatment induces c-Fos mRNA by quantitative RT-PCR. In chapter 4, we examine the role of NK3R in regulating transcription of c-Fos. In addition to transcriptional regulation, c-Fos RNA is known to be regulated at the level of RNA stability. Using transient transfection, we show that senktide treatment induces the transcriptional activity of a -1 Kb mouse c-Fos luciferase reporter. Thus, while these data do not rule out the potential for additional post-transcriptional effects, it indicates that NK3R activation in GnRH neurons induces c-Fos gene transcription.

Next, we employed truncations and *cis*-mutation of the -1Kb mouse c-Fos promoter luciferase reporter to map the region and binding sites necessary for induction by NK3R activation. Truncations identified the region between -400 and -200 bp upstream from the transcriptional start site as necessary for induction. This region is known to contain several binding sites, including STAT, AP-1, Ets, and SRF. Using *cis*-mutations, we find that both the STAT and SRF sites in the c-Fos promoter are required for activation of c-Fos.

The STAT binding site (TTCCCGTCAA) at -345 bp has been reported to contribute to c-Fos induction in response to TPA, EGF and serum (105). These stimuli induce signaling by the JAK-STAT pathway. The Tyr at the C-terminal of STAT is first phosphorylated and activated by JAK, which allows dimerization of STAT1 with STAT3. The STAT heterodimer complex then binds to the STAT site to induce c-Fos gene expression (54, 111). Therefore, we hypothesized that the induction of c-Fos by senktide may involve the activation of a JAK-STAT pathway downstream of NK3R. However,

senktide failed to induce activity of a STAT multimer, a luciferase reporter containing four copies of the STAT binding site. Therefore, although necessary, the STAT site alone is not sufficient for the senktide-mediated induction. It is possible that the STAT site acts in cooperation with another site between -400 and -200 bp of the c-Fos promoter. Both STAT and SRF/TCF have been shown to contribute to c-Fos promoter activation by granulocyte-macrophage colony stimulating factor (GMCF) in murine fibroblasts (112). Thus, STAT may contribute to c-Fos induction in response to NK3R activation in GnRH neurons.

The SRF site located at -310 bp in the c-Fos promoter contains a core sequence known as the CArG box (CCATATTAGG) (54, 102, 113). We find that senktide treatment induced the activity of a luciferase reporter containing four copies of the SRF binding site. These data indicate that the SRF site is sufficient for senktide-mediated induction. In this chapter, we employ what is known about the pathways that activate SRF to induce c-Fos promoter activity to examine pathways acting downstream from NK3R in GnRH neurons.

ERK activation can increase binding to SRF. β-arrestin, a protein originally shown to be involved in ligand-activated receptor desensitization, has been shown to also be involved in the activation of ERK. Upon ligand activation, 7-TM-GPCRs such as β2 adrenergic, serotonin 5-HT1_A, and muscarinic acetylcholine receptors have been shown to activate ERK1/2 via a β-arrestin-dependent pathway. Acting as a scaffold, β-arrestin brings Raf1 and ERK1/2 together to facilitate the activation of MEK1 and ERK2 (114). Also, β-arrestin in clathrin coated pits can enhance Ras-dependent phosphorylation of ERK1/2 (115). Because ligand-activated NK3R was shown to associate with β-arrestin

in enteric neurons (53) and GT1-7 cells express β-arrestin-1 and 2, we hypothesized that NK3R might induce activation of ERK1/2 by a β-arrestin-dependent pathway. However, knock-down of either β-arrestin-1 or β-arrestin-2 protein expression in GT1-7 cells did not block senktide-mediated induction of activity of the SRF multimer reporter. Therefore, neither β-arrestin-1 nor β-arrestin-2 is required for SRF induction.

c-Fos transcription can also be induced by activation of SRF binding in response to a receptor tyrosine kinase-RAS-Raf-MAPK signal pathway that leads to ERK phosphorylation and activation of ELK-1 binding to SRF sites (102). Alternatively, the activation of SRF binding can be induced by a Rho GTPase (Cdc42, Rac1, or RhoA) signaling pathway, which ultimately converges on ERK1/2 and p38 MAPK (102). Alternatively, activated Rac1 can activate c-Jun N-terminal kinase (JNK) to upregulate c-Fos transcription activity (116). Thus, ERK1/2, p38 MAPK and JNK appeared to be ideal candidates for pathways acting downstream from NK3R to regulated c-Fos gene transcription by SRF.

We first examined the involvement of ERK1/2 activation downstream of NK3R activation in GT1-7 cells using a time course with 30 nM senktide by Western blotting. Using a time course including 5, 15, 30, 60, and 90 minutes, senktide failed to induce phosphorylation of ERK1/2. These data suggest that NK3R does not activate ERK1/2. However, it is possible that the effect of senktide on ERK phosphorylation is not detectable by Western blot due to low transfection efficiency. Since only 10% of the cells were successfully transfected with NK3R, the effect of senktide on ERK phosphorylation in these transfected cells may have been diluted by protein from the

majority of the cells which do not express NK3R or respond to senktide and may have masked changes in ERK phosphorylation.

Next, as an alternate approach, the pathway inhibitor U0126 was used with transient transfection assay to test the role of MAPK pathway leading to ERK1/2 phosphorylation downstream from NK3R in GT1-7 cells. Using doses of up to 10 μM U0126 did not block senktide-mediated induction of the SRF multimer luciferase reporter. These data confirm our results obtained by Western blotting and further suggest that ERK1/2 activation is not involved in mediating c-Fos induction by NK3R activation. SRF can also be activated by the p38 MAPK and JNK1/2/3 pathways. However, the use of inhibitors to block either the p38 MAPK or JNK1/2/3 pathways also failed to block the induction of SRF multimer reporter activity by senktide. Thus, the activation of p38 MAPK and JNK1/2/3 pathways also do not appear to be required for induction of SRF by NK3R activation.

NK3R has been shown to be coupled to G_q/G_{11} (117, 118) which, when activated, hydrolyzes phosphoinositide (PIP_2) into inositol triphosphate (IP3) and diacylglycerol (DAG) and results in PKC activation. In dorsal root ganglia neurons, activation of NK3R was reported to stimulate PKC activity and inhibit N-type Ca^{2+} channels (119). PKD is a common downstream target of PKC and can also be activated by DAG (120, 121). In enteric neurons, senktide was shown to evoke an immediate and rapid translocation of PKC and PKD from the cytosol to the plasma membrane, and increase PKD phosphorylation. Interestingly, TPA, a PKC activator, has been shown to induce c-Fos transcription by activation of SRF (122). Also, like senktide, TPA was shown to cause repression of GnRH gene transcription in GT1-7 cells and acutely induce GnRH secretion

in GT1-7 cells (110, 123). Thus, we hypothesized that NK3R could signal through activation of PKC in GnRH neurons. The PKC pathway inhibitor Go6983 blocked the induction of SRF multimer transcription activity by senktide in transient transfection assay. Moreover, Go6983 also reduced SRF multimer activity in the vehicle treated cells, suggesting that PKC is involved in basal transcription of SRF multimer. Thus, we find that PKC activation is required for senktide-mediated induction of transcription at the SRF site. Importantly, this is the very first information available as to the cellular pathway acting downstream of NK3R in GnRH neurons.

To understand more about pathways downstream of NK3R in GnRH neurons, we sought to draw from what was already known about PKC and c-Fos promoter regulation. Previous studies of the c-Fos promoter have shown that PKC and RAS cooperate to induce transcription through the SRF site by a PKC-MAPK-TCF pathway (122). TCF can form a ternary complex with SRF to activate transcription at SRF site (124). Alternatively, PKC can also activate Raf, an effector protein of RAS, to enhance the activity of RAS-Raf-MAPK signaling pathway and activate transcription of the c-Fos promoter via the SRF site (102). In addition, some specific isoforms of PKC, such as PKCα and PKCε, have been shown to enhance the activity of a RhoA-SRF pathway and induce transcription independently from MAPK (116, 122). Since our data indicate that MAPK is not required for senktide induction of SRF, perhaps NK3R signals through PKCα, PKCε or RhoA to induce c-Fos transcription in GnRH neurons.

In summary, in this chapter, we show that NK3R induces cFos mRNA by increasing transcription. We map this activation to between -400 and -200 bp in the cFos promoter and show that a STAT site and a SRF site are required for induction. Using

multimers of these sites, we show that the SRF site is also sufficient for activation, while the STAT site is not. Finally, we identify the PKC pathway acting downstream from NK3R as responsible for activation of the SRF multimer. Three main questions remained to be answered to more fully elucidate the mechanism whereby NK3R activates cFos gene expression. First, we will determine if PKC is the main signaling pathway involved in NK3R-mediated induction of cFos by examining whether the blockade of PKC signaling with Go6983 is also sufficient to block the senktide-mediated increase in -1Kb cFos reporter activity by luciferase assay and induction of cFos mRNA by RT-PCR. Second, we would like to determine which isoform of PKC is activated by NK3R and is responsible for c-Fos induction. There are several isoforms of PKC, including PKCα, PKC_{β}, PKCε, PKCζ, and PKCδ, etc. Different PKC isoforms have different cell-specific functions and are involved in receptor desensitization, modulation of membrane structure, transcription regulation, cell growth and mediating immune response. Using dominant-negative constructs available from the Kazi laboratory, we will determine which isoform is required for senktide-mediated induction of the SRF multimer and -1 Kb cFos luciferase reporter activity (122). Third, we will identify which transcription factors bind to the SRF and STAT sites in the cFos promoter in response to senktide treatment using EMSA and supershift with specific antibodies. Using what is known about the regulation of cFos through these sites, we have chosen to perform supershift with antibodies against STAT1 and STAT3 with an oligonucleotide probe that includes the STAT site (125). For the oligo probe containing the SRF site, we will use antibodies to test the binding of SRF and ternary complex factors (TCF), including Elk-1, SAP-1, SAP-2/ERP/Net (113).

CONCLUSION

In conclusion, we propose a new model wherein NKB directly signals to NK3R-expressing GnRH neurons to modulate GnRH secretion and GnRH gene transcription (Fig. 4-13). Briefly, in response to NKB, NK3R activates a PKC signaling pathway to stimulate acute GnRH secretion and rapid induction of cFos gene transcription by effects mediated through SRF (-310) and STAT (-345) binding sites. c-Fos protein then represses GnRH gene transcription through formation of an AP-1 complex and binding to a novel AP-1 half-site within in enhancer 1. This model is in accord with previous studies that have shown that activation of PKC signaling by TPA treatment induces GnRH secretion and represses GnRH transcription in GnRH neurons by effects at enhancer 1 (58, 76, 77, 110, 123, 126). Therefore, compounds that can modulate PKC signaling in GnRH neurons may be useful therapies for the treatment of pubertal disorders, IHH and infertility.

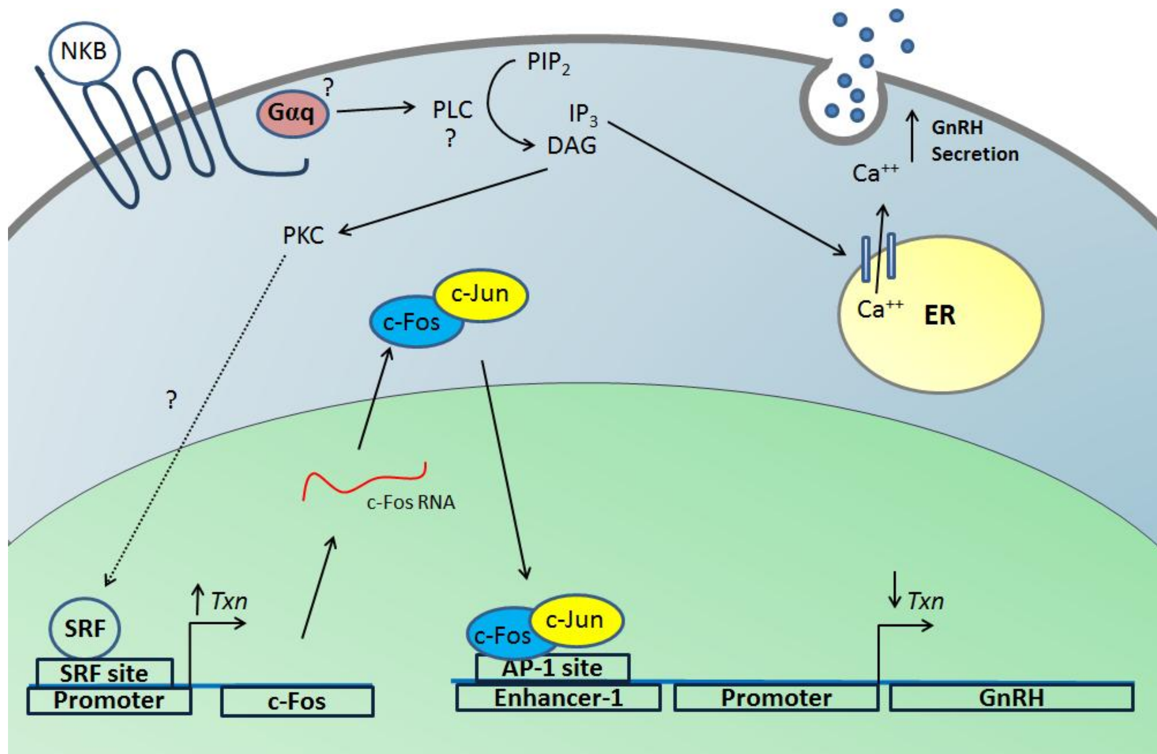

Figure 4-13: NK3R Activation Model in GnRH Neurons.

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