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60 Hz magnetic field exposure inhibits protein Kinase C dependent induction of Neuropeptide Y mRNA in PC-12 cells

William James Thomas

60 Hz MAGNETIC FIELD EXPOSURE INHIBITS PROTEIN KINASE C DEPENDENT INDUCTION OF NEUROPEPTIDE Y mRNA IN PC-12 CELLS

A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Biology

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by

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June 1994

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Approved by:

[redacted] 5/18/94

Dr. Jeffrey Thompson, Chair, Biology Date

[redacted]

Dr. Robert Stagg

[redacted]

Dr. Nicole Bournias-Vardiabasis

Abstract

Neuropeptide Y (NPY), discovered in 1982, is one of the most abundant neuropeptides found in the mammalian brain. It is also stored and secreted as a hormone from chromaffin cells in the body. It has been shown to regulate cardiac blood pressure, intracellular calcium concentrations in tissue culture, and pineal gland production and secretion of the hormone melatonin, all which have been shown to be affected by exposure to magnetic fields (MF). In this thesis, I examined the effects of MF exposure on the chemically induced production of NPY mRNA in the rat pheochromocytoma, PC-12 cell line. NPY mRNA production is regulated in these cells by at least three different signal transduction mechanisms, the phosphoinositide/protein kinase C (PKC) dependent, the cAMP dependent, and a tyrosine kinase dependent mechanism with a synergism occurring upon simultaneous stimulation of both the phosphoinositide/PKC and the cAMP dependent pathways.

There is a significant decline in the synergistic production of NPY mRNA in PC-12 cells stimulated with forskolin (20uM) and TPA (50nM) upon magnetic field exposure. This decline was seen at 8 and 24 hours of chemical plus MF exposure. To determine whether or not a specific signal transduction pathway was affected by MF exposure, PC-12 cells were exposed to TPA (12-O-tetradecanoylphorbol-13-acetate) (50nM), or forskolin (20uM) for 8 hours with and without MF exposure. No changes in NPY mRNA were seen between magnetic field and sham exposed unstimulated cells, or in those cells exposed to forskolin (20uM). In three of four experiments, those cells exposed to TPA (50nM) and magnetic field showed an average 27%, 36%,

and 24% ($p < 0.05$), decline in NPY mRNA. It has been shown that transcript stability of the GAP-43 gene in PC-12 cells can be affected by activation of PKC. To determine if the MF induced decline in NPY mRNA was at the posttranscriptional level, cells were incubated with TPA (50nM) for 8 hours at which time, DRB (5,6-Dichloro-1-B-D-Ribo-Furanosyl-Benzimidazole) (65uM), an inhibitor of transcription was added. RNA was extracted at various time points thereafter. The TPA-induced half-life was approximately 12 hours in both magnetic field and sham exposed cultures. These results indicate exposure to a 1 gauss, 60 Hz magnetic field affects a mechanism involving PKC that leads to an inhibition of the production of NPY mRNA in PC-12 cells and this effect does not appear to be at the posttranscriptional level.

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Acknowledgements:

I would like to thank my wife Debbie and my children, Bobby and Christopher who have endured my long weekend hours at the lab, for their never-ending support, and thanks to the understanding faculty at Cal State San Bernardino: Dr. Nicole Bournias-Vardiabasis, Dr. Jeffrey M. Thompson, and Dr. Richard Fehn. I would also like to thank the members of the Neurobiology department at the V.A. Hospital in Loma Linda, Ca.: Drs. Cain, Stagg, Haggren, Phillips, Bawin, and Walleczeck; Bob and Tami Jones for their excellent technical and EMF exposure advice. Special thanks goes to Dr. Steve Sabol at NIH for supplying the pBLNPY1 plasmid and PC-12 cells. Without those, this study could not have been completed. Many thanks go to Dr. W. Ross Adey for allowing me to continue this project in his laboratories, thanks.

This work was supported by a CSUSB ASI graduate research award and by DOE Department of Energy Contract No. AI 01-90CE35035.

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INTRODUCTION

Low-frequency electromagnetic field (EMF) interactions with biological systems have been recorded for the last twenty years. Only in the last decade or so, has exposure to these fields been implicated with higher incidences of cancer. Today, there is an overwhelming demand to clarify the issue of electromagnetic fields possibly interacting with biological systems to promote cancer.

Epidemiological studies have shown that a correlation exists between exposure to EMF's and increased incidences of cancer, e.g., childhood leukemia, brain, and breast cancers, and an increased incidence of miscarriage (Feychting M. *et. al.*, 1992; Savitz D. *et. al.*, 1990; Myers A. *et. al.*, 1990; Goldhabler M. *et. al.*, 1988; Wertheimer N. *et. al.*, 1979, 1989; Delgado J. *et. al.*, 1982). A current theory is that these EMF's are not promoting cancer by themselves, but are possibly enhancing the effects of exposure to low doses of known carcinogens, i. e. co-promotion (Adey, W., 1990). Recently, a cellular model of focus formation has been used to demonstrate an increase in focus formation of cells grown in the presence of a known tumor promotor, TPA (phorbol 12-myristate 13-acetate), and a 60 Hz, 1 gauss magnetic field (Cain C. *et. al.*, 1993). The mechanism responsible for this effect is not yet understood, although it has also been shown that expression of early activation genes can be affected in a specific manner by EMF exposure (Goodman R. *et. al.*, 1983, 1986, 1989; Phillips J. *et. al.*, 1992). It is possible that there exists similar mechanism(s) susceptible to magnetic field exposure within both of those findings. For instance, TPA has been shown to directly bind and activate protein kinase C which will in turn

phosphorylate transcription factors changing the rate of transcription of certain genes.

A molecular mechanism by which EMF's can interact with biological systems needs to be identified if this research is to have any real credibility. There is good evidence that the mechanism is associated with the cell membrane, perhaps at the level of signal transduction (Adey W.R., 1986, 1988). Evidence exists implying second messengers such as cAMP, and Ca2+ as the site of EMF-cellular interaction (Bawin S. and Adey W., 1976; Cain C. *et. al.*, 1987; Walleczek J. *et. al.*, 1990; Liburdy R. *et. al.*, 1993). The question of whether the alteration of this molecular mechanism leads to plausible susceptibility to various forms of cancer needs to be addressed. The focus formation model reported by Cain *et. al.*, 1993, answers the question of whether mechanism alterations caused by EMF exposure can lead to enhanced focus formation, while not yet identifying a mechanism.

Another possible answer to the question of whether exposure to EMF's changes the susceptibility to cancer is demonstrated by the finding of decreased pineal production of the hormone melatonin in animals exposed to EMF's (Reiter R. *et. al.*, 1992; Wilson B. *et. al.* 1981, 1986, 1990; Welker H. *et. al.*, 1983). There is some evidence that melatonin could be involved in the body's natural defense against certain cancers. Blood levels of circulating melatonin decline in patients with various forms of cancer. This observed decline in melatonin was attributed specifically to a decrease of synthesis and release from the brain (Bartsch C. *et. al.*, 1985,1989). As of yet, there is no identified molecular mechanism being affected by EMF exposure that would lead to declined production of melatonin. Natural regulation of melatonin

secretion is due partly to pineal gland adrenergic receptor stimulation by norepinephrine, which is released during dark periods of the circadian cycle (Moore R. *et. al.*, 1968).

Another known regulator of pineal production and secretion of melatonin is the neuropeptide, Neuropeptide Y (NPY) (Olcese J. *et. al.*, 1991; Vacas M. *et. al.*, 1987; Reuss S. *et. al.*, 1989). Neuropeptide Y (NPY) is a 36-amino acid peptide originally isolated from porcine brain in 1982 (Tatemoto K. *et. al.*,1982). It is among the most abundant neuropeptides found in the brain (de Quidt M. *et. al.*, 1986), and appears to be an important regulator in the mammalian nervous system. There is evidence this neuropeptide plays a significant role in the regulation of cardiac artery blood pressure (Lundberg J. *et. al.*, 1982), hormonal release from the brain (Kerkerian L. *et. al.*, 1985), and feeding behavior (Stanley B. and Leibowitz S., 1985; Stanley B. *et. al.*, 1993; Stanley B. and Thomas W., 1993). It is found co-localized with other catecholamines and appears to be involved in regulating their secretion (Guy J. and Pelletier G., 1988). NPY is also stored and secreted as a hormone from adrenal chromaffin cells (Allen J. *et. al.*, 1983), and is widely distributed throughout the body.

The gene expression of NPY is an ideal system to use for the study of biological effects of stimuli on cell membrane signal transduction because NPY gene expression appears to be dependent on at least three signal transduction mechanisms: the phosphoinositide/protein kinase C, the cAMP, and a tyrosine kinase dependent mechanism (Higuchi H. *et. al.*, 1988; Sabol S. *et. al.*, 1990). This regulation of NPY gene expression has been demonstrated in fetal aggregating brain cells (Magni P. *et.*

al., 1992) as well as in the rat PC-12 pheochromocytoma cell line (Higuchi H. *et. al.*, 1988; Sabol S. *et. al.*, 1990). A novel aspect of NPY gene regulation in PC-12 cells is that activation of both cAMP and protein kinase C dependent signal transduction mechanisms concurrently leads to a synergism in the production of NPY mRNA where the levels obtained are far greater than if the mechanisms were additive (Higuchi H. *et. al.*, 1988; Sabol S. *et. al.*, 1990). The mechanism(s) for this phenomena are unknown although similar effects have been seen with other genes in other cell lines (Comb M. *et. al.*, 1986; Anderson B. *et. al.*, 1988).

I chose to examine whether exposure to EMF's can alter the chemically induced production of NPY in the rat pheochromocytoma cell line PC-12 using NPY prepro mRNA as a marker. This cell line has been shown to be responsive to EMF exposure. The PC-12 cell line has been extensively used to study the process of neuronal differentiation. Upon exposure to Nerve Growth Factor, these cells begin to form neurite processes. This neurite forming process appears to be altered in the presence of a magnetic field (Blackman C. *et. al.*, 1993a, 1993b). Also, these cells are known to produce and secrete catecholamines. The secretion of the catecholeamines has also been shown to be affected upon exposure to EMF's (Dixey R. and Rein G., 1982).

In this study, PC-12 cells were exposed to a 60 Hz, 1 gauss sinusoidal magnetic field with the measurement of NPY mRNA expression used as an endpoint. The hypothesis was that exposure to the magnetic field would alter one or more of the before mentioned signal transduction pathways leading to an alteration in the production of NPY mRNA in PC-12 cells. It was demonstrated that exposure to this

magnetic field significantly inhibits the synergistic production of NPY mRNA induced by the combined actions of forskolin and TPA . It was also demonstrated that NPY mRNA is induced upon activation of PKC by TPA application in a time and dose-dependent manner. This PKC dependent induced NPY mRNA is also significantly inhibited by magnetic field exposure.

MATERIALS AND METHODS

Magnetic field (MF) exposure conditions. A cell culture incubator containing a modified Merrit coil consisting of 3 square coils 14" in diameter separated from each other by one-half the diameter with the number of wire turns in each coil in a 2:1:2 configuration (52:26:52) was used for MF exposures. Unless stated otherwise, all magnetic field exposures were 1 $gauss_{rms}$ (100uTesla) sinusoidal at a frequency of 60 Hz.

Materials. A plasmid pBLNPY1 containing the cDNA coding for rat brain prepro NPY was a generous gift from Dr. Steve Sabol (National Institutes of Health, Bethesda, Md.) Forskolin and TPA were from Sigma. Nerve Growth Factor (NGF) was obtained from Upstate Biotechnology Inc. Forskolin and TPA were dissolved in 95% ethanol; NGF was dissolved in water. In each experiment, the final concentration of ethanol in the cell culture dishes was < 0.2%. This alcohol concentration appeared to have no effect on cell morphology. Control dishes were also exposed to equal concentrations of ethanol.

Cell culture. PC-12 cells, obtained from Dr. Gordon Gurhoff through Dr. Steve Sabol (National Institutes of Health, Bethesda Md.) were grown in DME tissue culture medium containing 5% horse serum and 5% fetal calf serum in an incubator with a humidified atmosphere of 5% CO_2/95% air with a background MF of < 0.002 gauss. Cell culture medium was changed every two days. The cells were grown for 1 week

to 50-90% confluency prior to assay.

Preparation of total cellular RNA. Total cellular RNA was extracted with a previously described 4M Guanidinium Isothiocyanate procedure (Chomczynski P and Sacchi N., 1987), precipitated with ethanol and quantified by absorbance at 260nm (40μg RNA/unit A_{260}).

Northern blot analysis. Equal amounts of RNA (25-50μg) were electrophoresed in 1.2% agarose gels containing 3% formaldehyde and transferred to Magna (MSI) nylon membranes. The membranes were dried at 80 degrees and U.V. cross linked. The integrity of the RNA was determined visually by the staining of the membranes with methylene blue with no degradation noted. The membranes were hybridized with the random-prime labeled (2-3 X 10^8 cpm/ug) 511 basepair EcoR1 insert of the pBLNPY1 plasmid (Higuchi H. *et. al.*, 1988), and a linearized plasmid containing glyceraldehyde 3-phosphate dehydrogenase (GPDH) cDNA (for normalization) for 16-36 hours, washed per standard protocols with a final wash of .5 X SSPE, 1% SDS for 20 min. at 42° C. and exposed to x-ray film overnight. The GPDH gene does not seem to be regulated by the compounds used or by MF exposure (Sabol S. *et. al.*, 1990; Liburdy R. *et. al.*, 1993). Films were analyzed using the JAVA (Jandel Sci.) system of scanning densitometry. Results are expressed as Relative NPY mRNA (gray units NPY mRNA/ gray units GPDH mRNA). Statistics were done using a Student's T-test comparing, independently, relative NPY mRNA of sham exposed vs MF exposed.

RESULTS

Stimulation of PC-12 cells with forskolin, TPA, and forskolin + TPA increases levels of NPY mRNA. Previously published data (Higuchi H. *et. al.*, 1988) indicated increases in NPY mRNA in PC-12 cells upon a 24 hour exposure to forskolin (20uM), TPA (100nM), and forskolin (20uM) + TPA (100nM) with the combination of the two chemicals producing a synergistic increase in NPY mRNA levels. To replicate this previous data, cells were exposed to forskolin (20uM), TPA (100nM), and forskolin (20uM) + TPA (100nM) for 24 hours. Total RNA was extracted and analyzed for relative NPY mRNA. Confirming the previously published results, NPY mRNA levels were 6, 4, and 12 times those of controls, respectively after chemical stimulation (Figure 1).

MF exposure inhibits the cAMP/PKC dependent synergistic induction of NPY mRNA in cells exposed for 24 hours with varying TPA concentrations. After replicating previously published data, 2 identical experiments were performed where cells were exposed to forskolin (20uM) + TPA (12, 25, 50, and 100nM) +/- MF for 24 hours. A previous publication (Cain C. *et. al.*, 1987) has indicated that in some cases, magnetic field effects can only be seen upon sub-maximal stimulation of specific signal transduction pathways. Therefore, the stimulation of the protein kinase C signal transduction pathway was varied by varying the concentration of TPA in the presence of maximal forskolin. The maximal concentration of TPA in the presence of forskolin appears to be 50nM for the conditions employed in this laboratory. In experiment 1,

there was a decline in NPY mRNA of those cells exposed to the MF by 51, 40, 50, and 0 % corresponding to 12, 25, 50, and 100nM TPA in the presence of forskolin (20uM) (Figure 2a). In the second experiment, the levels of NPY mRNA declined in cells exposed to MF by 0, 28, 25, and 35% corresponding to 12, 25, 50, and 100nM TPA respectively in the presence of forskolin (20uM) (Figure 2b).

Kinetic study of forskolin plus TPA induced NPY mRNA. To determine when the maximal forskolin (20uM) plus TPA (50nM) synergistic induction of NPY mRNA occurs using the protocols employed by this laboratory, a kinetic study was performed. The synergistic induction of NPY mRNA begins at 2 hours (the earliest time taken) and reaches maximum levels (51-fold) at 8 hours. NPY mRNA level then begins to decline but never reaches basal levels by 24 hours of exposure (Figure 3). This data is somewhat contradictory to previously published data (Higuchi H. *et. al.*, 1988) that shows maximum levels at 24 hours of exposure.

MF exposure inhibits the cAMP/PKC dependent synergistic induction of NPY mRNA in cells exposed for 8 hours with varying TPA concentrations. Because maximum levels of forskolin (20uM) plus TPA (50nM) induced NPY mRNA occurs at 8 hours after chemical stimulation, cells were exposed to forskolin (20uM) plus TPA (12, 25, 50, and 100 nM) plus or minus MF exposure for 8 hours. The NPY mRNA levels in cells exposed to MF were 20, 20, 45, and 20 % less than unexposed cells corresponding to 12, 25, 50, and 100nM TPA + forskolin (20uM) respectively. A second experiment with n=4 showed an average 27% decline in NPY mRNA in cells exposed to forskolin (20uM) + TPA (50nM) + MF. This inhibition was statistically

significant at $p < 0.01$ (Figure 4).

Kinetic studies of forskolin and TPA induced NPY mRNA. In order to determine if the decline in forskolin plus TPA induced NPY mRNA by MF exposure was the result of MF effects on a specific signal transduction pathway, kinetic studies of both forskolin and TPA induced NPY mRNA were performed. Cells were exposed to forskolin (20uM) or TPA (50nM) for 2, 4, 8, 12, and 24 hours. Total RNA was extracted and analyzed for relative NPY mRNA. The forskolin induced NPY mRNA began at 2 hours and maximized at 8 hours (10-fold), staying relatively constant through 24 hours of chemical exposure. The TPA induced NPY mRNA began at 2 hours, maximized at 8 hours (20-fold) and began to decline by 24 hours although not quite reaching basal levels (Figure 5).

TPA induces NPY mRNA in a time and dose dependent manner by activation of PKC in PC-12 cells. Previous reports have shown either no change (Higuchi H. *et. al.*, 1988) or increases (Sabol S. *et. al.*, 1990) in NPY mRNA upon TPA application in PC-12 cells, with the increase only shown at 24 hours of chemical application. In this lab, I have shown that there is a marked increase in NPY mRNA upon TPA application. This increase is time dependent, beginning at two hours and maximizing at 8 hours of chemical exposure, and TPA dose dependent with increases beginning at 6nM and maximizing at 50nM (Figure 6a). In an attempt to show this PKC induction of NPY mRNA could be hormonally regulated, the previously published induction of NPY mRNA by Nerve Growth Factor (Sabol S. *et. al.*, 1990; Higuchi H. *et. al.*, 1992) was examined for PKC dependency. Cells were exposed to TPA (2uM) for 24 hours to

down-regulate the available PKC. The cells were then subsequently stimulated with either TPA (50nM) or Nerve Growth Factor (50ng/ml) for 6 hours. Total RNA was extracted and analyzed for relative NPY mRNA. Those cells that were exposed to the high concentration of TPA for 24 hours were unable to respond to subsequent TPA stimulation, but were able to respond to the NGF producing NPY mRNA levels comparable to those previously described (Sabol S. *et. al.*, 1990; Higuchi H. *et. al.*, 1992) (Figure 6b). Therefore, the production of NPY mRNA by exposure to TPA and NGF occur by two distinct mechanisms.

MF exposure inhibits, specifically, PKC dependent induction of NPY mRNA in PC-12 cells. To determine if a specific signal transduction pathway was affected by magnetic field exposure, PC-12 cells were exposed to TPA (50nM) and forskolin (20uM), plus or minus MF for 8 hours. There was a statistically significant decline in the production of NPY mRNA in cells exposed to TPA (50nM) and the magnetic field compared to sham exposed ($n = 4$; $p < .05$). No changes were seen in the control (no chemical) or forskolin induced conditions (Figure 7).

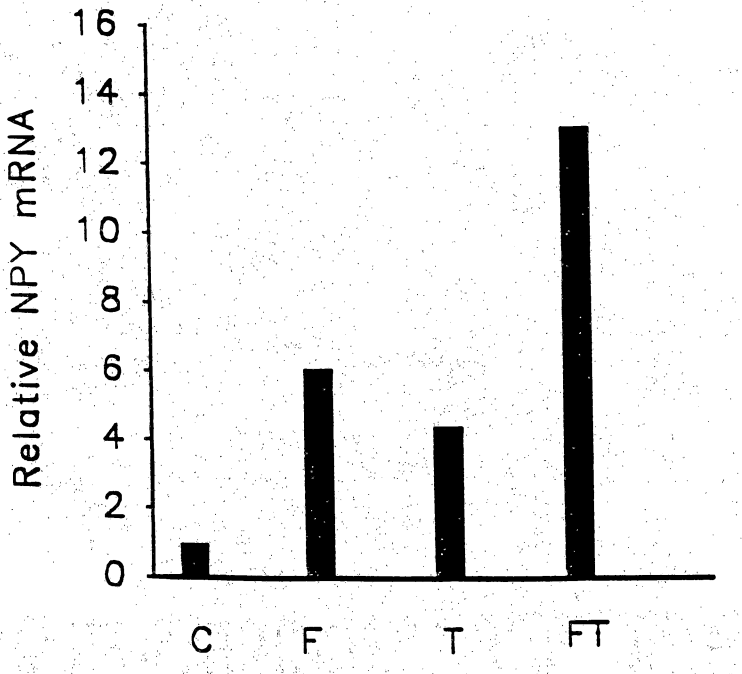
Determination of half-life of cAMP, PKC, and cAMP + PKC induced NPY mRNA. Previous reports have indicated the half-life of the forskolin/ TPA induced NPY mRNA in fetal brain cells to be at least 12 hours (Magni P. *et. al.*, 1992). The goal was to determine the half-life of the induced NPY mRNA in PC-12 cells. Cells were exposed to forskolin (20uM), TPA (50nM), and forskolin (20uM) + TPA (50nM), for 6 hours. After the six hour stimulation, DRB (5,6-dichloro-1-B-D-ribofuranosyl-benzimidazole) (65uM), an inhibitor of transcription (Tamm I., and

Kikuchi T., 1979), was added. Total RNA was isolated before, and 6, 11, 24, and 30 hours after the addition of DBR. The half-life was determined to be approximately 12 hours for all conditions (Figure 8).

MF exposure does not affect the half-life of TPA induced NPY mRNA. To determine if the half-life stability was affected by exposure to magnetic fields, cells were exposed to TPA (50nM) plus or minus MF exposure for 8 hours at which time DRB (65uM) was added to stop transcription. RNA was extracted before, and 8, 14, 20, and 32 hours after the addition of DRB. Those cells exposed to TPA and magnetic field produced 35% ($p < 0.02$) less NPY mRNA than did sham exposed cells in the first eight hours. The half-life was determined to be 12 hours. The half-life was not affected by exposure to magnetic fields (Figure 9).

Fig. 1. **Stimulation of PC-12 cells with forskolin, TPA, and forskolin + TPA increases levels of NPY mRNA.** PC-12 cells (80% confluent at time of chemical stimulation) were stimulated for 24 hours with **F**-forskolin (20uM), **T**-TPA (100nM), and **FT**-forskolin (20uM) + TPA (100nM) or **C**-control. Relative NPY mRNA is expressed as the ratio of gray units NPY mRNA/ gray units GPDH with the control ratio equal to 1.

Figure 1



C- control; F-forskolin (20uM); T-TPA (100nM)

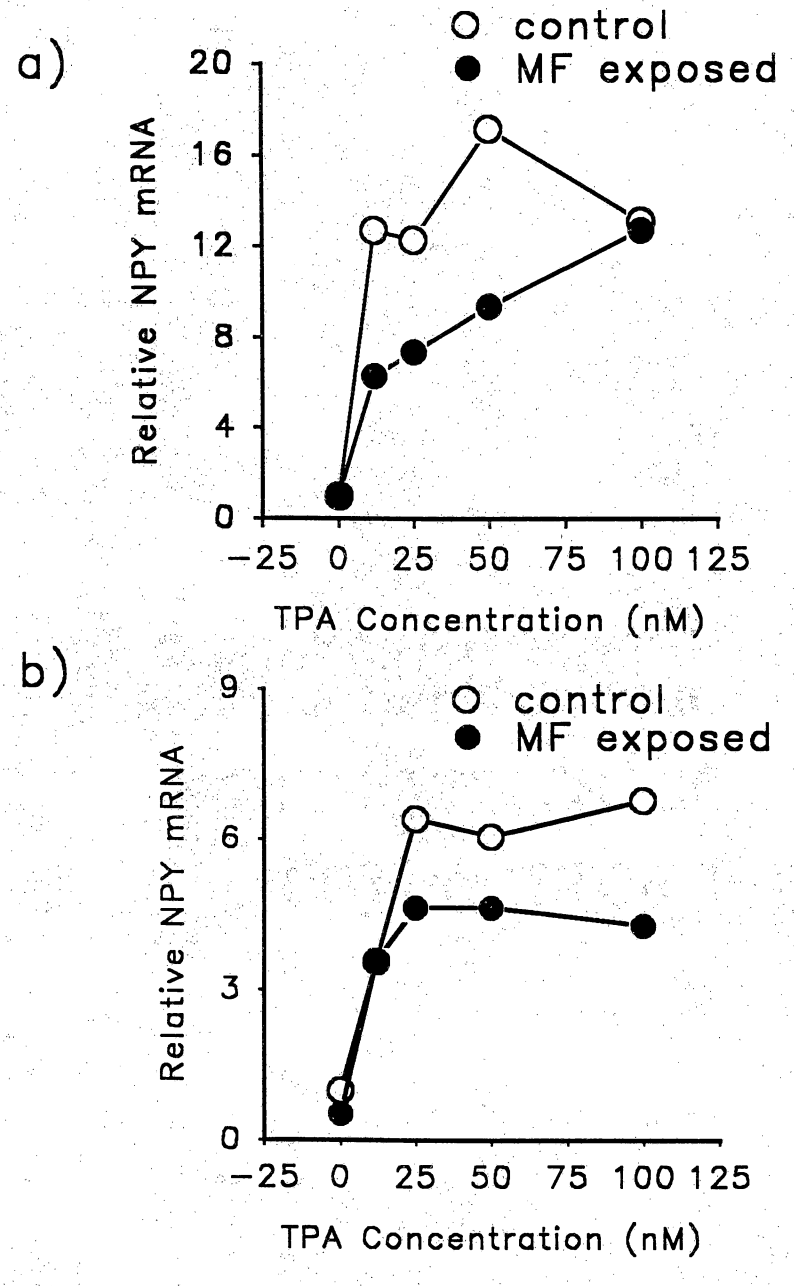
FT-forskolin (20uM) + TPA (100nM); all for 24 hours.

Stimulation of PC-12 cells with forskolin, TPA, and forskolin + TPA increases levels of NPY mRNA

Fig 2. **MF exposure inhibits cAMP/PKC dependent synergistic induction of NPY mRNA in cells exposed for 24 hours with varying TPA concentrations.** A) PC-12 cells (70-90% confluent) were exposed to forskolin (20uM), forskolin (20uM) + TPA (12nM), forskolin (20uM) + TPA (25nM), forskolin (20uM) + TPA (50nM), and forskolin (20uM) + TPA (100nM) with or without 1 G, 60 Hz magnetic field for 24 hours. Symbols: o-sham exposed; ●-MF exposed. Relative NPY mRNA is expressed as the ratio of gray units NPY mRNA/ gray units GPDH with the control ratio equal to 1. B) A replicate study with the same conditions as A.

Figure 2

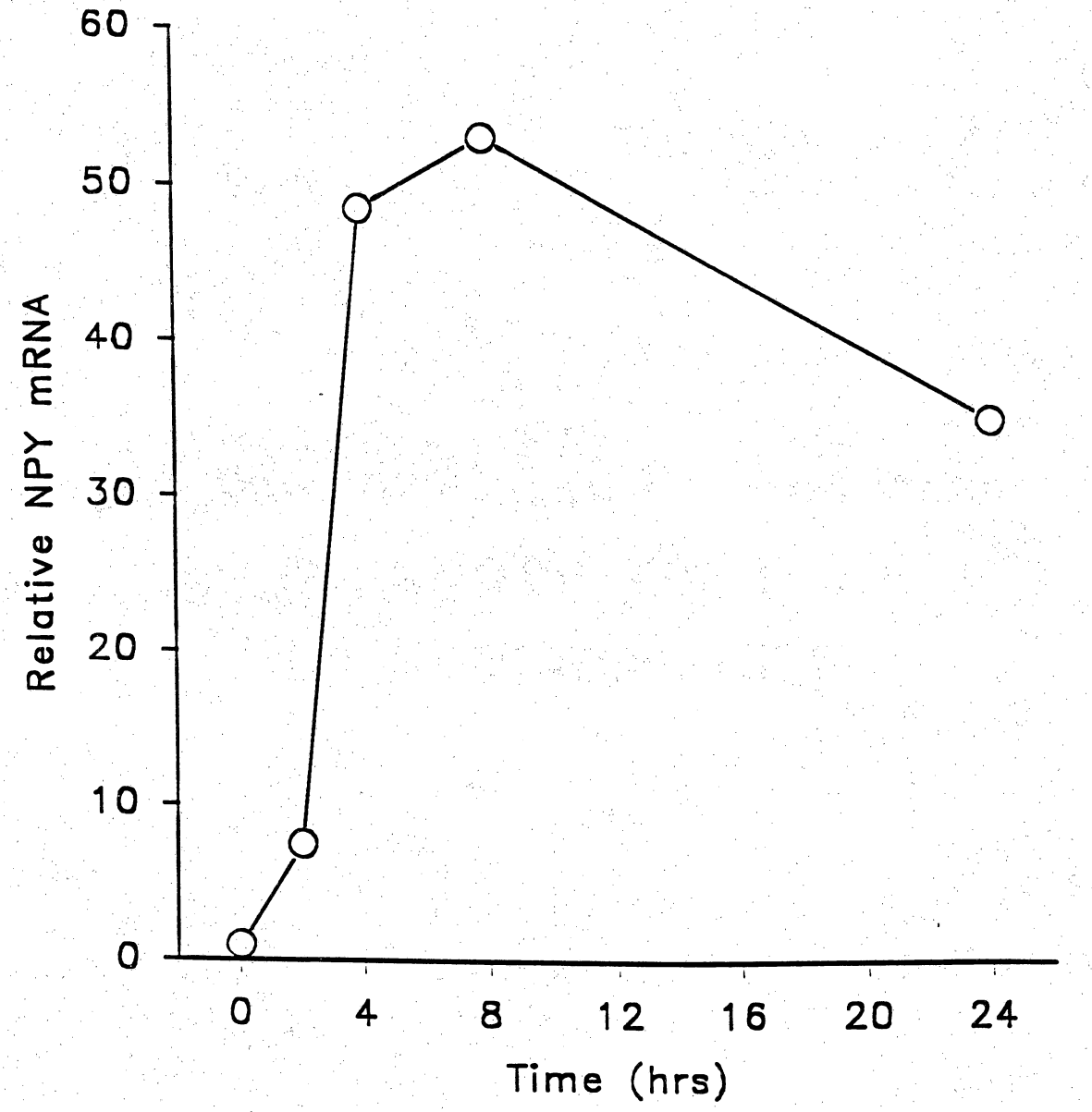
All contain forskolin (20uM)



MF Exposure Inhibits cAMP + PKC Induced NPY mRNA at 24 Hours.

Fig. 3. **Kinetic study of forskolin + TPA induced NPY mRNA.** A) PC-12 cells (70-90% confluent) were exposed to forskolin (20uM) + TPA (50nM) for 2, 4, 8, and 24 hours. Relative NPY mRNA is expressed as the ratio of gray units NPY mRNA/ gray units GPDH with the control ratio equal to 1.

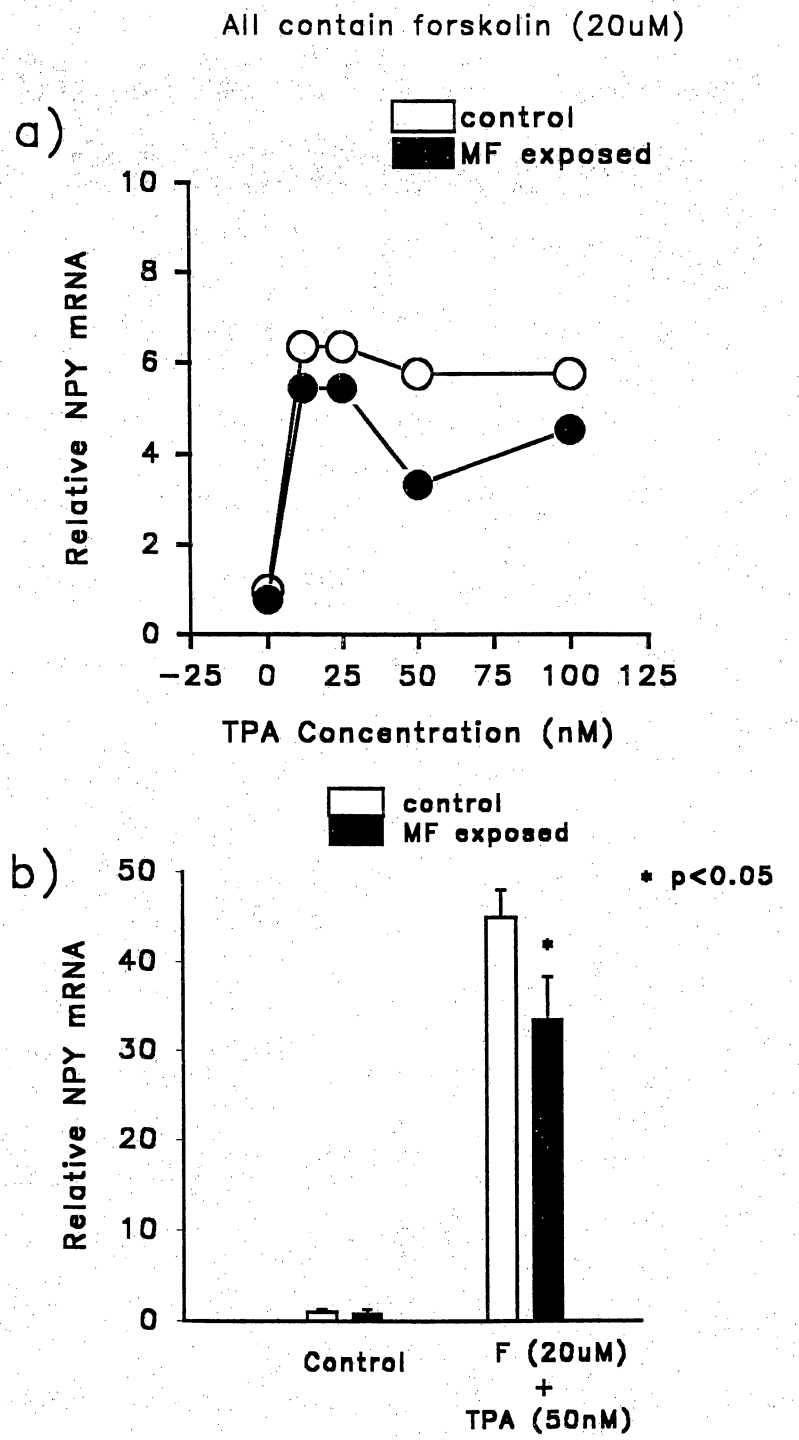
Figure 3



Kinetics Study of Forskolin (20uM) + TPA (50nM) Induced NPY mRNA

Fig. 4. **MF exposure inhibits the cAMP/PKC dependent synergistic induction of NPY mRNA in cells exposed for 8 hours with varying TPA concentrations.** PC-12 cells (70-90% confluent) were exposed to forskolin (20uM), forskolin (20uM) + TPA (12nM), forskolin (20uM) + TPA (25nM), forskolin (20uM) + TPA (50nM), and forskolin (20uM) + TPA (100nM) for 8 hours with and without 1 G, 60 Hz magnetic field exposure. Symbols: o -sham exposed; • -MF exposed. B) PC-12 cells (70-90% confluent) were exposed to forskolin (20uM) + TPA (50nM) for 8 hours with and without magnetic field exposure (n=4/condition). Symbols: o -sham exposed; • -MF exposed.

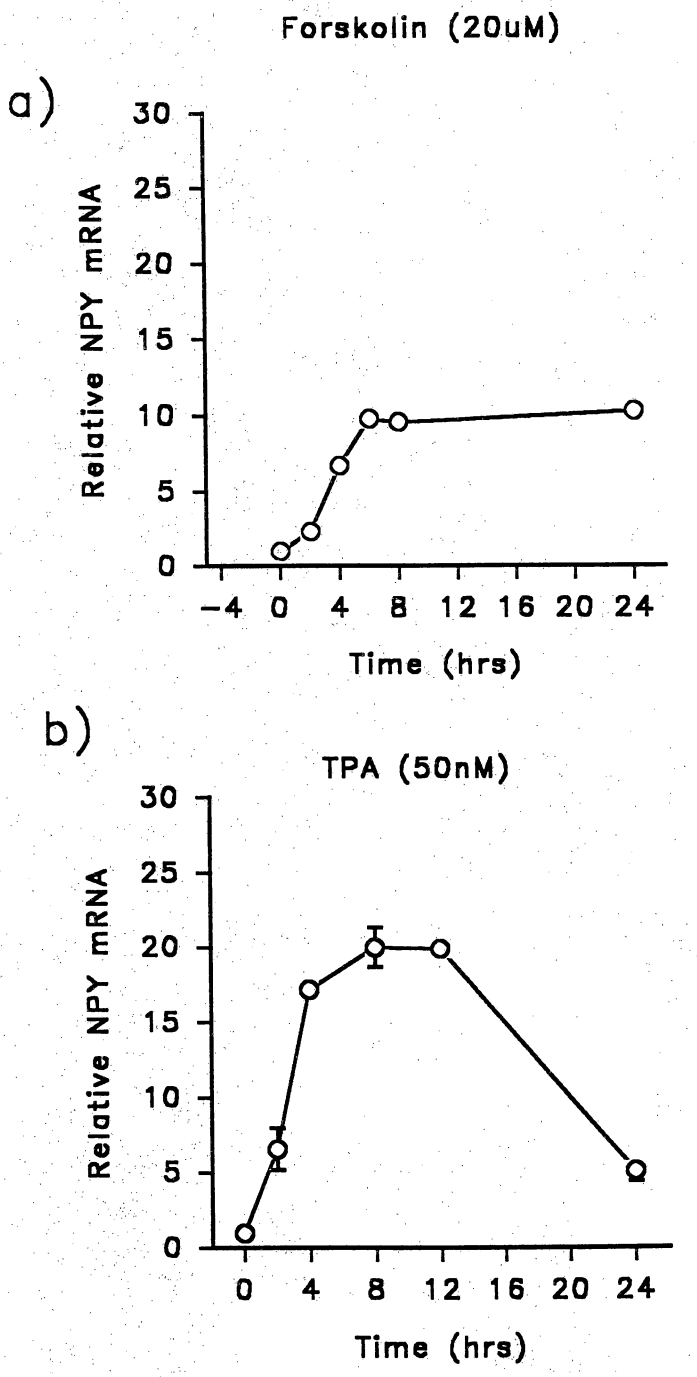
Figure 4



MF Exposure Inhibits cAMP + PKC Induced NPY mRNA at 8 Hours

Fig. 5. **Kinetic studies of forskolin and TPA induced NPY mRNA.** A) PC-12 cells (70-90% confluent) were exposed to forskolin (20uM) for 2, 4, 6, 8, and 24 hours. B) PC-12 cells (70-90% confluent) were exposed to TPA (50nM) for 2, 4, 8, and 24 hours. Relative NPY mRNA is expressed as the ratio of gray units NPY mRNA/ gray units GPDH with the control ratio equal to 1.

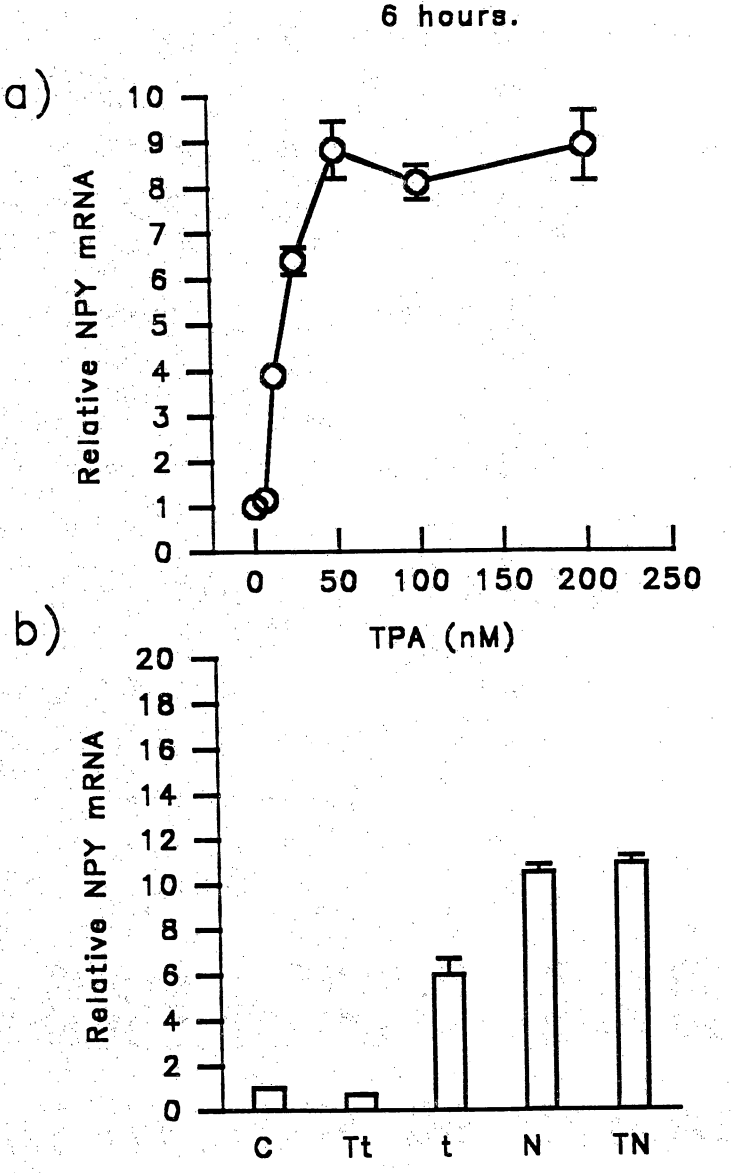
Figure 5



Kinetics Study of Forskolin or TPA Induced NPY mRNA

Fig. 6. **TPA induces NPY mRNA in a time and dose dependent manner by activation of PKC in PC-12 cells.** A) PC-12 cells (70-90% confluent) were exposed to TPA (6, 12, 25, 50, 100, and 200nM) for 6 hours. B) PC-12 cells were exposed to TPA (2uM) for 24 hours with subsequent exposure to TPA (50nM) for 6 hours (**Tt**), TPA (2uM) for 24 hours with subsequent exposure to Nerve Growth Factor (50ng/ml) for 6 hours (**TN**), TPA (50nM) for 6 hours (**t**), Nerve Growth Factor (50ng/ml) for 6 hours (**N**), and controls with no chemical exposure (**C**). Relative NPY mRNA is expressed as the ratio of gray units NPY mRNA/ gray units GPDH with the control ratio equal to 1.

Figure 6

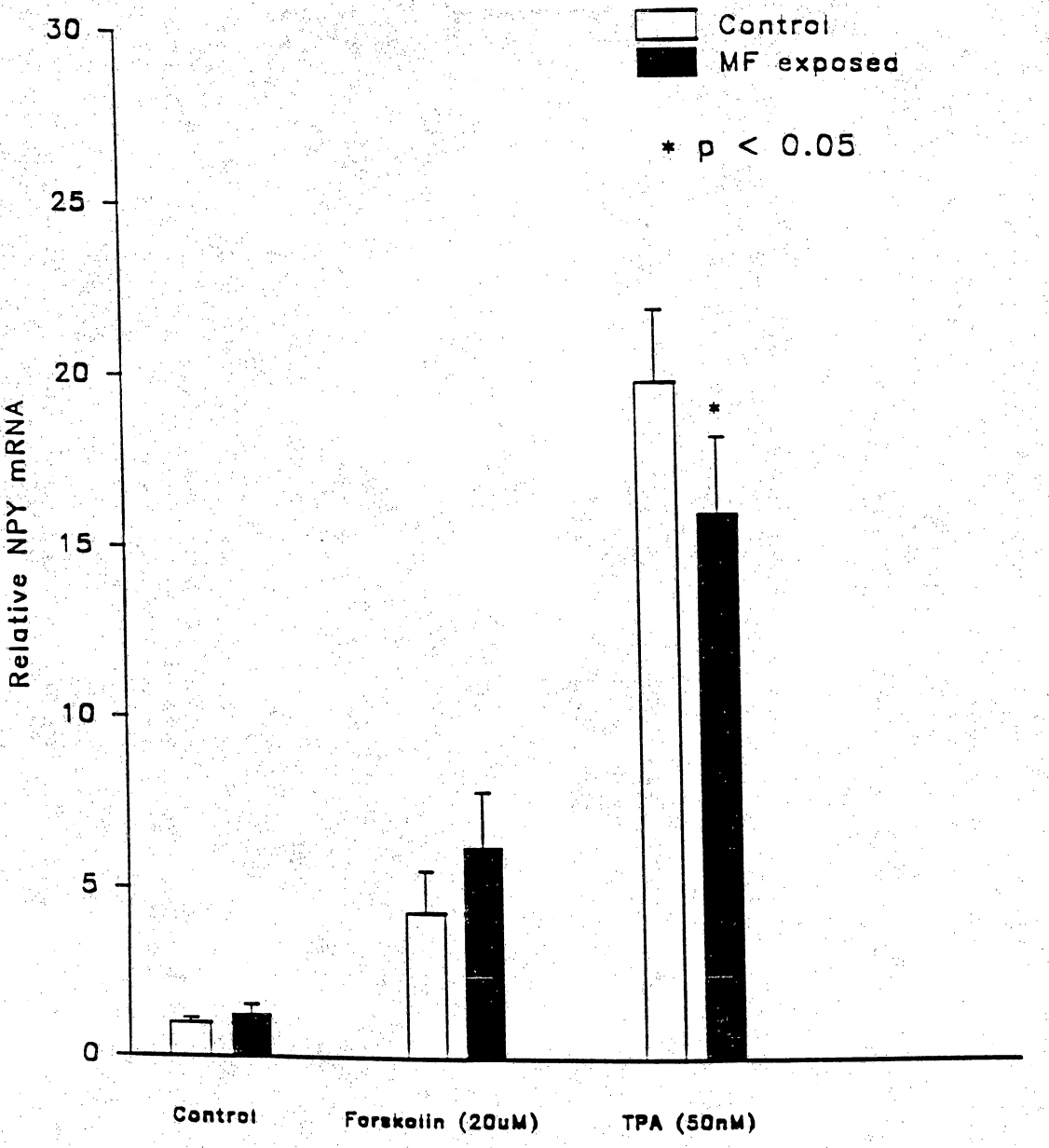


C– control
Tt– TPA (2uM) for 24 hrs. + TPA (50nM) for 6 hrs.
t– TPA (50nM) for 6 hrs.
N– NGF (50ng/ml)
TN– TPA (2uM) for 24 hrs. + NGF (50ng/ml) for 6 hrs.

TPA Induces NPY mRNA in a Time/Dose Dependent Manner by Activation of PKC in PC–12 Cells

Fig. 7. **MF exposure inhibits, specifically, PKC dependent induction of NPY mRNA in PC-12 cells.** PC-12 cells (70-90% confluent) were exposed to forskolin (20uM), and TPA (50nM) with without 1 G, 60 Hz magnetic field for eight hours (n=4/condition). Relative NPY mRNA is expressed as the ratio of gray units NPY mRNA/ gray units GPDH with the control ratio equal to 1. Symbols: ▭-sham exposed; ▬ -MF exposed.

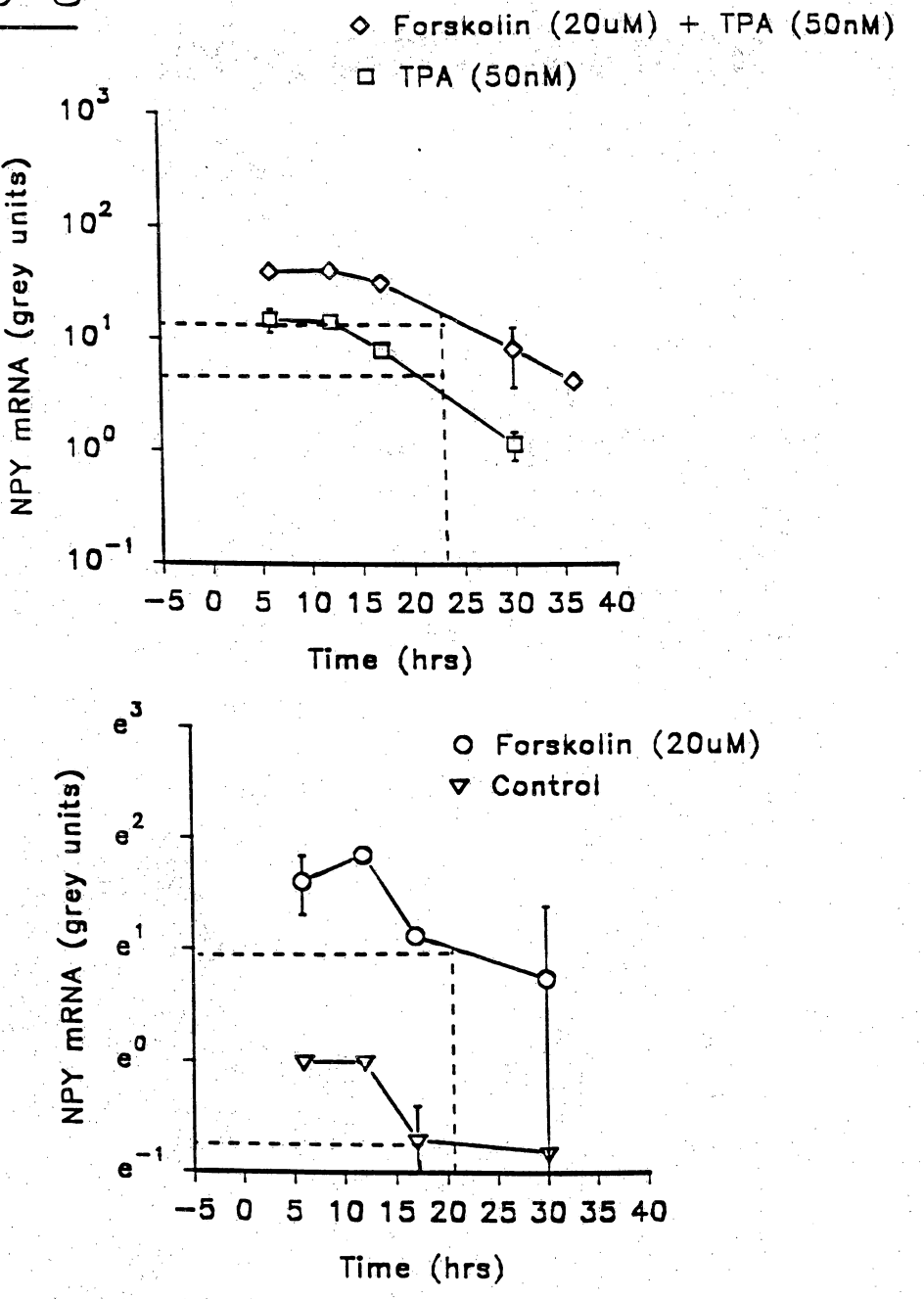
Figure 7



MF Exposure Inhibits, Specifically, PKC Dependent Induction of NPY mRNA in PC-12 Cells

Fig. 8. **Determination of half-life of cAMP, PKC, and cAMP + PKC induced mRNA.** PC-12 cells (70-90% confluent) were exposed to forskolin (20uM), TPA (50nM), and forskolin (20uM) + TPA (50nM), or control (unexposed) for 6 hours at which time DRB (65uM) (a transcriptional inhibitor) was added. RNA was extracted and analyzed at the indicated timepoints for NPY mRNA. NPY mRNA is expressed in grey units. Symbols: ○ -forskolin (20uM); □ -TPA (50nM); ◇ -forskolin (20uM) + TPA (50nM); ▽ -control.

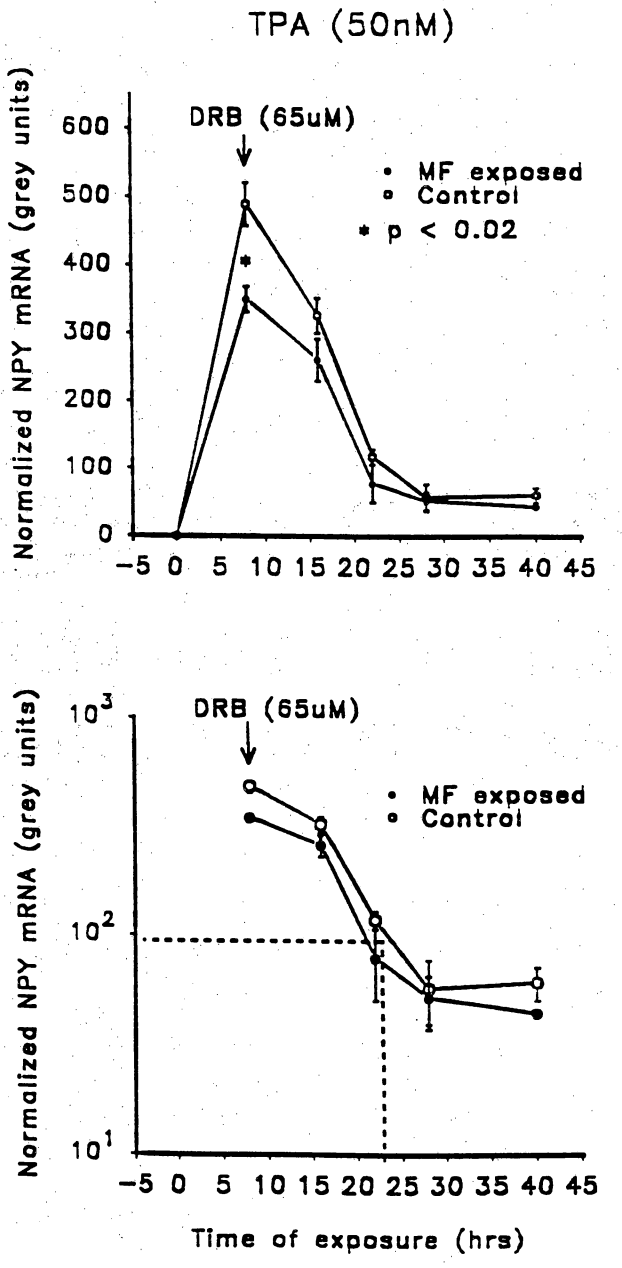
Figure 8



Determination of Half-life of cAMP, PKC, and cAMP + PKC Induced NPY mRNA

Fig. 9. **MF exposure does not affect the half-life of TPA induced NPY mRNA.** PC-12 cells (70-90% confluent) were exposed to TPA (50nM) with or without 1 G, 60 Hz magnetic field for 6 hours at which time DRB (65uM) was added. Total RNA was extracted and analyzed for NPY mRNA at the timepoints indicated. NPY mRNA is expressed in grey units. Symbols: o -sham exposed; ● -MF exposed.

Figure 9



MF Exposure Does Not Affect the Half-life of TPA Induced NPY mRNA

DISCUSSION

In this study, previous reports of chemically induced NPY mRNA in PC-12 cells were replicated. Also, it was demonstrated that NPY mRNA is regulated in PC-12 cells by PKC in a time and dose dependent manner. This PKC dependent induction of NPY mRNA appears to be physiologically relevant as exposure to TPA causes significant increases in NPY secretion from fetal rat brain cells (Magni P. *et. al.*, 1992) and increases in NPY mRNA in 5-6 day old rat brain primary cell cultures (unpublished data). I conclude that from the above data, the forskolin + TPA and TPA induced production of NPY mRNA are significantly inhibited in PC-12 cells exposed to a 1 gauss, 60 Hz magnetic field for 8, and 24 hours. It must be noted that not in all cases was there a decline in chemically induced NPY mRNA in MF exposed cells. In the case of the forskolin + TPA induced NPY mRNA, 11 of 15 experiments (75%), showed declines from 20% - 50%, while 3 showed no changes, and one showed an increase of 50%. In the case of the TPA induced NPY mRNA, 5 of 8 experiments (63%) showed declines from 20% - 40%, while the others showed no changes. In every case, when both forskolin + TPA and TPA conditions were employed in the same experiment, when there was no change in induced NPY mRNA upon magnetic field exposure for one condition, there was no change for the other. This could suggest that the state of the cell population, for example, growth rate, confluence, etc... at time of exposure may be extremely important for this observed effect of magnetic field exposure. Some variation in the magnitudes of NPY mRNA induction exists between similar experiments. I attribute these variations to possible

differences in cell populations, freshness of reagents, and differences in basal levels of NPY mRNA at the time of assay.

This data indicate that the observed decline in forskolin + TPA induced NPY mRNA in MF exposed cells is likely due to the effects on the phoshoinositide/PKC dependent signal transduction pathway as indicated by the decline of NPY mRNA in cells exposed to only TPA and MF. It has been shown that the stabilization of other transcripts in PC-12 cells can be increased by the activation of PKC (Perrone-Bizzozero N. *et. al.*, 1993). According to our data, PKC activation does not lead to stabilization of the NPY mRNA. There is no change in chemically induced NPY mRNA half-life upon exposure to magnetic fields. I can only conclude that the observed decline in PKC induced NPY mRNA upon exposure to magnetic fields is not at the posttranscriptional level. The effect is perhaps at a PKC dependent event occurring at the level of transcription.

Because of the many physiological roles of NPY, changes in circulating blood and brain levels could have significant clinical implications. Do relationships exist between changes in NPY or changes in the mechanisms of NPY production and reported biological effects associated with exposure to magnetic fields? There exists a wide array of published data that suggest a magnetic field/physiological interaction. Many of the physiological changes associated with exposure to magnetic fields are in part regulated by NPY. For instance, there have been reports of changes in blood pressure (Cook M. *et. al.*, 1992), intracellular calcium levels (Walleczek J. *et. al.*, 1990; Liburdy R. *et. al.*, 1993), catecholamine secretion (Dixey R. and Rein G., 1982)

and production and secretion of the pineal hormone, melatonin in subjects exposed to magnetic fields (Reiter R. *et. al.*, 1992; Wilson B. *et. al.* 1981, 1986, 1990; Welker H. *et. al.*, 1983). All of these phenomena appear to be regulated by NPY (Lundberg J. *et. al.*, 1982; Olcese J. *et. al.*, 1991; Vacas M. *et. al.*, 1987; Reuss S. *et. al.*, 1989; Erdbrugger W. *et. al.*, 1993; Motulsky H. and Michel M., 1988; Michel M. *et. al.*, 1990; Guy J. and Pelletier G., 1988). It is possible that exposure to magnetic fields affects the natural brain and body production of NPY which could lead to the before mentioned physiological changes associated with magnetic field exposure. Alterations in PKC dependent signal transduction mechanism by MF exposure may also help to explain previously published MF effects. In PC-12 cells, tyrosine hydroxylase, the rate-determining enzyme involved in the production of norepinephrine, appears to be regulated by events involving protein kinase C (Vyas S. *et. al.*, 1990). Norepinephrine appears to be the primary regulator of melatonin production (Moore R. *et. al.*, 1968, Reiter R., 1992). Neurotransmitter release from sympathetic neurons appears to be regulated by PKC (Heinrich J. *et. al.*, 1987).

The question still remains, "Is there a relationship between NPY and cancer?" There is one plausible connection to exposure to EMF's, changes in NPY levels, and cancer. This connection can be hypothesized two different ways. First, it has been demonstrated that pineal melatonin concentrations are lowered upon exposure to EMF. Melatonin has been shown to play a role in some cancers associated with EMF exposure. In patients with either prostate or breast cancer, blood levels of melatonin are 40%-70% below normal (Bartsch C. *et. al.*, 1985, 1989). This decline is

specifically due to a decline in brain secretion which is also the case with exposure to EMF. It is currently unknown why melatonin levels are suppressed in the presence of EMF, although this dissertation could shed some light. Neuropeptide Y appears to be a positive regulator of melatonin production by the pineal gland. This dissertation has shown specific declines in the production of NPY mRNA in PC-12 cells exposed to EMF. The connection to pineal cells is only speculative, but plausible. The second hypothesis involves the levels of NPY itself. In children diagnosed with acute leukemia, a cancer associated with EMF exposure, blood levels of NPY were significantly higher than those without the cancer. Of the children tested, those with highest levels of NPY had a significantly better survival rate than those with lower levels (Kogner P. *et. al.*, 1992). NPY immunoreactivity was detected in tumors of children with neuroblastoma. Again, those with the highest immunoreactivity showed better survival rates. (Cohen P. *et. al.*, 1990) These data suggest that NPY may itself play a role in the immune response to certain forms of cancer. It has been suggested that the presence of NPY be used as a marker to determine between benign and malignant pheochromocytoma tumors. In that study, NPY was present in all (9 of 9) benign tumors, but in only 4 of 11 malignant tumors (Helman L. *et. al.*, 1989).

For this inhibition of induced NPY mRNA by MF exposure to have any real significance, the resultant NPY levels must also change. Because NPY can be stored in vesicles and secreted upon command, this effect of MF exposure could be happening earlier than what is shown here. If chemical application to the cells causes immediate release of the stored protein, the results I have shown could be due to

changes in possible feedback mechanisms. For instance, if the MF exposure causes a suppression in the immediate chemically induced neuropeptide secretion, this might show up in declined levels of production to replace the stored NPY. Also, does this decline in NPY production upon MF exposure result in a decline of stored peptide which in turn would cause a decline in subsequent release? Is there a natural hormone in the body that is capable of inducing cAMP and PKC in similar chromaffin cells within the body, and does this stimulation lead to changes in circulating NPY levels?

The exact part of the PKC dependent pathway leading to the production of NPY mRNA that is affected by MF exposure needs to be determined, as well. This mechanism could involve declined levels of TPA induced PKC enzymatic activity, perhaps by inhibiting the effects of TPA on cytoplasmic PKC. Is there an inhibition of the phosphorylations by PKC upon MF exposure? Many more experiments need to be performed to identify the exact mechanism in this system that is affected by MF exposure.

NPY may play a role in cancer defense by regulating the secretion of brain melatonin and by its own presence in the blood and tissues. If the decline in protein kinase C induced NPY mRNA upon magnetic field exposure occurs in the living system and, if this decline leads to a subsequent decline in brain and blood levels of the neuropeptide, the findings we have just reported could lead to physiological explanations of some magnetic field induced phenomena.

REFERENCES

Adey, W.R., Electromagnetic fields, cell membrane amplification and cancer promotion. *Proceedings of National Council on Radiation Protection and Measurements Annual Meeting, National Academy of Sciences, Washington, D.C.* pgs. 80-110, 1986.

Adey, W.R., The cellular microenvironment and signaling through cell membranes. *Electromagnetic Fields and Neurobehavioral Function.* pgs. 81-106, 1988.

Adey, W.R., Introduction: effects of electromagnetic radiation on the nervous system. *Annals New York Academy of Sciences.*

Adey, W.R., Joint actions of environmental nonionizing electromagnetic fields and chemical pollution in cancer promotion. *Environmental Health Perspectives.* **86**:297-305, 1990.

Allen, J.M., Adrian, T.E., Polak, J.M., Bloom, S.R., Neuropeptide Y in the adrenal gland. *J. Auton. Nerv. Syst.* **9**:559-563, 1983.

Anderson, B., Milsted, A., Kennedy, G., Nilson, J.H., Cyclic AMP and phorbol ester interact synergistically to regulate expression of the chorion gonadotropin genes. *J. Biol. Chem.* **263**:15578-15583, 1988.

Bartsch, C., Bartsch, H., Fluchter, S.H., Attanasio, A., Gupta, D., Evidence for modulation of melatonin secretion in men with benign and malignant tumors of the prostate: relationship with the pituitary hormones. *J. Pineal Res.* **2**:121-132, 1985.

Bartsch, C., Bartsch, H., Fuchs, U., Lippert, T.H., Bellman, O., Gupta, D., Stage-dependent depression of melatonin in patients with primary breast cancer, *Cancer* **64**:426-433, 1989.

Bawin, S.M., Adey, W.R., Sensitivity of calcium binding in cerebral tissue to weak environmental electric fields oscillating at low frequency. *Proc. Natl. Acad. Sci. USA,* **73**:1999-2003, 1976.

Blackman C.F., Benane S.G., House D.E., Pollock M.M., Action of 50 Hz magnetic fields on neurite outgrowth in pheochromocytoma cells. *Bioelectromagnetics* **14**:273-286, 1993.

Blackman, C.F., Benane, S.G., House, D.E., Evidence for direct effect of magnetic fields on neurite outgrowth. *FASEB J* **7**:801-806, 1993.

Cain, C., Adey, W.R., Luben, R.A., Evidence that pulsed electromagnetic fields inhibit coupling of adenylate cyclase by parathyroid hormone in bone cells. *J. Bone and Min. Res.* **2**:437-441, 1987.

Cain, C.D., Thomas, D.L., Adey, W.R., 60 Hz magnetic field acts as a co-promoter in focus formation of C3H/10/T1/2 cells. *Carcinogenesis* **14**:950-960, 1993.

Chomczynski, P., Sacchi, N., Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156-159, 1987.

Cohen, P., Cooper, M.J., Helman, L.J., Thiele, V.J., Seeger, R.C., Israel, M.A., Neuropeptide Y expression in the developing adrenal gland and in childhood neuroblastoma tumors. *Cancer Research* **50**:6055-6061, 1990.

Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E., Goodman, H.M., A cyclic AMP- and phorbol ester-inducible DNA element. *Nature* **323**:353-356 1986.

Cook, M.R., Graham, C., Cohen, H.D., Gercovich, M.M., A replication study of human exposure to 60-Hz fields: Effects on neurobehavioral measures. *Bioelectromagnetics* **13**:261-285, 1992.

de Quidt, M.E., Emson, P.C., Distribution of neuropeptide Y-like immunoreactivity in the rat central nervous system. II Immunohistochemical analysis. *Neuroscience* **18**:545-618, 1986.

Delgado, J.M.R., Leal, J., Monteagudo, J.L., Gracia, M.G., Embryological changes induced by weak, extremely low frequency electromagnetic fields. *J. Anat.* **134**:533-551, 1982.

Dixey, R., Rein, G., ^{3}H-noradrenaline release potentiated in a clonal nerve cell line by low-intensity pulsed magnetic fields. *Nature* **296**:253-256, 1982.

Erdbrugger, W., Vischer, P., Bauch, H., Michel, M., Norepinephrine and neuropeptide Y increase intracellular Ca^{2+} in cultured porcine aortic smooth muscle cells. *J. Cardiovas. Pharmocol.* **22**:97-102, 1993.

Feychting, M., Anders, A., Magnetic fields and cancer in people residing near Swedish high voltage power lines. Abstract: IMM-rapport 6/92, 1992.

Goldhaber, M.K., Polen, M.R., Hiatt, R.A., The risk of miscarriage and birth defects among women who use visual display terminals during pregnancy. *Am. J. Indust. Med.* **13**:695-706, 1988.

Goodman, R., Henderson, A.S., Sine waves enhance cellular transcription. *Bioelectromagnetics* **7**:23-29, 1986.

Goodman, R., Lin-Xiang, W., Jing-Chu, X., Henderson, A., Exposure of human cells to low-frequency electromagnetic fields results in quantitative changes in transcripts. *Biochim. et. Biophys. Acta* **1009**:216-220, 1989.

Goodman, R., Basset, C.A., Henderson, A., Pulsing electromagnetic fields induce cellular transcription. *Science* **220**:1283-1285, 1983.

Guy, J., Pelletier, G., Neuronal interactions between neuropeptide y (NPY) and catecholaminergic systems in the rat arcuate nucleus as shown by dual immunocytochemistry. *Peptides* **9**:567-570, 1988.

Helman, L.J., Cohen, P.S., Averbuch, S.D., Cooper, M.J., Keiser, H.R., Israel, M.A., Neuropeptide Y expression distinguishes malignant from benign pheochromocytoma. *J. Clin. Oncol.* **7**:1720-1725, 1989.

Higuchi, H., Yang, H.Y., Sabol, S.L., Rat neuropeptide Y precursor gene expression, mRNA structure, tissue distribution, and regulation by glucocorticoids, cAMP and phorbol ester. *J. Biol. Chem.* **263**:6288-6295, 1988.

Higuchi, H., Nakano, K., Miki, N., Identification of NGF-response element in the rat neuropeptide Y gene and induction of the binding proteins. *Biochem. Biophys. Res. Comm.* **189**:1553-1560, 1992.

Kerkerian, L., Guy, J., Lefevre, G., Pelletier, G., Effects of neuropeptide Y (NPY) on the release of anterior pituitary hormones in the rat. *Peptides* **6**:1201-1204, 1985.

Kogner, P., Ericsson, A., Barbany, G., Persson, H., Theodorsson, E., Bjork, O., Neuropeptide Y (NPY) syntheses in lymphoblasts and increased plasma NPY in pediatric B-cell precursor leukemia. *Blood* **80**:1324-1329,1992.

Liburdy, R.P., Callahan D.E., Harland J., Dunham E., Sloma T.R., Yaswen P., Experimental evidence for 60 Hz magnetic fields operating through the signal transduction cascade: Effects of calcium influx and c-MYC mRNA induction. *FEBS Lett.* **334**:301-8, 1993.

Lundberg, J.M., Tenenius, L., Hokfelt, T., Martling, C.R., Tatemoto, K., Mutt, V., Polak, J., Bloom, S., Goldstein, M., Neuropeptide Y (NPY) -like immunoreactivity in peripheral noradrenergic neurons and effects of NPY on sympathetic function. *Acta. Physiol. Scand.* **116**:477-480, 1982.

Magni, P., Barnea, A., Forskolin and phorbol ester stimulation of neuropeptide Y (NPY) production and secretion by aggregating fetal brain cells in culture: evidence for regulation of NPY biosynthesis at transcriptional and posttranscriptional levels. *Endocrinology* **130**:976-984, 1992.

Matthies, H., Palfrey, H., Hirning, L., Miller, R., Down regulation of protein kinase C in neuronal cells: Effects on neurotransmitter release. *J. Neurosci.* **4:**1198-1206, 1987.

Michel, M., Schlicker, E., Fink, K., Boublik, J., Gothert, M., Willette, R., Daly, R., Hieble, J., Rivier, J., Motulsky, H., Distinction of NPY receptors in vitro and in vivo. I. NPY-(18-36) discriminates NPY receptor subtypes in vitro. *Endocrinol. Metab.* **22**:E131-E139, 1990.

Moore, R.Y., Heller, A., Bhatnager, R.K., Wurtman, R.J., Axelrod, J., Central control of the pineal gland: visual pathways. *Arch. Neurol.* **18**:208-218, 1968.

Motulsky, H., Michel, M., Neuropeptide Y mobilizes Ca^{2+} and inhibits adenylate cyclase in human erythroleukemia cells. *Am. J. Physiol.* 255 (Endicrinol. Metab. **18**):E880-E885, 1988.

Myers, A., Clayden, A.D., Cartwright, R.A., Cartwright, S.C., Childhood cancer and overhead power lines: a case-control study. *Br. J. Cancer* **62**:1008-1014, 1990.

Olcese, J., Neuropeptide Y: An inhibitor of Norepinephrine-stimulated melatonin in the rat pineal gland, *J. Neurochem.* **57**:943-947, 1991.

Perrone-Bizzozero, N.I., Cansino, V.V., Kohn, D.T., Posttranscriptional regulation of GAP-43 gene expression in PC-12 cells through protein kinase C-dependent stabilization of the mRNA. J. of Cell Biology **120**:1263:1270, 1993.

Phillips, J.L., Haggren, W., Thomas, W.J., Ishida-Jones, T., Adey, W.R., Magnetic field-induced changes in specific gene transcription. *Biochem. et Biophys. Acta* **1132**:140-144, 1992.

Reiter, R., Changes in circadian melatonin synthesis in the pineal gland of animals exposed to extremely low frequency electromagnetic radiation: A summary of observations and speculation on their implications. *Electromagnetic Fields and Circadian Rhythmicity* pgs. 13-27, 1992.

Reiter, R.J., Richardson, B.A., Magnetic field effects on pineal indoleamine metabolism and possible biological consequences. *FASEB* **6**:2283-2287, 1992.

Reuss, S., Moore, R.Y., Neuropeptide Y-containing neurons in the rat superior cervical ganglion: projections to the pineal gland. *J. Pineal Res.* **6**:307-316, 1989.

Sabol, S.L., Higuchi, H., Transcriptional regulation of the neuropeptide Y gene by nerve growth factor: antagonism by glucocorticoids and potentiation by adenosine 3',5' monophosphate and phorbol ester. *Mol. Endocrinol.* **4**:384-392, 1990.

Savitz, D.A., John, E.M., Klecker, R.C., Magnetic field exposure from electric appliances and childhood cancer. *Am. J. Epidemiol.* **131**:763-73, 1990.

Shinohara, K., Tominaga, K., Isobe, Y. Inouye, S.T., Photic regulation of peptides located in the ventrolateral subdivision of the suprachiasmatic nucleus of the rat: daily variations of vasoactive intestinal peptide, gastrin-releasing peptide, and neuropeptide Y. *J. of Neurosci.* **13**:793-800, 1993.

Stanley, B.G., Leibowitz, S.F., Neuropeptide Y injected in the paraventricular hypothalamus: a powerful stimulant of feeding behavior. *Proc. Natl. Acad. Sci. USA* **82**:3940-3943, 1985.

Stanley, B.G., Magdalin, W., Seirafi, A., Thomas, W.J., Leibowitz, S.F., The perifornical area: the major focus of (a) patchily distributed hypothalamic neuropeptide Y-sensitive feeding system (s). *Brain Research* **604**:304-317, 1993.

Stanley, B.G., Thomas, W.J., Feeding responses to perifornical hypothalamic injection of neuropeptide Y in relation to circadian rhythms of eating behavior. *Peptides* **14**:475-481, 1993.

Tamm, I., Kikuchi, T., Early termination of heterogeneous nuclear RNA transcripts in mammalian cells: Accentuation by 5,6-dichloro-1-B-D-ribofuranosylbenzimidazole. *Proc. Natl. Acad. Sci. USA* **76**:5750-5754, 1979.

Tatemoto, K., Carlquist, M., Mutt, V., Neuropeptide Y: complete amino acid sequence of the brain peptide. *Proc. Natl. Acad. Sci. USA* **79**:2513, 1982.

Vacas, M.I., Sarmiento, M.I.K., Pererya E.N., Etchegoyen, G.S., Cardinali, D.P., In vitro effect of neuropeptide Y on melatonin and norepinephrine release in rat pineal gland. *Cell Mol. Neurobiol.* **7**: 309-315, 1987.

Vyas, S., Faucon Biguet, N., Mallet, J., Transcriptional and post-transcriptional regulation of tyrosine hydroxylase gene by protein kinase C. *EMBO J.* **9**:3707-3712, 1990.

Walleczek, J., Liburdy, R.P., Nonthermal 60 Hz sinusoidal magnetic-field exposure enhances 45Ca2+ uptake in rat thymocytes: dependence on mitogen activation. *FEBS Lett.* **271**(1-2):157-160, 1990.

Wertheimer, N., Leeper, E., Electrical wiring configuration and childhood cancer. *Am. J. Epidemiol.* **109**:273-284, 1979.

Wertheimer, N., Leeper, E., Fetal loss associated two seasonal sources of electromagnetic field exposure. *Am. J. Epidemiol.* **129**:220-224, 1989.

Welker, H.A., Semm, P., Willig, R.P., Commentz, J.C., Wiltschko, W., Vollrath, L., Effects of an artificial magnetic field on serotonin N-acetyltransferase activity and melatonin content of the rat pineal gland. *Exp. Brain Res.* **50**:426-432, 1983.

Wilson, B.W., Anderson, L.E., Hilton, I., Phillips, R.D., Chronic exposure to 60 Hz electric fields: Effects on pineal function in the rat. *Bioelectromagnetics* **2**:371-380, 1981.

Wilson, B.W., Chess, E.K., Anderson L.E., 60 Hz electric-field effects on pineal melatonin rhythms. *Bioelectromagnetics* **7**:239-242, 1986.

Wilson, B.W., Wright, C.W., Morris, J.E., Buschbom, R.L., Brown, D.P., Miller, D.L., Sommers-Flannigan, R., Anderson, L.E., Evidence for an effect of ELF electromagnetic fields on human pineal gland function. *J. Pineal Res.* **9**:259-269, 1990.