Transcriptional profiling of brain-derived-neurotrophic factor-induced neuronal plasticity: a novel role for nociceptins in hippocampal neurite outgrowth

Transcriptional Profiling of Brain-Derived Neurotrophic Factor-Induced Neuronal Plasticity: A Novel Role for Nociceptin in Hippocampal Neurite Outgrowth

Running title: BDNF-induced nociceptin enhances neurites

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ABSTRACT

BDNF exhibits a sequence of actions on neurons ranging from acute enhancement of transmission to long-term promotion of neurite outgrowth and synaptogenesis associated with learning and memory. The manifold effects of BDNF on neuronal modifications may be mediated by genomic alterations. We previously found that BDNF treatment acutely increases transcription of the synaptic vesicle protein, *Rab3A*, required for trophin-induced synaptic plasticity, as well as the peptide, *VGF*, which increases during learning (Thakker-Varia et al., 2001; Alder et al., 2003). To elucidate comprehensive transcriptional programs associated with short and long-term BDNF exposure, we now examine mRNA abundance and complexity using Affymetrix GeneChips in cultured hippocampal neurons. Consistent with the modulation of synaptic plasticity, BDNF treatment (3-6 hr) induced mRNAs encoding the synapse-associated proteins *synaptojanin 2, neuronal pentraxin 1, septin 9* and *ryanodine receptor 2.* BDNF also induced expression of mRNAs encoding neuropeptides (6-12 hr), including *prepronociceptin, neuropeptide Y* and *secretogranin.* To determine whether these neuropeptides induced by BDNF mediate neuronal development, we examined their effects on hippocampal neurons. The four mature peptides derived from post-translational processing of the ppNociceptin propeptide induced the expression of several *immediate early genes* in hippocampal cultures, indicating neuronal activation. To examine the significance of activation, the effects of nociceptin (orphanin FQ) and nocistatin on neurite outgrowth were examined. Quantitative morphometric analysis revealed that nociceptin significantly increased both average neurite length and average number of neurites per neuron, while nocistatin had no

effect on these parameters. These results reveal a novel role for nociceptin and suggest that these neuropeptide systems may contribute to the regulation of neuronal function by BDNF.

Keywords: BDNF, nociceptin, neurite outgrowth, microarray, synaptic plasticity

INTRODUCTION

Neurotrophins exert multiple neuronal actions ranging from rapid effects on activity to long-lasting alterations of synaptic organization associated with learning and memory. Within milliseconds of BDNF exposure, membrane potential is altered, revealing excitatory properties of BDNF (Kafitz et al., 1999). A subsequent phase of BDNF actions, increased synaptic charge, occurs within minutes and involves both pre- and postsynaptic processes (Levine et al., 1995; Lessmann and Heumann, 1998; Li et al., 1998; Schinder et al., 2000; Tyler and Pozzo-Miller, 2001). BDNF also plays critical roles in the early and late phases of long-term potentiation (LTP) which requires transcription and protein synthesis over hours (Frey et al., 1996; Nguyen and Kandel, 1997). Finally, the trophin mediates synaptic scaling and intrinsic excitability of neurons over days (Rutherford et al., 1998). In addition to these electrophysiological changes, BDNF modulates morphologic synapse development over the same time-frame (Cohen-Cory and Fraser, 1995; Patel and McNamara, 1995; Wang et al., 1995; Labelle and Leclerc, 2000). These alterations include enhanced neurite outgrowth which may result in synaptogenesis associated with learning and memory (Geinisman, 2000; Muller et al., 2002; Shors, 2004). It remains to be revealed how a single trophic molecule can exert diverse effects spanning a temporal continuum. It is likely that molecules induced by BDNF mediate downstream actions. Therefore, by elucidating BDNF-induced changes in gene expression, we may gain insight into the molecular pathways leading to enhanced synaptic strengthening by BDNF.

Traditionally, a candidate gene approach has been employed to delineate molecular pathways in neuronal systems. However, this method is limited by bias and the number of genes that can be interrogated. Large-scale transcriptional profiling approaches (e.g. cDNA or oligonulceotide microarrays, differential display, SAGE) have emerged and proven useful in revealing novel roles for known genes in biological systems (Angelastro et al., 2000; Huh et al., 2000), as well driving the discovery of unknown genes (Wen et al., 2002; Farkas et al., 2004). Although transcriptional profiling has been performed following LTP (Tang et al., 2001; Thompson et al., 2003) and several learning paradigms (Luo et al., 2001; Donahue et al., 2002; D'Agata and Cavallaro, 2003; Leil et al., 2003), the full spectrum of transcriptional changes induced specifically by BDNF in the hippocampus has not been investigated.

We have previously identified important roles for BDNF-regulated genes using basic profiling techniques combined with functional studies. A critical role for synaptic proteins in BDNF-induced plasticity was identified using PCR-based differential display of hippocampal cultures exposed to BDNF. Analysis revealed increased transcription and translation of the GTP-binding protein *Rab3A*, which was shown to be required for an early phase of BDNF-enhanced synaptic activity (Thakker-Varia et al., 2001). In a separate study, we demonstrated induction of the neuropeptide VGF by BDNF using a cDNA microarray. A novel role for *VGF* in augmenting synaptic plasticity was discovered, suggesting that neuropeptides and neurotrophins act in concert to modulate synaptic function (Alder et al., 2003). Collectively, these observations suggest that

transcriptional profiling can help reveal molecular mechanisms underlying the BNDF effects on synaptic strengthening, which have been associated with learning and memory.

To elucidate genome-wide events spanning the temporal continuum of BDNF actions, we have now performed comprehensive profiling of 9,109 unique mRNA transcripts utilizing Affymetrix oligonucleotide (GeneChip) arrays. Using this platform, profiles of RNA abundance and complexity were generated by interrogating RNA samples from hippocampal cultures exposed to BDNF at numerous time points along a 48-hour time course. Comparative analysis of these profiles revealed the induction of mRNAs encoding many synapse-associated proteins and neuropeptides by BDNF, elaborating upon our earlier studies. Among the most robustly induced neuropeptide mRNAs was that encoding ppNociceptin, which has not previously been implicated in BDNF plasticity. A peptide derivative, nociceptin, induced *IEG* expression and promoted neurite outgrowth *in vitro*. These findings link neuropeptides with neurotrophin signaling involved in the structural dynamics of developing neurites. Our studies are developing a molecular timetable of BDNF actions on hippocampal cells with the long-term goal of understanding trophic regulation of synaptic plasticity and synaptogenesis, which is associated with learning and memory.

METHODS

Cell Culture Preparation. Time-mated pregnant mice were killed by CO_2 asphyxiation in accordance with institutional guidelines for care and use of animals. Fetuses were

removed by caesarean section and transferred to a sterile petri dish with phosphate-buffered saline. Fetal hippocampi were dissected from surrounding brain tissue and meninges were completely removed. Low-density cultures of dissociated embryonic day 16 mouse hippocampi (C57BL/6, from Hilltop Laboratories, Scottsdale, PA) were prepared as described (Thakker-Varia et al., 2001). Briefly, pooled tissue from each litter was mechanically dissociated in nutrient medium containing 7.5% fetal bovine serum and plated on poly-D-lysine-coated culture dishes at 350,000 cells/dish. Cultures were maintained in serum-free medium (Thakker-Varia et al., 2001) for 10 days. A single, continuous treatment with 50 ng/ml BDNF was used for all experiments because that dose is within the physiologic range and was effective in previous electrophysiological and transcriptional profiling studies (Thakker-Varia et al., 2001; Alder et al., 2003).

RNA Isolation. Total cellular RNA was prepared from 10 days *in vitro* neuronal cultures treated with either a single continuous BDNF (50 ng/ml) or vehicle (water) for 20 min, 1, 3, 6, 12, 24 and 48 hours (n = 10 culture dishes/time point) in 3 separate experiments by the guanidine isothiocyanate method followed by cesium chloride gradient (Chomczynski and Sacchi, 1987). RNA used for all experiments was further purified using RNeasy Mini-Spin Columns (Qiagen, Valencia, CA) incorporating a DNase I (Qiagen) treatment step to remove any genomic DNA carryover.

Oligonucleotide Microarray (GeneChip) Experiments. Double-stranded cDNA was synthesized from the 3 separate samples for each time point of 10 μg total RNA using the SuperScript System (Invitrogen, Carlsbad, CA). Briefly, the RNA was mixed with 100

pmol oligonucleotide GGCCATGGAATTGTAATACGACTCACTATAGGGAGGCGG $(dT)^{24}$ in 20 µl water, annealed at 70 °C for 10 min, and quick-chilled. Buffer, dithiothreitol, and dNTP mix were then added and incubated at 37 °C for 2 min. SuperScript II reverse transcriptase was added and the 50°C incubation was continued for 60 min. Second-strand synthesis was performed by adding reaction buffer, dNTPs (200 M), DNA ligase (10 U), DNA polymerase (40 U), ribonuclease H (2 U), and water (to a final volume of 150 µl), and the reaction was incubated for 2 h at 16°C. This was followed by addition of 10 U T4 DNA polymerase and incubation at 16°C for 5 min. The cDNA was purified by phenol/chloroform extraction, precipitated, and transcribed in vitro using T7 RNA polymerase. Biotinylated cRNA was generated using the BioArray HighYield RNA Transcription Kit (Enzo Diagnostics, Inc., Farmingdale, NY). The cRNA was purified by RNeasy mini-spin columns and fragmented by incubation in 40 mM Tris (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate buffer at 94°C for 35 min. Fragmented cRNA was hybridized to the mouse U74Av2 GeneChip® (Affymetrix, Santa Clara, CA) at 45°C for 18 hours as recommended by the manufacturer. Triplicate sample of cRNA from each time point was hybridized to individual chips (n = 3 x 7 time points = 21 chips each for BDNF and control). Hybridized chips were washed and stained using Affymetrix Fluidics Station 400 and EukGE-WS1 Standard Format as recommended by the manufacturer. The staining was performed using streptavidin–phycoerythrin conjugate (SAPE; Molecular Probes, Eugene, OR), followed by biotinylated antibody against streptavidin (Vector Laboratories, Burlingame, CA), and then SAPE. The chips were scanned using a Hewlett-Packard GeneArray Scanner and analyzed using Affymetrix MAS 5.0 software.

Hybridization intensities were normalized using a method featuring a pool of 11 biotin-labeled cRNA control transcripts, derived by *in vitro* transcription of 11 cloned Bacillus subtilis genes, which are spiked into each hybridization experiment. This normalization method has been described in detail previously (Hill et al., 2001). The 5'/3' ratio for *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* and for *beta-actin* ranged from 0.8 to 1.1.

Transcriptional Profiling Analysis. Expression data was analyzed using Genesis 2.0 (GeneLogic, Gaithersburg, MD) comparative analysis software tool. Raw expression values from Genechip experiments were calculated using the MAS 5.0 algorithm, and normalized to spike-ins to generate absolute expression values. For each gene fragment, the tool calculates the ratio of the geometric means of expression intensities in untreated control samples and all time points following BDNF treatment as the fold change. Confidence limits and *P*-values were calculated using a two-sided Welch modified two-sample *t*-test on the difference of the means of the logs of the intensities. For the purposes of this study, differential expression was considered real when the fold change (delta) obtained from any given probeset met all three of the following criteria: 1) expression level for treated sample versus its time-matched control was greater than or equal to 1.5 fold, signed in either direction (up- or down-regulated), 2) the p-value (t-test) of the delta was ≤ 0.05, $n = 3$) a minimum of 66% present calls (detection p-value < 0.05) among samples making up the higher expression level of a fold change.

Quantatative RT-PCR Analysis (RT-qPCR). Quality control evaluation of RNA samples was performed by spectrophotometric analysis (260nm/280nm UV absorption ratio > 1.8) and visual inspection of rRNA integrity (28S, 18S) using 2100 BioAnalyzer (Agilent, Palo Alto, CA). Reverse transcription was performed using the SuperScript™ Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) with 1 μg of total RNA and oligo(dT)$^{12\text{-}18}$. 1/100th of each cDNA synthesis reaction was used to template each qPCR reaction. Final concentration of qPCR components: 30 U/ml (1.5 U total) Platinum® *Taq* DNA polymerase, 20 mM Tris-HCL (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 200 μM each (dGTP, dATP, dCTP), 400 μM dUTP, 20 U/ml (1.0 U total) uracil DNA glycosylase, 500 nM each of forward and reverse primer, and 200 nM of fluorogenic probe. Gene-specific qPCR primer/probe sequences for mouse *ppNociceptin, NPY, c-fos,* and *Arc* were designed from reference sequences in GenBank using Primer Express® 2.0 (Applied Biosystems, Foster City, CA) and are shown in Table 1. Gene-specific primers and 5'-FAM/3'-BHQ-1 labeled probes were synthesized by Biosearch Technologies, Novato, CA. Predesigned primers and TaqMan® probes were used to assay mouse *Egr-2* (Cat# Mm00456650, Applied Biosystems) and *GAPDH* expression (Applied Biosystems). qPCR was performed on a ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). The fluorescent signal from each well was first normalized using data generated from a standard concentration curve of murine genomic DNA, as previously described (Macdonald et al., 2001). Relative expression for each target was then calculated by dividing the normalized expression level for the target by the normalized expression value of the internal passive reference (GAPDH). From this

relative expression level, fold change calculations were made between treated and untreated samples.

IEG Induction by Nociceptin Peptides. Mouse hippocampal cultures (10 days *in vitro,* DIV) were treated with 1µM nociceptin, nocistatin, PNP-4, PNP-5 (Phoenix Pharmaceuticals, Belmont, CA) for 1 hr. 100 µl of cDNA was prepared from 2 µg of RNA from control or peptide-treated samples using random primers and Superscript II reverse transcriptase (Invitrogen). 25 µl PCR reactions were then carried out using gene specific primers [*c-fos, early growth response gene 2 (Egr-2), and activity-regulated cytoskeletal associated protein (Arc)*] designed by Primer Express software and TaqMan MGB probes (Table 1). Duplicate wells were included for each condition and primer pair. Primers specific to the housekeeping gene, *GAPDH* were used as an internal control. Data analysis was performed according to the protocol provided by Applied Biosystems.

Quantitative Morphometry. Hippocampal cells were plated at 9,000 cells per well in a 96 well Biocoat plate (Becton Dickinson, Bedford, MA) and treated immediately with peptides at varying concentrations from 0.1 to 10 µM final concentration. Cells were fixed after 24 hr with 4% paraformaldehyde for 30 min. Neurons were immunostained with an antibody specific for the neuronal Class III ß-tubulin (TuJ1) (1:500, Covance, Berkeley, CA) followed by Alexa Fluor 488 goat anti-mouse IgG (1:1000, Molecular Probes, Eugene, OR) and counterstained with the membrane permeable nucleic acid stain, Hoechst 33342 (1:1000, Molecular Probes, Eugene, OR). Hippocampal cell

cultures, fixed and stained as described above, were imaged using an ArrayScanII® HCS Reader (Cellomics, Pittsburgh, PA). Duplicate digital images were captured from a total of 10 fields per well using two separate fluorescent filters. Channel 1 (blue) captured Hoechst 33342 staining, while Channel 2 (green) captured FITC labeling of neuronal tubulin (TuJ1). Analysis of cell morphometry from ArrayScanII® HCS images was performed using the algorithms of the Extended Neurite Outgrowth (ENO) BioApplication software (Cellomics, Pittsburgh, PA). A valid neuron was defined as TuJ1-positive cell body that contains a valid nucleus (Hoechst 33342 positive). From this, the overall percentage of cells per field counted as a valid neuron (% valid neuron) was calculated. Peptide treatment effects on neurite outgrowth in valid neurons were captured by five measurements of neurite morphometry: 1) % valid neuron with neurite is the percentage of valid neurons exhibiting at least one neurite; 2) total neurite length is the mean of the calculated total length of all neurites per valid neuron; 3) average neurite length is the mean of the average length of individual neurites per valid neuron; 4) average neurite # is the mean of the average number of neurites per valid neuron; 5) average branch point # is the average number of branch points per valid neuron. If the criterion for neurite inclusion was set at >12.6 µm (calculated average diameter of a valid neuron), the percentage of valid neurons possessing at least one valid neurite as well as peptide effects on neuritogenic parameters displayed similar trends (data not shown).

RESULTS

Transcriptional Profiling

Profiles of mRNA transcript abundance and complexity were generated for hippocampal neuronal cultures (10 DIV) following a single, continuous treatment with BDNF (50 ng/ml), over a time course comprised of seven time points (20 min, 1, 3, 6, 12, 24, 48 hrs) along with time-matched, vehicle-treated controls using 3 chips for each time point. 50 ng/ml BDNF was used because that dose is within the physiologic range and was effective in previous electrophysiological and transcriptional profiling studies (Thakker-Varia et al., 2001; Alder et al., 2003). In each sample, expression levels for 9,109 unique mRNAs were interrogated using the 12,422 probesets of the MG_U74Av2 Genechip because it is the most highly annotated rat chip. Complete annotation of these probesets, and the mRNAs they interrogate is publically available from Affymetrix (http://www.affymetrix.com/analysis/index.affx). The discrepancy between mRNAs and probesets is accounted for by the fact that numerous transcripts have multiple associated probesets.

Comparative analysis of expression profiles for untreated versus BDNF samples identified 306 transcripts exhibiting significant differential expression in at least one of the seven time points using criteria described in Methods section. The majority of differentially expressed transcripts (201 transcripts, 67%) were cases of transcriptional induction, indicating a general stimulatory effect of BDNF. Those genes that were downregulated by BDNF appeared less relevant to the process of synaptic enhancement and were not pursued for functional studies. Based on current annotation, eighty-five percent of differentially expressed transcripts encode proteins of known function, while the remainder represents expressed sequence tags (ESTs), which have no known

function. A complete list of all differentially expressed mRNAs from this study is provided in the Supplemental Table 1 (available at www.jneurosci.org).

Classes of Induced Genes: IEGs, Synapse-Associated Molecules and Neuropeptides

Consistent with previous observations, results of this study indicate that BDNF treatment rapidly and transiently activates a complex program of *IEG* expression in hippocampal cells, as evidenced by the characteristic and robust induction of *IEG* mRNAs during the first hour following BDNF treatment (Figure 1). The *IEGs* featured in the BNDF response here include members of the *Fos* (*c-fos*, *fosB*), *Jun* (*jun*, *junB*), and *Krox* (*Egr-1, Egr-2*) families of transcription factors, as well as members of the *nuclear hormone receptor* family (*NR4A1 and NR4A2*). Evidence has clearly implicated *IEG* induction in the modulation of various behavioral, sensory and pathological conditions associated with memory formation (Clayton, 2000). Based on this body of evidence, the induction of *IEGs* by BDNF in this study indicate that the immediate effects of BDNF on transcription in neurons overlap with those observed for other stimuli linked to learning and memory.

Temporally downstream, BDNF activates numerous functionally related groups of transcripts (Figure 2). Examples of these families include: A) membrane bound receptors/transporters including G protein coupled receptors (GPCR), B) molecules involved in intracellular signal transduction such as kinases and protein phosphatases, and C) structural molecules such as extracellular matrix related proteins, cytoskeletal molecules and metabolic enzymes (Figure 2). These genes cluster in functional

categories and exhibit similar temporal profiles within the families, suggesting that they work in concert to mediate BDNF effects. There are also a few downregulated genes that fall into the above categories. Specifically, the RNA levels of A) *ß3 subunit of the voltage dependent Ca^{2+} channel* and *rho GTPase activating protein 5*, B) *protein kinase inhibitor alpha* and the *regulatory subunit B of protein phosphatase 2*, as well as C) *cytoplasmic ß actin* and *myosin heavy chain* are all decreased by BDNF treatment (see Supplemental Table 2). The downregulated genes may themselves lend insight into the mechanism of BDNF action.

Three to 6 hours after BDNF treatment, an increase in expression of molecules localized to the synapse was evident (Figure 3). *Neuronal pentraxin (NPTX1)* and *stathmin (STMN4*), both markers of neurite sprouting (Ujike et al., 2002), were the most robustly induced by BDNF. A number of other transcripts induced by BDNF encode proteins that are synapse-associated. For example, *ryanodine receptor 2 (RYR2*) may regulate Ca^{2+} levels at presynaptic terminals (Bouchard et al., 2003). *Seven in absentia 2 (SIAH2)* and *synaptojanin 2 (SYNJ2)* may be involved in synaptic vesicle formation (Cremona et al., 1999; Luthi et al., 2001; Wheeler et al., 2002) and *septin 9 (SETP9*) (Beites et al., 1999) may play a role in exocytosis. *Brain-specific angiogenesis inhibitor 1 (BAI2*) may regulate postsynaptic receptor clustering (Lim et al., 2002). Finally *sialyltransferase 8c (SIAT8c*) is associated with neuronal cell adhesion molecule (NCAM) and may be involved in synapse stabilization (Dey et al., 2000). These data suggest that this group of proteins may play roles in BDNF's effect on synapse development and function.

Additionally, neuropeptide precursors featured prominently among the functionally related groups of BDNF-responsive genes (Figure 4). Induction of several of the genes peaked between 6 and 12 hours following BDNF treatment, whereas the other neuropeptides displayed different patterns of temporal expression. Among this group were mRNAs encoding *ppNociceptin, NPY, BDNF, somatostatin (SST), substance P (SP), amphiregulin (AREG), CXC chemokine ligand 12 (CXC12)* and *SCG2*. This is the first documentation of the induction of several of these neuropeptides by BDNF, including *ppNociceptin, AREG and CXC12*. *ppNociceptin* is translated into a precursor protein that is processed into four smaller peptides (nociceptin, nocistatin, PNP-4 and PNP-5) (Okuda-Ashitaka et al., 1998). Nociceptin is an endogenous ligand for the opioid receptor-like 1 (ORL-1) receptor and has been implicated in a variety of CNS mediated-behaviors including pain modulation, anxiety, memory, food intake and drug addiction (Zaveri, 2003). Nocistatin exhibits effects on pain transmission that are opposite, and in some cases antagonistic, to those observed for nociceptin without demonstrating any affinity for ORL1 (Okuda-Ashitaka et al., 1998). There were several genes related to neuropeptides which were downregulated including the ORL-1 and NPY receptors (see Supplemental Table 2). To further elucidate the roles of nociceptin, nocistatin and NPY, we explored the effects of these peptides on hippocampal neurons.

Confirmation of Microarray Results for ppNociceptin and NPY

BDNF induction of mRNA encoding both *ppNociceptin* and *NPY* detected by oligonucleotide microarray was confirmed using qRT-PCR (Figure 5). qRT-PCR

(TaqMan) experiments were performed on separate aliquots of total RNA (n=3) used in the original microarray interrogation. Results from the qRT-PCR experiments demonstrate a time-dependent, and statistically significant increase in absolute expression levels of *NPY* mRNA by BDNF when compared to time-matched, untreated cultures (Figure 5A). Similarly, BDNF induction of *ppNociceptin* mRNA, although less robust than *NPY*, was statistically significant but with a peak induction between 6 and 12 hours following treatment (Figure 5B).

Downstream Actions of Peptides Derived from ppNociceptin: Induction of IEG mRNAs

To determine whether peptides processed from ppNociceptin (nociceptin, nocistatin, PNP-4 and PNP-5) and NPY could activate downstream events within hippocampal neurons, we examined *IEG* induction in cultures following exposure to these neuropeptides. *IEG* expression is indicative of activation of cellular signaling pathways which may lead to morphologic and functional alterations. Each of the ppNociceptin peptides and NPY induced expression of the IEG *c-fos*. Nociceptin and NPY were the most effective in inducing *Egr-2* and *Arc* expression (Figure 6). In contrast, nocistatin, PNP-4 and PNP-5 did not induce *Egr-2* or *Arc* expression, rendering nociceptin the most potent cellular activator (Figure 6). Although the function of *Arc* remains not well defined, *Arc* mRNA has been shown to translocate to dendrites where it is translated and incorporated into the postsynaptic density (PSD) (Steward and Worley, 2001). We have previously defined a correlation between BDNF-induced synaptic plasticity and *Arc* expression in single cells (Alder et al., 2003), suggesting that Arc is involved in

downstream changes following synaptic plasticity. Moreover, Arc has been implicated in neurite extension, one aspect of synaptic development (Donai et al., 2003). Based on these observations, we chose to further investigate the effects of nociceptin, nocistatin and NPY on a more biologically relevant readout in neurons with respect synaptic development, neurite outgrowth.

Effects of Nociceptin, Nocistatin, and NPY on Neurite Morphology

Morphometric analysis of nociceptin, nocistatin and NPY effects on various measures of neurite morphology in freshly-plated primary hippocampal cell cultures was assayed on the ArrayScanII® HCS Reader. Neurons were identified as a valid if positive for neuron-specific ß-III tubulin (TuJ1), and also contained a valid nucleus (Hoechst 33342 positive). The average percent valid neurons did not differ significantly after peptide treatments (data not shown), suggesting no effects on survival.

Morphometric measurements were calculated after 24 hr to examine whether the three peptides exhibited effects on neurite formation (Figure 7). Nociceptin and NPY evoked similar effects on all parameters examined, while nocistatin was generally without effect. Nociceptin was most effective at 10 µM whereas NPY reached signficance at 1 µM and the following values represent statistically significant data ($p < 0.05$, one way ANOVA, Dunnett's Test, $n = 3$) for those concentrations (Figure 7A-E). Both nociceptin and NPY enhanced 1) the percentage of neurons exhibiting at least one neurite (control: 81.5 ± 2.2, nociceptin: 89.4 ± 1.3, NPY: 87.4 ± 2.3, %). In addition, nociceptin and NPY increased 2) the average total length of neurites per neuron (control:

35.3 ± 1.1, nociceptin: 49.7 ± 0.9. NPY: 47.9 ± 3.8, µm) and 3) the average length of neurites per neuron (control: 11.1 ± 0.3, nociceptin: 14.6 ± 0.2, NPY: 14.4 ± 1.3, µm). Finally, nociceptin and NPY increased 4) the average number of neurites per neuron (control: 2.83 ± 0.06, nociceptin: 3.23 ± 0.08, NPY: 3.22 ± 0.05, #) and 5) the branch points per neuron (control: 0.94 ± 0.05, nociceptin: 1.28 ± 0.04, NPY: 1.27 ± 0.14, #) (Figure 7A-E). In contrast, nocistatin only altered the average total length of neurites per neuron. These data suggest that nocistatin has a relatively weaker effect overall on neurite outgrowth, consistent with its counteractive effects to nociceptin.

The effects of BDNF on hippocampal neurite outgrowth were similar to those observed with nocistatin and NPY and were consistent with published findings (Patel and McNamara, 1995; Labelle and Leclerc, 2000). The two parameters induced the most by nociceptin were also enhanced the most by BDNF. Total neurite length was highly upregulated relative to control (nociceptin: 110, BDNF: 110, % of control, n = 3). Moreover, average branch points per neuron were increased by both treatments (nociceptin: 136, BDNF: 120, % of control, n = 3). In a representative experiment, BDNF (100 ng/ml) increased the average total length of neurites per neuron (control: 106.7 ± 9.0, BDNF: 122.0 ± 4.4, µm) and the average length of neurites per neuron (control: 24.8 ± 1.9, BDNF: 25.9 ± 0.7, µm). In addition, BDNF increased the average number of neurites per neuron (control: 4.19 ± 0.16, BDNF: 4.59 ± 0.08, #) and the branch points per neuron (control: 2.78 ± 0.36, BDNF: 3.59 ± 0.39, #). Only the percentage of neurons exhibiting at least one neurite (control: 95.1 ± 1.4, BDNF: 95.5 ± 0.6, %) did not show a dramatic change. In addition to nocistatin, several other BDNF-

regulated neuropeptides including somatostatin, substance P, cortistatin, Met-enkephalin, Leu-enkephalin, PNP-4, and PNP-5 did not have effects on neurite outgrowth in preliminary screens (data not shown). These experiments reveal that nociceptin and NPY selectively increase neurite outgrowth in freshly-plated hippocampal cultures, thereby enhancing their development.

DISCUSSION

Transcriptional Profiling of BDNF Effects on Hippocampal Neurons

We combined oligonucleotide microarray analysis of BDNF-treated hippocampal neurons with functional studies of the identified genes to elucidate molecular pathways related to synaptic strengthening. Our results reveal that BDNF initiates a complex transcriptional program in hippocampal neurons featuring several classes of mRNAs relevant to synaptic plasticity. These classes include transcriptional regulatory genes, receptors and transporters, signal transduction molecules, structural molecules and metabolic enzymes. Two important categories of upregulated genes included synapse-associated molecules and neuropeptides. This is consistent with our earlier findings of induction of *Rab3A* and *VGF* by BDNF both at the transcriptional and translational level (Thakker-Varia et al., 2001; Alder et al., 2003). A novel role for one neuropeptide, nociceptin, in promoting neurite outgrowth validates the use of microarray screens to identify potential effectors. The transcriptional response of hippocampal neurons to BDNF suggests that a complex genomic program underlies the well-known physiological changes associated with BDNF effects on trkB activation.

Our previous studies have defined several signaling pathways that are required for BDNF-mediated transcription. In addition to trkB, the MAP kinase, CaMK and PLC-γ cascades are necessary for transcriptional regulation of both *IEGs* and the neuropeptide *VGF* by BDNF treatment (Alder et al., 2003). Since additional IEGs and neuropeptides were identified in this study, it is highly likely that these same signaling pathways are involved in transcription of the classes of genes induced by BDNF in this screen. Our results do not necessarily indicate that transcription of the BDNF-induced genes is required for their function. For example, we have previously shown that transcription of *Rab3A* was not detected until hours of BDNF exposure but that *Rab3A* function is required for acute synaptic plasticity observed within minutes of BDNF treatment (Thakker-Varia et al., 2001). Therefore, *de novo* transcription may not be necessary for the function of these molecules. Rather, it is possible that these molecules are transcribed by BDNF in response to increased turnover and the need to replenish these molecules important in neuronal function.

Several studies have employed transcriptional profiling to catalog genes differentially regulated in the hippocampus during development (Mody et al., 2001), following induction of LTP and learning (Luo et al., 2001; Donahue et al., 2002; D'Agata and Cavallaro, 2003; Leil et al., 2003) and within regional hippocampal compartments (Lein et al., 2004). However, this study is the first to establish a functional role for BDNF-induced genes in neuronal development. Our study employed rigorous criteria, resulting in approximately 3.4% differentially expressed transcripts with high confidence

(see Methods). In addition, the temporal patterns of gene expression observed by qPCR were highly similar to those of the microarray, validating our use of stringent criteria. A complete list of all genes examined is cataloged in Supplemental Table 1 (available at www.jneurosci.org). In this study, all genes selected for analysis were upregulated. In general, transcripts downregulated by BDNF appeared less relevant to the process of synaptic enhancement. However, several membrane receptors, signal transduction molecules and cytoskeletal elements were decreased, suggesting a delicate interplay between proteins during BDNF-mediated events. Two receptors for BDNF-induced neuropeptides including the *ORL-1* and *NPY* receptors were also downregulated. These data suggest that there is a negative feedback loop in which increased neuropeptide expression and binding to receptor result in a compensatory decrease in the number and presumptive activity of the receptors. Future experiments will explore the role of all types of regulated genes including unknown ESTs in BDNF-mediated synaptic strengthening.

Roles for Synapse-Associated Molecules and Neuropeptides in Neuronal Function

A number of synapse-associated transcripts induced by BDNF emerged in the results of this study. The regulation of other synapse-associated proteins by BDNF has been observed at the translational and post-translational levels (Takei et al., 1997; Pozzo-Miller et al., 1999; Jovanovic et al., 2000; Tartaglia et al., 2001; Schratt et al., 2004). In our previous study using differential display, *Rab3A*, a synaptic vesicle trafficking protein was upregulated by BDNF at the mRNA and protein levels, and was essential for BDNF-enhanced plasticity (Thakker-Varia et al., 2001). It is therefore likely that some

of the synapse-associated proteins identified in this study also have important functions in BDNF-mediated synaptic strengthening. Three of these genes potentially play roles in synaptic vesicle trafficking. For example, *synaptojanin 2* is a major presynaptic protein associated with endocytic-coated intermediates and is involved in synaptic vesicle recycling (Cremona et al., 1999; Luthi et al., 2001). *Septin 9,* binds to syntaxin and may have a role in regulated exocytosis (Beites et al., 1999). Finally, *seven in absentia 2* co-localizes with synaptophysin on synaptic vesicles (Wheeler et al., 2002). In addition, *ryanodine receptor 2* has been implicated in regulation of intracellular Ca^{2+} concentration at synaptic terminals (Bouchard et al., 2003). Two other genes associated with synaptic membrane are *brain-specific angiogenesis inhibitor 1* which co-immunoprecipitates with postsynaptic density protein 95 kD (PSD-95) (Lim et al., 2002) and *sialyltransferase 8c* which is associated with NCAM (Dey et al., 2000). Lastly, *neuronal pentraxin* and *stathmin* are markers of neurite sprouting (Ujike et al., 2002). Future studies will employ genetic manipulations to define the roles of these genes in synapse function.

Neuropeptides and neurotrophins are emerging as important regulators of neuronal function. *BDNF* itself was the only neurotrophin regulated by BDNF treatment. Other neurotrophins and their receptors such as *trkB, neurotrophin 3 (NT3), nerve growth factor (NGF), glial-derived neurotrophic factor (GDNF)* and *ciliary neurotrophic factor (CNTF)* were present on the microarray, but were not altered by BDNF treatment, indicating the specificity of the BDNF effect. However a number of other neuropeptides, not belonging to the neurotrophin family were induced by BDNF in our study. Induction by BDNF of message and protein encoding several neuropeptides, including *NPY,* has

already been demonstrated (Nawa et al., 1994; Arenas et al., 1996; Croll et al., 1999; Reibel et al., 2000). To our knowledge however, *ppNociceptin, AREG*, and *CXC12* have not previously been linked to BDNF/trkB signaling. Expression of another neuropeptide *VGF* by BDNF was demonstrated using cDNA Clontech Atlas Array in our earlier studies. Moreover, a VGF peptide regulated synaptic activity in dissociated hippocampal cells and *VGF* mRNA was induced during an *in vivo* learning model (Alder et al., 2003). Other reports suggest that neuropeptide expression is correlated with learning and activity paradigms such as seizures and exercise (Neeper et al., 1995; Reibel et al., 2003; Altar et al., 2004). Since neuropeptides serve as central regulators of neuronal function, we have explored the role of neuropeptides identified in this study in developing hippocampal cultures.

Neurite Outgrowth Induced by Nociceptin and NPY

To identify neuropeptides that are potentially involved in neuronal function, we chose to use the activity-dependent *IEG* transcripts *c-fos Egr-2*, and *Arc* as indices of activation. *Arc* mRNA is translocated to dendrites following neuronal activity and may play a role in initiation of signaling pathways involved in neurite extension (Donai et al., 2003). Here we show that nociceptin and NPY peptides induce high levels of *IEG* expression. Nocistatin, on the other hand, did not induce *Arc* or *Egr-2,* consistent with the known opposing actions of nociceptin and nocistatin (Okuda-Ashitaka et al., 1998) and indicating peptide specificity. *ppNociceptin* is translated into a precursor protein that is processed into four smaller peptides (nociceptin, nocistatin, PNP-4 and PNP-5) (Okuda-Ashitaka et al., 1998)). Nociceptin, the endogenous ligand for ORL-1 is a widely

expressed heptadecapeptide originally identified as a potent anti-analgesic neuropeptide although other roles have been proposed (Mogil and Pasternak, 2001; Zaveri, 2003). Moreover, studies investigating the functional role of nociceptin in behavior or physiology remain controversial (Heinricher, 2003). NPY traditionally modulates appetitive behavior and blood pressure regulation (Thorsell and Heilig, 2002; Pedrazzini et al., 2003). To date, little evidence has linked nociceptin or NPY with neuronal development associated with neurotrophins in the hippocampus.

Neurite extension is one of the many effects elicited by BDNF on developing neurons (Cohen-Cory and Fraser, 1995; Patel and McNamara, 1995; Wang et al., 1995; Labelle and Leclerc, 2000). We have now examined whether neuropeptides induce neuritogenesis in the hippocampus. Previously, there were no reports demonstrating neuritogenic effects of nociceptin or NPY on primary neurons derived from the central nervous system (Saito et al., 1997; White, 1998). Our report indicates that both nociceptin and NPY stimulate all parameters of neuritogenesis in freshly-plated hippocampal cells. Both nociceptin and NPY increased the total length, average length and number of neurites per neuron, number of branch points per neurite as well as number of neurons with at least one neurite. This last parameter suggests that there is *de novo* neurite formation in the neuropeptide-treated cultures, however extension of existing neurites may also occur. Nocistatin exhibited a significant effect on total neurite length at the highest concentration tested, but was without effect on the other parameters, indicating a different effect on neurite outgrowth. Several other BDNF-regulated neuropeptides including the other ppNociceptin derivatives did not alter neurite

outgrowth, demonstrating the specificity of nociceptin and NPY's effects. Together, these data indicate that nociceptin and NPY may play a neuritogenic role when released within the hippocampus. In sum, this study demonstrates that BDNF increases expression of neuropeptides which induce neurite outgrowth in developing neurons. Future studies will examine the possible roles of nociceptin and NPY in synaptogenesis. In addition, a number of molecules identified in our screen may come into play for stabilization and functioning of synapses leading to enhanced synaptic transmission. Our study is therefore developing a molecular timetable of neuronal development initiated by BDNF.

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FIGURE LEGENDS

Figure 1 BDNF induces an immediate early gene program in hippocampal cultures. Differential expression (fold change) of immediate early gene transcripts across the study's 7 time points following BDNF challenge are shown, including a table featuring actual fold change values for all time points with their associated p-values (*p-value ≤ 0.05, t-test). Symbol key (Unigene Cluster ID): *EGR1, early growth response 1* (Mm.181959); *EGR2, early growth response 2* (Mm.290421); *TIEG1, TGFB inducible early growth response 1* (Mm.4292); *IER2, immediate early response 2* (Mm.399); *NR4A2, nuclear receptor subfamily 4, group A, member 2* (Mm.3507); *NR4A1, nuclear receptor subfamily 4, group A, member 1* (Mm.119); *c-fos, FBJ osteosarcoma oncogene* (Mm.246513); *junB, jun-B oncogene* (Mm.1167); *fosB, FBJ osteosarcoma oncogene B* (Mm.248335); *jun, jun oncogene / AP-1 transcription factor* (Mm.275071); *BTG2, B-cell translocation gene 2/PC3* (Mm.239605); *IFRD1, Interferon-related developmental regulator 1/PC4* (Mm.168).

Figure 2 BDNF induces transcription of mRNAs encoding functionally related molecules. (A) Receptors and Transporters: *SSTR-2*, *somatostatin receptor 2* (Mm.4375); *MAS1*, *MAS1 oncogene* (Mm.57182); *CXCR4*, *chemokine (C-X-C) receptor 4* (Mm.1401); *IL-17R*, *interleukin 17 receptor* (Mm.4481); *GABAAb3*, *gamma-aminobutyric acid (GABA-A) receptor, subunit β3* (Mm.8004); *GDNFR1*, *glial cell line derived neurotrophic factor receptor α1* (Mm.88367); *ADRB2*, *adrenergic receptor, β2* (Mm.5598); *GLUT-3*, *SLC2A3/glucose transporter 3* (Mm.269857); *CRT*,

SLC6A8/creatine transporter (Mm.274553); *Pit-1*, *SLC20A1/phosphate transporter* (Mm.27267); *RGS4*, *regulator of G-protein signaling 4* (Mm.41642); *RGS16*, *regulator of G-protein signaling 16* (Mm.181709); *ADCY8*, *adenylate cyclase 8* (Mm.1425); *CREM, cAMP responsive element modulator* (Mm.5244); *RRAD, ras-related associated with diabetes* (Mm.29467); *RAP2B, member of RAS oncogene family* (Mm.7548); *GNG2, guanine nucleotide binding protein (G protein), γ2 subunit* (Mm.46767). (B) Signal Transduction Molecules: *SGK-1, serum/glucocorticoid regulated kinase* (Mm.28405); *PKCb*, *protein kinase C, beta* (Mm.4182); *MAP2K3*, *mitogen activated protein kinase kinase 3* (Mm.18494); *MAP3K5*, *mitogen activated protein kinase kinase kinase 5* (Mm.6595); *Pi4K2a*, *phosphatidylinositol 4-kinase type 2α* (Mm.117037); *DUSP1, dual specificity phosphatase 1* (Mm.239041); *DUSP6*, *dual specificity phosphatase 6* (Mm.1791); *PPP1R15B, protein phosphatase 1, regulatory (inhibitor) subunit 15b* (Mm.27987); *PTPN5, protein tyrosine phosphatase, non-receptor type 5* (Mm.4654); *TYRO3, protein tyrosine kinase 3* (Mm.2901); *ACVR1, activin A receptor, type 1* (Mm.689); *PTPRN*, *protein tyrosine phosphatase, receptor type, N* (Mm.2902); *PTPRG*, *protein tyrosine phosphatase, receptor type, G* (Mm.9010). (C) Structural Molecules and Metabolic Enzymes: *TLL-1, tolloid-like* (Mm.5076); *PAI-2, plasminogen activator inhibitor 2* (Mm.271870); *Np1, neuropilin 1* (Mm.271745); *MP3, matrix metalloproteinase 3* (Mm.4993); *ADAM19*, *disintegrin and metalloproteinase domain 19* (Mm.89940); *TN-C*, tenascin C (Mm.980); *NP25, neuronal protein Np25 or transgelin 3* (Mm.24183); *NEFL*, *neurofilament, light polypeptide* (Mm.1956); *SPNB3, spectrin β3* (Mm.7363); *NUSAP1, nucleolar and spindle associated protein 1* (Mm.27584); *RB3, stathmin-like 4* (Mm.35474); *Sc5d*, *sterol-C5-desaturase* (Mm.13081); *Hsd11b1,*

hydroxysteroid 11β dehydrogenase 1 (Mm.28328); *Sptlc2*, *serine palmitoyltransferase, long chain base subunit 2* (Mm.565); *Lypla2, lysophospholipase 2* (Mm.34302); *Pafah1b2, platelet-activating factor acetylhydrolase, isoform 1b, α2 subunit* (Mm.24199).

Figure 3 BDNF induces transcription of genes encoding synapse-associated proteins. Differential expression (fold change) of synapse-associated transcripts across the study's 7 time points following BDNF challenge are shown, including a table featuring actual fold change values for all time points with their associated p-values (*p-value ≤ 0.05, t-test). Symbol key (Unigene Cluster ID): *NPTX1, neuronal pentraxin 1* (Mm.5142); *STMN4, stathmin-like 4* (Mm.35474); *SYNJ2, synaptojanin 2* (Mm.30717); *SEPT9, septin 9* (Mm.38450); *RYR2, ryanodine receptor 2* (Mm.195900); *BAI2, brain-specific angiogenesis inhibitor 1* (Mm.43133); *SIAH2, seven in absentia 2* (Mm.2847); *SIAT8c, sialyltransferase 8 C* (Mm.41726).

Figure 4 BDNF enhances transcription of genes encoding neuropeptides. Differential expression (fold change) of neuropeptide transcripts across the study's 7 time points following BDNF challenge are shown, including a table featuring actual fold change values for all time points with their associated p-values (*p-value ≤ 0.05, t-test). Symbol key (Unigene Cluster ID): *ppNociceptin, preproNociceptin* (Mm.16347); *NPY, neuropeptide Y* (Mm.154796); *BDNF, brain-derived neurotrophic factor* (Mm.1442);

SST, somatostatin (Mm.2453); *SP, substance P* (Mm.1440); *AREG, amphiregulin* (Mm.8039); *SCG2, secretogranin 2* (Mm.5038).

Figure 5 Quantitative RT-PCR (Taqman) confirms differential expression of mRNAs encoding *NPY* and *ppNociceptin* observed in original microarray experiment. Closed circles: BDNF-treated cultures, open circles = time matched, untreated cultures. Both microarray and qRTPCR results are shown for each neuropeptide mRNA (A) *NPY* (B) *ppNociceptin* (average ± SE, n = 3). Statistics: * p-value < 0.05; ANOVA.

Figure 6 Peptides derived from the ppNociceptin peptide induce immediate early genes in hippocampal cultures. Cells were treated with vehicle, nociceptin, nocistatin, PNP4 or PNP5 at 1μM for 1 hr. *C-fos, EGR2* and *Arc* expression was examined using quantitative RT-PCR (TaqMan). All data was normalized to *GAPDH* and expressed as a fold of vehicle control (average ± SE, n = 3, 4). Statistics: * p-value < 0.05; ANOVA.

Figure 7 Morphometric analysis of nociceptin, nocistatin, and NPY effects on neuritogenic parameters in primary cultures of hippocampal neurons. Hippocampal cells treated with vehicle (A) or 10 μM nociceptin (B) for 24 hr and double labeled for TuJ1 (green) and Hoeschst (blue). Neurites are indicated by the arrows. Note the longer neurites in the nociceptin treated cells. Scale bar = 50 μm. (C) Percentage of valid neurons with at least one neurite. (D) Average total length (microns) of all neurites per valid neuron. (E) Mean of the average neurite length for each valid neuron. (F) Average

number of neurites per valid neuron. (G) Average number of branch points per valid neuron. (n = 3) Statistics: *p-value < 0.05; One way ANOVA, Dunnett's Test.

Table 1 Oligonucleotide sequences of primers and probes used for quantitiative PCR detection of target mRNAs (*ppNociceptin, pNPY, c-fos*, and *Arc*). All primer and probe sequences were designed from publicly available reference sequences (Ref Seq). Reporter (5') and quencher (3') dye-labeling of probe oligonucleotides are shown in parentheses.

Supplemental Table 1 Summary of mRNAs up-regulated by BDNF across all time points. Summary list of all mRNAs (n=201) that demonstrated a significant increase in expression (BDNF/untreated control), as defined in methods section, at a minimum of one time point following BDNF challenge. Affymetrix identifiers (Affy ID), or qualifiers, refer to the specific probeset on the U74Av2 GeneChip used to interrogate expression of each transcript. Table features annotation for each probeset, if known, along with actual fold change (FC) values and associated p-values (*p-value ≤ 0.05) at all time points in the study.

Supplemental Table 2 Summary of mRNAs down-regulated by BDNF across all time points. Summary list of all mRNAs (n=105) that demonstrated a significant decrease in expression (untreated control/BDNF), as defined in methods section, at a minimum of one time point following BDNF challenge. Affymetrix identifiers (Affy ID), or qualifiers, refer to the specific probeset on the U74Av2 GeneChip used to interrogate

expression of each transcript. Table features annotation for each probeset, if known, along with actual fold change (FC) values and associated p-values (*p-value ≤ 0.05) at all time points in the study.