THE EFFECT OF NEUROPEPTIDE Y ON PROLIFERATION AND SYNAPTOGENESIS OF HIPPOCAMPAL CELLS

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ABSTRACT

Brain-derived neurotrophic factor has been implicated in learning and memory which are attributed to the cellular processes of neurogenesis and synaptogenesis. Additionally, the transcription of Neuropeptide Y (NPY) is induced by BDNF. We explored the link between NPY, neurogenesis, and synaptogenesis in the hopes of learning more about learning and memory. The first step was to explore neurogenesis and proliferation via ^{3}H-Thymidine incorporation assays. Then the effect of NPY on specific individual cell types was examined by labeling cells with BrdU and specific cell type markers. Real time reverse transcriptase PCR was used to find a potential mechanism by examining the transcription of a group of cell cycle genes. We studied synaptogenesis by observing the expression of synapse-associated molecules at the nucleic acid and protein level. Towards the end of our experiment, we were able to develop a protocol that allowed for the visualization of presynaptic and postsynaptic proteins, thus signifying the presence of synaptic sites. Our results confirmed our hypothesis that NPY would positively affect the two processes. Hippocampal cell cultures treated with NPY were found to show an increase in proliferation, and an increase in expression of cell cycle gene, cdc2. The results concerning synaptogenesis are somewhat preliminary, but promising. There was a trend of increase in the transcription of a group of synapse-associated proteins. By taking advantage of the immunocytochemistry of the synapse-associated proteins, we can elucidate NPY's effect on the formation of synapses. The extension of this data can help us understand how BDNF exerts its effects, and whether NPY is perhaps a mediator of its effects. This can be done through the use of pharmacological agents and NPY knock-out mice. The long-term goal of this work is to better understand the mechanisms and cellular processes of learning and memory so that effective treatments for neurodegenerative diseases can be developed.

INTRODUCTION

The neurotrophins are a family of neuropeptides that promote survival and differentiation of neurons. Brain-derived neurotrophic factor (BDNF) is a member of this group which exerts multiple electrophysiological and morphological effects associated with learning and memory. Previous studies have shown that chronic exposure to BDNF leads to increased neurogenesis. Additionally, neurotrophin treatment of hippocampal cells has also increased the number of excitatory and inhibitory synapses (Vicario-Abejon et al., 1998). The hippocampus is the site of the highest BDNF expression in the brain (Russo-Neustadt 2003). BDNF binds to trkB with affinity and exerts its effects (Lu 2003; Levine et al., 1995).

Previously in our lab, cDNA microarray analysis of BDNF-treated rodent hippocampal cells has revealed induction of many classes of genes including immediate early genes, neuropeptides, and synapse-associated genes (Alder et al., 2002). We wanted to focus on the effect of one of the neuropeptides whose transcription BDNF has been found to enhance. To elucidate the relationship between BDNF, neuropeptide Y, and learning and memory, we chiefly explored two main processes: neurogenesis and synaptogenesis. There have been previous studies that have worked with NPY that have shown that it may also have the some of the same effects as BDNF. One such study reported that Neuropeptide Y stimulates neuronal precursor

proliferation in the post-natal and adult dentate gyrus (Howell et al., 2003). We explored this process in embryonic rat hippocampus.

To determine NPY's effect on proliferation, we examined ^{3}H-thymidine and BrdU incorporation. To see if NPY exerted its effects on certain types of cells we used double labeling with specific cell type markers to detect any changes. To pinpoint a possible mechanism by which NPY exerts its effect we performed RT-PCR on a panel of cell cycle genes to identify any changes induced by NPY.

Additionally, previously in our lab, we found that exposure to the BDNF-induced peptides Neuropeptide Y and Nociceptin enhanced neurite outgrowth. Furthermore, other studies have shown that highly dynamic protein synthesis at synaptic endings after NPY treatment has been visualized (Russo-Neustadt 2003). To see whether this increased neurite outgrowth and dynamic protein synthesis were signs of the formation of synapses, we examined the transcription of some synapse-associated proteins as well as their protein level expression. At embryonic day 18, the majority of precursor cells are mitotically active and form synapses spontaneously (Vicario-Abejon et al., 1998). The expression of synapse-associated proteins, synaptophysin and postsynaptic density protein 95kD (PSD95) following NPY exposure, was determined by Western blot. Colocalization of synaptophysin and PSD95 was also examined by performing immunocytochemistry. Our findings suggest that NPY increases neural precursor cell proliferation and upregulates synapse-associated proteins. The extension of these results may be helpful in studying and treating neurodegenerative disorders where these processes of cell proliferation and synapse formation are disrupted.

MATERIAL AND METHODS

Hippocampal cell culture: Time-mated pregnant rats were sacrificed by CO_2 asphyxiation in accordance with institutional guidelines for care and use of animals. Fetal hippocampi were prepared as described (Thakker-Varia et al., 2001). They were dissected in phosphate-buffered saline solution and cells were mechanically dissociated in nutrient medium containing 7.5% fetal bovine serum and plated on poly-D-lysine-coated dishes with 350,000 cells per dish. The cells were maintained in serum-free medium for 10 to 14 div.

RNA Isolation.   Total cellular RNA was prepared from rat hippocampal cultures by the guanidine isothiocyanate method followed by CsCl gradient. RNA for the RT-PCR for the cell cycle genes was collected from cells treated from 0-48 hr with NPY at a final concentration of 1μM. RNA for the synapse-associated genes was harvested from 14 div cells, treated with 1μM NPY from 7 to 14 div, and retreated every 3 days. All negative control cultures were treated with vehicle. RNA used for all the subsequent experiments was treated with DNase to remove any DNA contamination. Quality control of RNA samples was performed by spectrophotometric analysis (260nm/280nm UV absorption ratio > 1.8).

Real time RT-PCR: cDNA was synthesized from independent pooled sources of total RNA using random primers. 100 μl of cDNA was prepared from 2 μg control or peptide-treated RNA using random primers and Superscript II reverse transcriptase (Invitrogen). 25 μl PCR reactions were then carried out using gene specific primers. Real time PCR analysis was performed using SYBR green master mix and primers for cdc2, cyclinE, and pCNA designed by Primer Express (ABI) for cell cycle experiment.   Synaptophysin, synaptophysin-4, synaptobrevin, synaptotagmin, PSD95 and AMPA primers were used for synapse-associated gene experiment. Duplicate wells were included for each condition and primer pair. Primers specific to the housekeeping gene, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH)* were used as an internal control. Data analysis was performed according to the protocol provided by Applied Biosystems.

^{3H}-Thymidine Incorporation Assay: 100,000 cells per well were plated in 24-well plates, and treated with NPY at concentrations of 10, 3, 1, 0.1, 0.03, and 0.01 μM in triplicate for 24 hours and 48 hours. ^{3}H-Thymidine was added and cells were incubated for 4 hours before being treated with trypsin-EDTA for 20 min. Cells were then harvested by a Skatron harvester and radioactivity (counts per minute) was recorded by a liquid scintillation counter.

BrdU double-labeling immunocytochemistry: Cells were treated with NPY at time of plating. 48 hr later, cells were then incubated with 10μM BrdU for 4 hours. Plates were fixed in cold 4% paraformaldehyde after 4hr or 6d and blocked in 30% Normal Goal Serum (NGS) in PBS/0.3% TX-100. They were treated separately with primary antibodies overnight: Nestin (1:250) from Hybridoma, TuJ1 (1:500) from Covance; GFAP (1:400) from Chemicon, and Tau (1:500) from Sigma. The dishes were then treated with 2° antibody 1:1000 AlexaFluor 594(red) goat anti-mouse or goat anti-rabbit and re-fixed with 4% paraformaldehyde. They were then treated with 1° antibody rat monoclonal for BrdU, then 2° antibody 1:1000 AlexaFluor 488(green) goat anti-rat. Plates were then coverslipped with Fluoromount G and staining was visualized on a Leica inverted fluorescence microscope at 40X. Six to eight fields per dish were counted, totaling approximately 200 cells.

Western blot: Treatment groups include 1μM NPY, 4μM of NOC, and 50ng/mL of BDNF for 7 –14 days. Protein was harvested in lysis buffer (20mM Tris, pH 8, 0.5% Triton X-100, 0.5% SDS, protease inhibitor tablet, 1mM PMSF, 1mM vanadate). Samples of equal amounts of protein were denatured in 4XSSB. Equal amounts were loaded onto a 10-20% gradient acrylamide gel. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) that were then blocked for 1 hr with 5% dry milk powder. Antibodies specific to PSD95 (1:2000) from Affinity Bioreagents Cat# MA1-046 and synaptophysin (1:2000) from Zymed were added overnight followed 2° antibody anti-mouse HRP-conjugated or anti-rabbit HRP-conjugated (1:5000) for 1 hr, respectively. Monoclonal GAPDH antibody (1:1000) from Biodesign Inc. was used for normalization. Quantitation was done by developing in chemiluminescent solution and analyzed using a GelDoc (Biorad) using local background substraction.

Immunocytochemistry for synapse associated proteins: Treatment groups include 1μM NPY, 4μM of NOC, and 50ng/mL of BDNF for 7 –14 days. Cells were rinsed twice in cold PBS. They were fixed in ice-cold methanol for 15 minutes at -20° C. Cells were washed three times with PBS for 5 min each. Cells were blocked for 1 hr at room temp in 2% NGS in PBS 0.1% Triton X-100. Primary antibodies to PSD95 (1:200) from Affinity Bioreagents Cat# MA1-045 and synaptophysin (1:200) from Zymed simultaneously in 2% NGS in PBS 0.1% Triton X-100 were applied overnight. The next day, the cells were washed three times with PBS for 10 min each. Secondary antibodies AlexaFluor 488 green goat anti-rabbit (1:1000) and AlexaFluor 594 red goat anti-mouse (1:200) in PBS 0.1% Triton X-100 were added for 1 hr. The cells were finally washed three times with PBS for 10 min each. The bottoms of plates were then cut with a hot scalpel blade and coverslipped with Fluromount-G. Staining was visualized on a Leica inverted fluorescence microscope at 60X with oil immersion.

RESULTS

I. Neurogenesis

A. NPY Enhances ^{3}H-Thymidine Incorporation in Hippocampal Cultures

We examined the potential effect that NPY may have on cell proliferation via a ^{3}H-Thymidine incorporation assay (Figure 1). The cells showed a significant increase for all NPY concentrations.
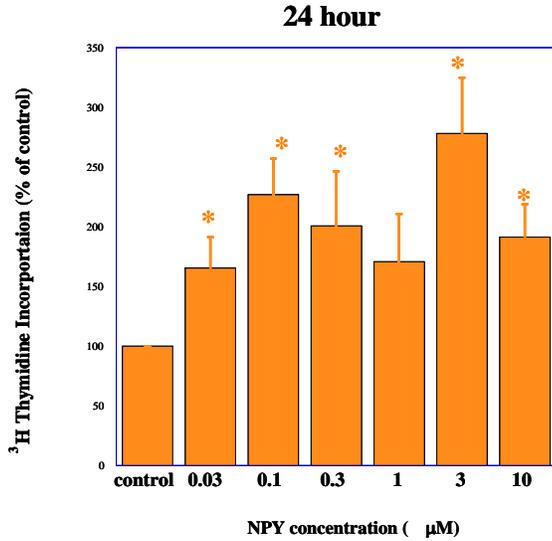
Figure 1. NPY Enhances ^{3}H-Thymidine at 24 hour. 24 hour cells treated with varying concentrations of NPY at time of plating were harvested after a 4 hr incubation period with ^{3}H-Thymidine. Each trial was done in triplicate with the indicated concentration of NPY. A concentration dependent increase in ^{3}H-Thymidine incorporation was seen after 24 hour NPY treatment. A statistical increase is seen even at 0.03 M NPY which persists up to 10 µM (p < 0.05, ANOVA). This indicates the proliferative effects of NPY.
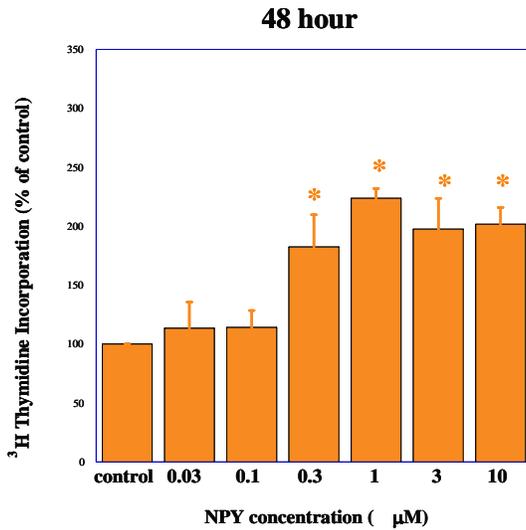
Figure 2. NPY Enhances ^{3}H-Thymidine Incorporation In Hippocampal Cultures at 48 hour. 48 hour cells treated with varying concentrations of NPY at time of plating were harvested after a 4 hr incubation period with ^{3}H-Thymidine. Each trial was done in triplicate with the indicated concentration of NPY. A similar increase in ^{3}H-thymidine incorporation was seen however an approximate 2-fold increase was seen at 0.3µM and continued to be significant up to 10mM (p < 0.05, ANOVA).

The same incorporation assay was done at a later time point (Figure 2). Again, the data suggest the same finding.

B. NPY Increases BrdU+ Cells In Vitro
Further to examine cell division and proliferation at a cellular level, the effect of NPY on BrdU incorporation was examined. As shown by Figure 3, there was an approximate 2-fold increase in the percentage of cells that were BrdU positive after both 48 hours and 6 days of differentiation.
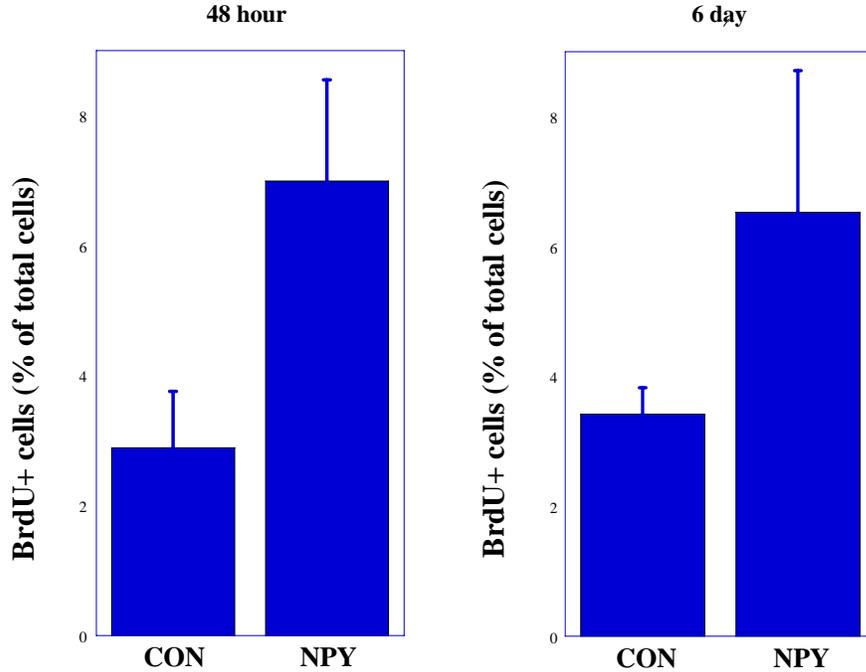
Figure 3. NPY Increases BrdU+ Cells In Vitro. Hippocampal cells were treated with control and NPY at time of plating. 48hr later, BrdU was added to cells and were fixed 4hr or 6d later. The bars represent the average percentage of total cells that were BrdU+ for all the trials performed. Each time period had 4 sets of control and NPY dishes per trial. Approximately 200 cells were counted on each dish, noting the number that were BrdU+. In total, for each bar, approximately 2400 cells were counted. Error bars diagram the standard error (n = 3).

C. BrdU+ Cells Express Markers of Neural Precursors After 6 days
To determine the identity of the newly dividing cells, we immunostained with various different cell-specific markers. Figure 4 shows a representative image of neurons immunolabeled with BrdU in the nucleus and cytoskeletal proteins labeled by specific cell markers. Table I shows the quantification.
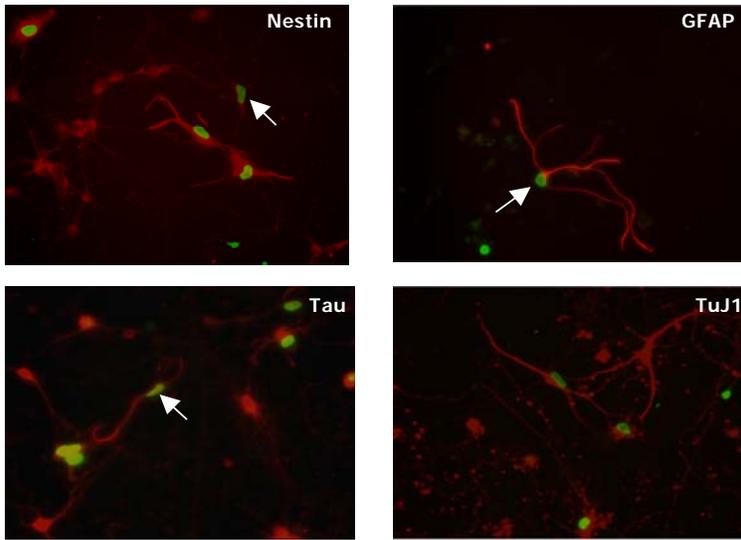
Figure 4. 6day Double-labeled cells. Cells were treated with NPY at time of plating. 48hr later BrdU was added and cells were fixed in 4% paraformaldehyde 6d later. They were treated immunolabeled for BrdU (green) and cell specific markers (red). Markers used were Nestin, a neural precursor marker; GFAP (glial fibrillary acidic protein); Tau (a cytoskeletal element of mature neuron) and TuJ1 (class III β-tubulin). Arrows indicate to double labeled cells, n = 3.

	48 hour		6 day	
	CON	NPY	CON	NPY
Nestin	77.78	75.46	32.38	42.59
GFAP	28.70	12.96	34.44	24.91
Tau	20.00	42.42	16.19	36.11
TuJ1	5.56	13.89	0.00	4.00

Table 1: % BrdU+ Cells Expressing Markers
The table shows the quantification of the staining. At 48 hr, majority of cells are precursor molecules as indicated by nestin-labeled cells. NPY treatment of GFAP showed a decrease at 48hr and 6 d. NPY increased the percentage of cells that were Tau and TuJ1 at 48hr and 6 d, n = 3.

D. Cdc2, A Cell Cycle Gene, Is Upregulated by NPY
To pinpoint a possible mechanism by which NPY exerts it effects, RT-PCR was performed on a panel of cell cycle genes: cdc2, cyclinE, and pCNA (Figure 5). Hippocampal cells were treated with NPY for 48 hr. All samples were first normalized to GAPDH and then represented as a ratio of control (n = 3).
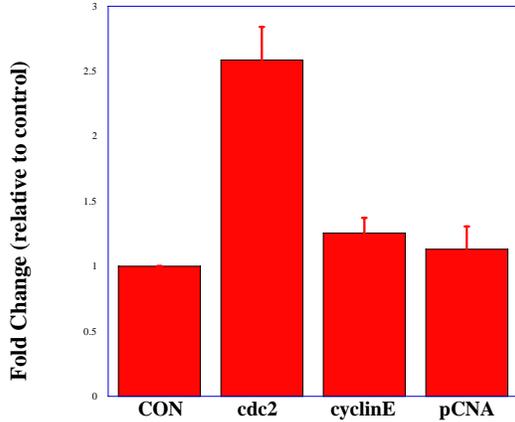
Figure 5. Cdc2 Is Upregulated by NPY. *Bars represent the fold change relative to control after NPY treatment for 48 hours. MRNA for cdc2 was increased approximately 2.5-fold; this increase was significant. Standard error is denoted by error bars ($p < 0.05$, ANOVA), n = 3*

II. Synaptogenesis
A. Expression of Synapse-Associated Genes Are Increased by NPY
To determine the effect of NPY on synaptogenesis, we examined the transcription levels of a group of presynaptic (synaptophysin, synaptophysin-4, synaptotagmin, and synaptobrevin) and postsynaptic (PSD96 and AMPA) proteins (see Figure 6). The trend however exists that PSD95, AMPA, synapotagmin, and synaptobrevin showed a marked increased in their transcription after being treated with NPY.
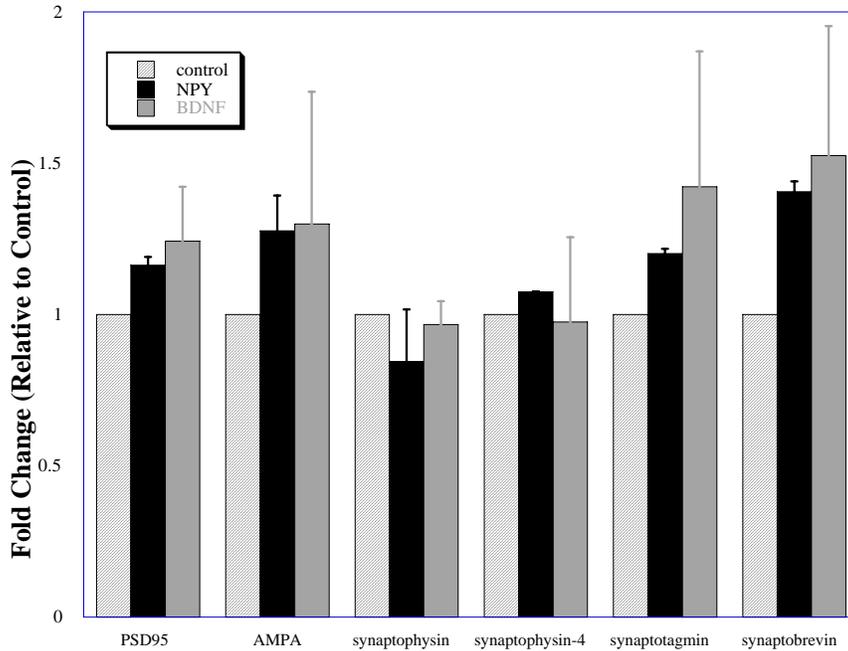
Figure 6. NPY increases transcription of some synapse-associated genes after 7-14 div treatment. Bars represent the fold change relative to control after 7-14 div NPY treatment, n = 2.

B. Expression of synapse associated proteins are enhanced by NPY

We examined the possible change in synapse-associated markers at the protein level via Western Blot. Cells were treated with NPY, Noc, BDNF for 7-14 div (see Figure 7). PSD95, a postsynaptic protein was shown to increase by NPY. BDNF was used as positive control. Synaptophysin, a presynaptic protein, did not show any change. GAPDH was used to normalize protein levels and data are expressed as fold of expression in untreated sister cultures.
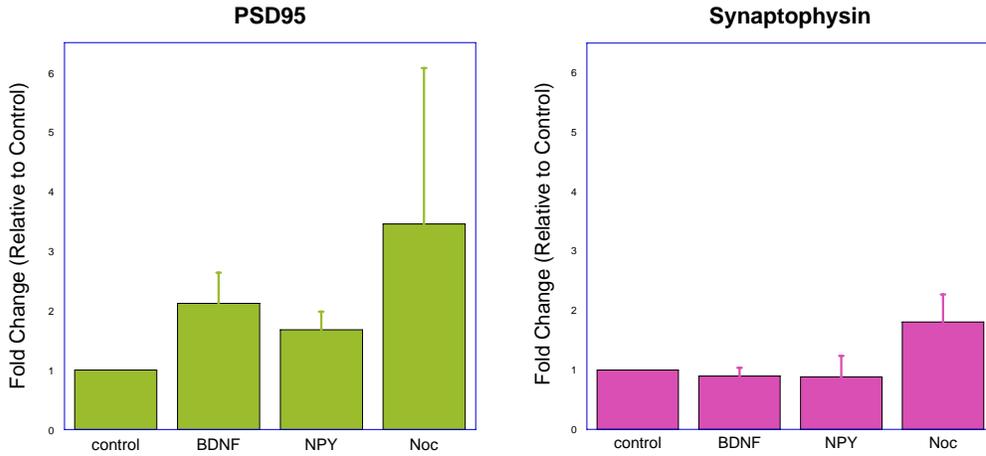
Figure 7. Expression of Synapse-associated Proteins are Enhanced by NPY. Graphs show the fold change in each of the designated proteins when treated with the specified treatment. Bars represent the average change in the amount of the protein with respect to the untreated plates, n = 4.
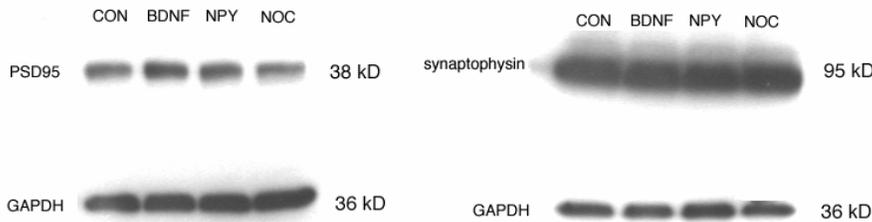
Figure 8. Western Blot of PSD95 and Synaptophysin. Image shows representative western blot gels for the 7-14 div treatment of NPY, Noc, BDNF.

C. Visualization of synapses
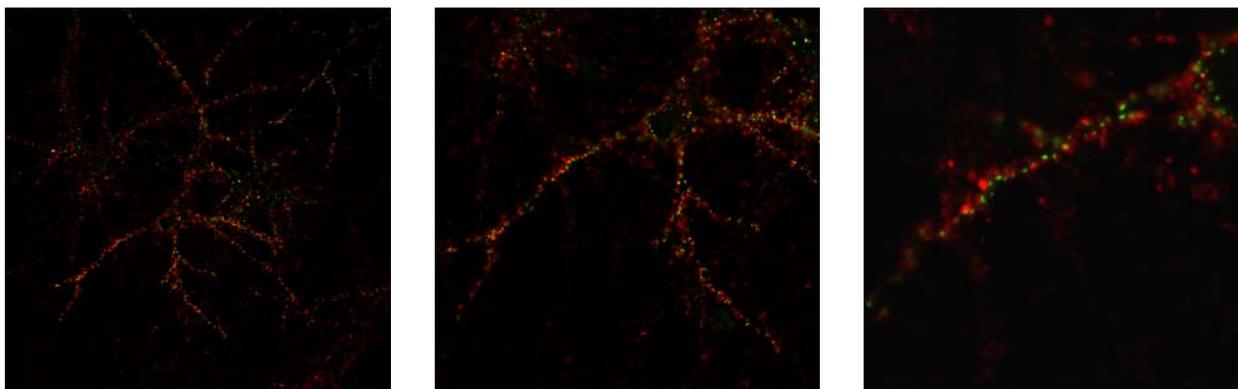
Figure 9. These are representative images from confocal microscope that show a cell labeled for the postsynaptic and presynaptic markers. By counting the number of opposing red (PSD95) and green (synaptophysin) sites, we can quantitate the number of synapses.

DISCUSSION

In summary, our results support the possibility NPY may mediate BDNF's effects on neurogenesis and synaptogenesis. In this study, we demonstrated that NPY has a positive effect on proliferation of cells. The data from the ^{3}H-thymidine incorporation assays showed that cells treated with the higher concentrations had a significantly higher percentage of ^{3}H-thymidine incorporation compared to the control. There can be some debate as to whether this increase in incorporation resulted from proliferative effect attributed to a change in the number of surviving cells. The BrdU staining confirmed that the effect was proliferative because the number of cells per dish did not change for the cultures treated with NPY. NPY induced a two-fold increase in cell number as exhibited by the ^{3}H-thymidine incorporation assay and also the BrdU staining experiment, thus signifying that NPY has a positive proliferative effect on neurogenesis.

Of the cells that we found to be BrdU-positive, many of them were precursor cells as shown by the number that were nestin-positive.   At 48 hr, we saw that approximately 75% of cells that were BrdU-positive were nestin-positive also. There was no change in cells that were treated with NPY compared to control at that time point, perhaps because the cells had not enough time to feel the effect of NPY after 48 hr. At 6 days, we did see an increase, but it was not enough to be significant. Also, the overall percentage of cells that were nestin-positive decreased. This accords with the fact that many of the cells may have differentiated within 6 days, and so many of them were no longer precursor cells at 6 days. NPY did show an increase in the percentage of Tau and TuJ1 labeled cells compared to control. Tau and TuJ1 are cytoskeletal elements of mature neurons. This implies that NPY may induce neuronal precursors to divide and then differentiate. These results are confirmed by a previous study that showed that NPY had a proliferative effect on both nestin-positive and β-tubulin-positive cells (Howell et. al 2003). The effect of NPY on GFAP labeled cells showed a decrease on the number of GFAP-positive cells. There could be an unexplored effect of NPY on specifically glial cells that has not yet been discovered. However, one has to be careful with the interpretation of the BrdU labeling experiment as there was a relatively small population size. The changes observed in the data could be attributed to simple errors in counting. Further repetitions could give more reliable data and lend it to more substantial interpretations.

A panel of cell cycle genes was studied to pinpoint a possible mechanism by which NPY causes its effects. NPY increase transcription of the cell cycle gene cdc2 2.5-fold. The protein encoded by this cdc2 is a member of the serine/threonine protein kinase family. This protein is a catalytic subunit of the highly conserved protein kinase complex known as M-phase promoting

factor (MPF), which is essential for G1/S and G2/M phase transitions of eukaryotic cell cycle. Further study into the mechanisms by which NPY affects cdc2 specifically can lend additional insight to the exact effect of NPY.

The work done to examine NPY's effect on synaptogenesis was preliminary. We assumed that by detecting the change in transcription levels of certain synapse-associated proteins, we could infer whether synaptogenesis is changing. We were able to see changes in certain presynaptic and postsynaptic proteins as shown by the RT-PCR results in Figure 6. However, we were not able to detect a change in synaptophysin at nucleic aid or protein level. This may have resulted from some inherent problem with the Western blot method, or antibody, as previous studies have used synaptophysin before and have shown better results. We could also look at different presynaptic markers that showed a more favorable increase, such as synaptotagmin or synaptobrevin and look at additional time points.

Still by eventually quantitating the colocalization of PSD95 and synaptophysin, we can answer whether NPY increases the number of synapses formed. Electron microscopy would be the optimal way to determine if a synapse has truly formed. However, this method of counting the adjacent presence of a presynaptic and postsynaptic protein is also reliable. Previous work performed to reveal the time course of synaptogenesis has established that presynaptic vesicle components (such as synaptophysin) begin to accumulate at presynaptic sites immediately after contact. The clustering of postsynaptic markers such as PSD95 follows the functional and morphological differentiation of presynaptic structures (Cohen-Cory 2002). Thus, it follows that given enough time, the simultaneous imaging of pre- and postsynaptic components is indicative of a synapse. By using the data that is obtained from the immunocytochemistry, we can gain a better idea of the effect of NPY on synapse formation.

Previous studies have shown that there is a decrease in proliferation in Y1 knockout animals, which is one NPY receptor (Howell et al., 2003). We can further elucidate the effects of NPY by doing *in vivo* studies. The main goal can be to see whether NPY mediates BDNF's effects. Specifically, does BDNF exert its effects via NPY. This can be done by using NPY knock-out mice and studying how the processes of neurogenesis and synaptogenesis are affected.

As the hippocampus is known as the gateway to learning and memory, studying the effects of NPY in the hippocampus on neurogenesis and synaptogenesis can facilitate the development of more efficacious and specific treatments of neurodegenerative diseases.

ACKNOWLEDGEMENTS

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