Identification of genes co-upregulated with *Arc* during BDNF-induced long-term potentiation in adult rat dentate gyrus *in vivo*

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Abstract

Brain-derived neurotrophic factor (BDNF) is a critical regulator of transcription-dependent adaptive neuronal responses, such as long-term potentiation (LTP). Brief infusion of BDNF into the dentate gyrus of adult anesthetized rats triggers stable LTP at medial perforant path-granule synapses that is transcription-dependent and requires induction of the immediate early gene *Arc*. Rather than acting alone, Arc is likely to be part of a larger BDNF-induced transcriptional program. Here, we used cDNA microarray expression profiling to search for genes co-upregulated with *Arc* 3 h after BDNF-LTP induction. Of nine cDNAs encoding for known genes and up-regulated more than four-fold, we selected five genes, *Narp*, *neuritin*, ADP-ribosylation factor-like protein-4 (*ARL4L*), TGF-β-induced immediate early gene-1 (*TIEG1*) and *CARP*, for further validation. Real-time PCR confirmed robust up-regulation of these genes in an independent set of BDNF-LTP experiments, whereas infusion of the control protein cytochrome C had no effect. *In situ* hybridization histochemistry further revealed up-regulation of all five genes in somata of post-synaptic granule cells following both BDNF-LTP and high-frequency stimulation-induced LTP. While Arc synthesis is critical for local actin polymerization and stable LTP formation, several of the co-upregulated genes have known functions in excitatory synaptogenesis, axon guidance and glutamate receptor clustering. These results provide novel insight into gene expression responses underlying BDNF-induced synaptic consolidation in the adult brain *in vivo*.

Introduction

Long-term adaptive responses in the nervous system are thought to require co-ordinate changes in gene expression. A critical time-dependent role of new gene expression has been demonstrated in memory formation as well as in activity-dependent synaptic plasticity such as long-term potentiation (LTP). Recently, the neurotrophin brain-derived neurotrophic factor (BDNF) has emerged as a major regulator of LTP induced by high-frequency stimulation (HFS–LTP) of excitatory synapses (Bramham & Messaoudi, 2005). BDNF is released from glutamatergic synapses following HFS and is capable of signaling pre- and post-synaptically through TrkB receptor tyrosine kinase receptors (Drake *et al*., 1999; Hartmann *et al*., 2001; Nawa & Takei, 2001; Balkowiec & Katz, 2002). The maintenance of LTP is typically divided into a transient early phase that does not require transcription and a stable late phase that is transcription-dependent (Bliss & Collingridge, 1993; Nguyen & Kandel, 1996). Genetic and pharmacological approaches indicate that BDNF signaling at excitatory synapses promotes the development of late-phase LTP (Kang *et al*., 1997; Messaoudi *et al*., 2002; Minichiello *et al*., 2002; Tang *et al*., 2002).

Exogenous application of BDNF induces a lasting potentiation of synaptic efficacy (BDNF-LTP) at medial perforant-path granule cell synapses in the dentate gyrus (Messaoudi *et al*., 1998). Induction of BDNF-LTP is blocked by inhibitors of RNA synthesis and occluded by prior expression of late-phase, but not early-phase, HFS-LTP (Messaoudi *et al*., 2002; Ying *et al*., 2002). Like HFS-LTP, BDNF-LTP is associated with the induction and dendritic transport of mRNA encoded by the immediate early gene activity-regulated cytoskeleton-associated protein *Arc* (also known as Arg3.1) (Link *et al*., 1995; Lyford *et al*., 1995; Ying *et al*., 2002). Furthermore, a recent study using antisense knockdown provides evidence that Arc synthesis is necessary for the induction of BDNF-LTP and its time-dependent consolidation (Soule *et al*., 2005). These studies suggest that BDNF activates a process of synaptic consolidation dependent on Arc transcription and translation (Bramham & Messaoudi, 2005).

Consolidation of HFS-LTP is associated with actin polymerization and expansion of synapses and dendritic spines (Fukazawa *et al*., 2003; Matsuzaki *et al*., 2004). We have recently obtained evidence that Arc synthesis during a critical window lasting between 2 and 4 h after HFS is necessary for local actin polymerization and consolidation of LTP (Soule *et al*., 2005; also E. Messaoudi, T. Kanhema, J. Soule, A. Tiron, G. Dagyte, B. da Silva and C.R. Bramham, unpublished observations). Rather than working alone, Arc is likely to be part of a co-ordinate process of synapse growth involving multiple gene products. Here, we used cDNA microarray expression profiling to screen for genes that are co-upregulated with Arc mRNA 3 h after

BDNF-LTP induction in the dentate gyrus of anesthetized rats. Of nine genes up-regulated more than four-fold, five (*Narp*, *Neuritin*, *TIEG1*, *ARL4L* and *CARP*) were selected for independent confirmation by real-time PCR and *in situ* hybridization. PCR analysis demonstrated strong up-regulation of all five genes in response to BDNF-LTP induction, and *in situ* hybridization revealed enhanced expression of these genes in dentate granule cells following both BDNF-LTP and HFS-LTP. Previous studies in primary neuronal cell cultures have shown that BDNF elicits a transient potentiation associated with induction of the secretory neuropeptide VGF (non-acronymic) and vesicular trafficking protein Rab3a (Thakker-Varia *et al.*, 2001; Alder *et al.*, 2002, 2003). We report that *VGF*, but not *Rab3a*, is induced following BDNF-LTP in the adult dentate gyrus. These results give insight into gene expression responses underlying BDNF-activated synaptic consolidation. Consistent with a process of synaptic growth and remodeling, Arc is co-upregulated with several genes (*Narp*, *Neuritin*, *TIEG1*, *N4WBP4*) involved in synaptogenesis, axon guidance and glutamate receptor clustering.

Materials and methods

These experiments were approved by the Norwegian Committee for Animal Research in accordance with European Community Council Directives.

Animals

Thirty male Mol: SD rats (Møllegaards Avls-laboratorium, Denmark) weighing 250–320 g were housed in a temperature- and light-controlled vivarium (23 ± 0.5 °C, lights on 07:00–19:00 h) and supplied with food (12–14 pellets per rat per day) and water for at least 1 week prior to the experiments.

Electrophysiology and intrahippocampal infusion

Rats were anesthetized with urethane (1.4–1.8 g/kg, i.p) and positioned in a stereotaxic frame with the upper incisor bar 2 mm below the interaural line (skull flat position). Supplemental doses of urethane were given as required to maintain a surgical level of anesthesia. Rectal temperature was maintained at 36 °C with a thermostatically controlled electric heating pad.

Stereotaxic coordinates relative to Bregma were 7.9 mm posterior, 4.2 mm lateral for stimulation, and 3.9 mm posterior, 2.2 mm lateral for recording. An outer (guide) cannula (24-gauge, PlasticsOne, Roanoke, VA, USA) was beveled sharp at the tip to facilitate tissue penetration. A Teflon-coated stainless steel wire recording electrode (coated diameter 112 μm) was glued (cyanoacrylate, Mega-G base, Mega Metal, Oslo, Norway) to the shaft of the outer cannula. The electrode was then cut so that it extended 900 μm from the end of the cannula. A concentric bipolar stimulating electrode (tip separation 500 μm; SNEX 100, Rhodes Medical Instruments) was lowered into the dorsomedial aspect of the angular bundle for stimulation of the medial perforant path. After making a small slit in the dura, the guide cannula and attached recording electrode was slowly lowered into the dorsal hippocampus until a positive-going field excitatory post-synaptic potential (fEPSP) of maximum slope was obtained in the dentate hilus. The final depth of the recording electrode ranged between 200 and 300 μm below the level of the maximum negative-going fEPSP sink recorded in the middle third of the dentate molecular layer. An inner infusion cannula (31-gauge) was then inserted so that it protruded 300 μm below the end of the guide. The tip of the infusion cannula was located in deep stratum lacunosum-moleculare of field

CA1, 700 μm above the hilar recording site and 300–400 μm above the medial perforant path synapse.

Biphasic rectangular pulses of 150-μs duration were applied every 30 s throughout the experiment. The stimulation intensity for test pulses was set to elicit a population spike amplitude of 30% of the maximal response. The infusion cannula was connected via PE50 polyethylene tubing to a 5-μL Hamilton syringe. Solutions were delivered by an infusion pump at a rate of 80 nL/min. BDNF-LTP was induced by infusing BDNF (2 μg in 2 μL PBS) for 25 min. The paradigm for HFS-LTP induction consisted of eight pulses at 400 Hz, repeated four times, at 10-s intervals. Three sessions of HFS were given at intervals of 5 min.

Signals from the dentate hilus were amplified, filtered (1–3 kHz), digitized (25 kHz) and stored on computer disk. Acquisition and analysis of field potentials were accomplished using DataWave Technologies WorkBench software (Longmont, CO, USA). The maximum slope of the fEPSP and the amplitude of the population spike measured from its negative-going apex to the tangent line joining the first two positive peaks were measured, and averages of four consecutive responses were obtained. Analysis of variance (ANOVA) for repeated measures followed by a *post-hoc* Scheffé test was used for statistical analysis of group effects (STATISTICA package, StatSoft Inc., Tulsa, OK, USA). Statistics were based on values obtained during the 5 min at the end of baseline recording and at the end of post-infusion recording. A *P*-value of 0.05 was chosen as the level of statistical significance.

Tissue microdissection and poly(A) RNA preparation

After 3 h of electrophysiological recording the rats were killed by decapitation and the dentate gyrus was bilaterally dissected on ice, immediately frozen on dry ice and stored at −80 °C until use.

Poly(A) RNA was isolated using the Dynabeads mRNA direct kit (Dynal, Oslo, Norway) according to the manufacturer's protocol. Minor modifications were that 70-μL magnetic beads were used per sample and that the isolated poly(A) RNA fraction was dissolved in 3×30 μL of 1 M Tris/HCl, pH 8.0. The yield and quality of the poly(A) RNA were determined by measuring the absorbance at 260/280 nm. In order to minimize inter-individual variation, RNA from six rats was pooled after poly(A) RNA preparation.

cDNA microarray screening

Fluorescence labeling of mRNA was performed using a Fairplay Aminoallyll labeling kit (Stratagene) with Cy-dyes from Amersham. Cy5- and Cy3-labeled cDNA from BDNF-treated and untreated dentate gyrus was then co-hybridized to a microarray representing ∼13 800 sequence-verified rat cDNA probes (Research Genetics, Huntsville, AL, USA; http://www.resgen.com/) printed in duplicate on amino-silane-coated slides (CMT GAPS II, Corning Life Sciences, Corning, NY, USA). A dye swap experiment was included in order to exclude dye bias from the experiment. Microarrays were provided by the Norwegian Microarray Consortium (http://www.mikromatrise.no).

Semi-quantitative real-time PCR

The cDNA microarray data for selected genes were confirmed by real-time PCR. Aliquots of pooled poly(A) RNA were analysed on a Roche LightCycler using the FastStart DNA Master SYBR Green I mix (Roche Norge AS). An aliquot of 30 ng poly(A) RNA was reversed transcribed using the Superscript First-Strand Synthesis Kit (Invitrogen, Norway). The cDNA was diluted 30-fold, and 5 μL was added to the PCR reaction mix to yield a total volume of 20 μL.

In addition, PCR confirmation was carried out on cDNA generated from individual animals using an iCycler (Bio-Rad) and the iQ™ SYBR® Green Supermix. Total RNA was isolated from single dentate gyrus using TRIzol Reagent (Gibco BRL, Norway). To remove genomic DNA, the RNA was treated with DNAse (Invitrogen) prior to reverse transcription. The integrity of the RNA and the absence of genomic DNA was checked by gel electrophoresis and the yield determined by measuring the absorbance at 260/280 nm. A 1-μg sample of total RNA was reverse transcribed using the Superscript First-Strand Synthesis Kit (Invitrogen). The cDNA was diluted 30-fold, and 5 μL was added to the PCR reaction mix to yield a total of 25 μL.

PCR quantification was performed in triplicate. The fluorescence signal was quantified by the second derivative maximum method using the LightCycler Data Analysis software or the iCycler iQ Real-Time Detection System software. The samples were normalized for variations in mRNA content and the efficiency of cDNA synthesis with the internal control gene *tubulin*. The threshold cycle for each sample was chosen in the linear range. Changes in concentration between the PCR products obtained were detected as differences in threshold cycle (ΔC_T) between samples. A single PCR product was verified by observing a single peak during melting-point analysis. Primer sequences in 5′ to 3′ direction and the annealing temperatures used are given in Table 1.

Probe preparation and *in situ hybridization*

Riboprobes were prepared from cDNA inserts from *bdnf* (gb | M61175.1), *bdnf exon3* (ref | X67107.1), *narp* (ref | XM_221901.2), *neuritin* (ref | NM_053346.1), *carp* (gb | AF030089.2), *tieg-1* (ref | NM_031135.1) and *arfl4l* (ref | XM_220933.1) cloned into the pCR®II-TOPO® vector (Invitrogen). Antisense and sense probes were transcribed from linearized plasmids using T7 and SP6 polymerase in the presence of digoxigenin labeling mix according to the manufacturer's instructions (Roche).

After transcardial perfusion with 4% paraformaldehyde, the brain was removed and 50-μm-thick coronal sections were cut on a vibratome. Floating sections were placed in PBS for 5 min, permeabilized with proteinase K (10 μg/mL) for 5 min at 37 °C, and post-fixed (5 min with 4% paraformaldehyde /PBS). After fixation, sections were treated with 0.25% acetic anhydride in 0.1 M TEA (pH 8) for 10 min, washed twice in 2× SSC, and placed for 10 min in a prehybridization buffer. Probes were applied to the sections and hybridization was performed in a humified chamber at 60 °C for at least 16 h. Sections were washed twice with 2× SSC at room temperature for 30 min, once at 65 °C, once with 50% formamide in 2× SSC at 65 °C, rinsed in 2× SSC at 37 °C, incubated with 20 μg/mL RNase A at 37 °C for 30 min and incubated in RNase A buffer at 65 °C for 30 min. After blocking in 2% blocking reagent (Roche) for 2 h at room temperature, alkaline coupled anti-digoxigenin antibody (1 : 2000; Roche) was applied. Visualization was achieved with the chromogene substrates XP and NCIB.

The *in situ* hybridization for the high-magnification images was carried out on cryosectioned snap-frozen brains. After decapitation the brains was rinsed in ice-cold PBS and immediately frozen in cold 2-methyl-butane (−80 °C). Sections 10 μm in thickness were cut using a LeicaCM350 cryostat and thaw-mounted onto Super Frost slides. Briefly, the sections were defrosted, allowed to air-dry for 15 min at room temperature and dipped for 10 min in PBS containing 4% paraformaldehyde. The rest of the protocol was the same as for the floating sections except that a dehydration step including 1 min of treatment with 100% chloroform was carried out before adding the probes.

TABLE 1. Primers used for PCR and their annealing temperature

Gene	Primer sequence	Annealing temperature (C°)
α-tubulin	F:GGGAGCTCTACTGCCTGGAACATG	62
	R:GGAGACAATTTGGCTGATGAGGCG	
enolase	F:CGCCATGGCAAATACGACTTG	57
	R:CATGGGGCACCAGTCTTGATC	
arc	F:GGGCAAGCTGGAGAACAACTTG	58
	R:GGTGCCCACCACATACTGAATG	
zif268	F:GAGCCAAGTCCTTCTAGTCAGTAG	57
	R:TGTGAGAGTTACAGTCGAGCAGTA	
bdnf	F:TGGGACTCTGGAGAGCGTGAATGG	62
	R:CGGGACTTTCTCCAGGACTGTGAC	
exon III bdnf	F:TGCGAGTATTACCTCCGCCAT	58
	R:AGGATGGTCATCACTCTTCTC	
narp	F:GGCAAGATCAAGAAGACGTTG	62
	R:TCCAGGTGATGCAGATATGGT	
neuritin	F:GGGACTTAAGTTGAACGGCA	62
	R:ACCCAGCTTGAGCAAACAGT	
tieg1	F:TAGTGTCTCAGTGCTCCGTCTG	57
	R:TGTGCTCCCTCTTTGGACTTTTC	
carp	F:CAGGCAACCTACCAGCATTT	62
	R:TAACACTCCAACAGGCAGCA	
ARL4L	F:CTTCCCTTCTTTACCGCCTCA	56
	R:ACCCCCAACATCCCACACTT	
rab3a	F:TCTCGATATGGGCAGAAGGAGT	59
	R:GTAGTAGGCTGTGGTGATGGTT	
vgf	F:TGTCCGAAAACGTTCCCCT	59
	R:ACACTCCTTCCCCGAACTGAT	
c-fos	F:CTCCAAGCGGAGACAGATCAAC	59
	R:AGGAACCAGACAGGTCCACATC	

SDS-PAGE and Western blot analysis

At the end of electrophysiological recording, rats were decapitated and the brains were rapidly dissected on ice. Tissues were hand-homogenized in SDS sample buffer. Homogenates were boiled for 5 min, aliquoted and stored at −80 °C until use. Equal amounts of protein were separated on SDS-PAGE gels (10%) and Western blotting was performed. The membranes were blocked for 1 h at room temperature in 5% bovine serum albumin. Narp (polyclonal antibody 1 : 1000; gift from Richard O'Brien, Johns Hopkins University) and β-actin (monoclonal antibody, 1 : 5000, A-5441, Sigma) were analysed by sequential immunoblotting. The secondary peroxidase-conjugated antibodies used were goat anti-rabbit IgG (1 : 20 000, 401393, Calbiochem) and goat anti-mouse IgG (1 : 5000, AP124P, Chemicon). The proteins were visualized using enhanced chemiluminescence. Autoradiographs were quantified using Image J software (NHI, USA). Optical density values obtained for Narp were normalized to the value obtained for β-actin. The normalized value from the treated dentate gyrus was then compared against that from the contralateral dentate gyrus.

Results

Up-regulation of Arc mRNA following BDNF-LTP

Local infusion of BDNF resulted in LTP of medial perforant path-granule evoked fEPSPs (Fig. 1A). The magnitude and kinetics of this increase were similar to those of previous reports (Messaoudi *et al.*,
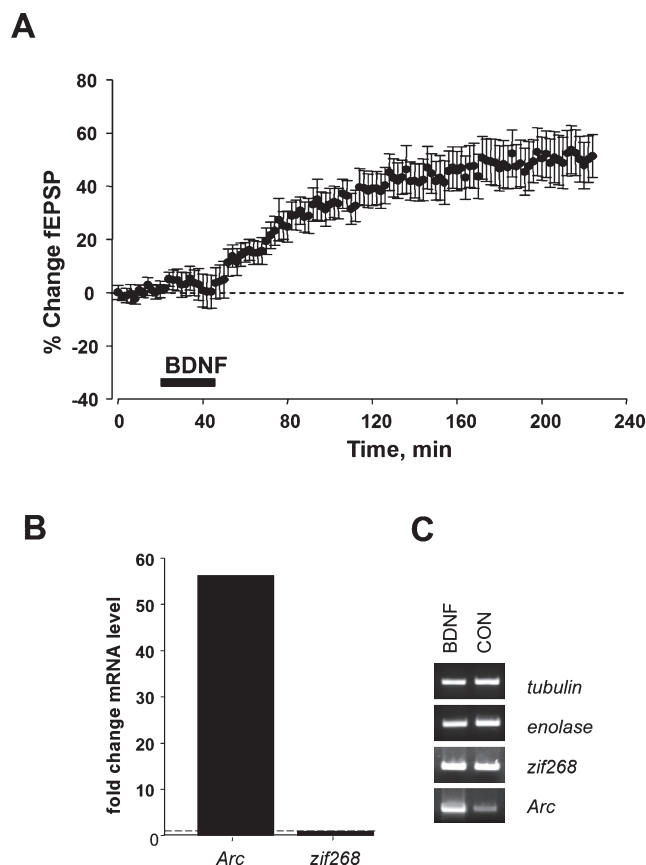
A

B

C

FIG. 1. *In vivo* BDNF-LTP is associated with up-regulation of *Arc*, but not *zif268*, in dentate gyrus samples used for microarray expression profiling. (A) Time course plots of medial perforant path-evoked fEPSP responses. Test pulses were applied at a rate of one every 30 s. BDNF (2 μg/μL) was locally infused during the time indicated by the bar. Values are means ± SEM expressed as a percentage of baseline ($n = 5$). (B) Threshold real-time PCR analysis in pooled dentate gyrus samples collected 3 h after BDNF infusion. Values are normalized to *tubulin* mRNA and expressed as the fold change between the BDNF-infused and contralateral (CON) dentate gyrus. *Arc*, but not *zif268*, expression was elevated 3 h after BDNF infusion. (C) Semi-quantitative real-time PCR endpoint analysis. Transcripts were amplified by real-time PCR and the respective endpoint products were separated by electrophoresis (1.9% agarose gel) and detected by ethidium bromide staining. The levels of *tubulin* and neuron-specific *enolase* were unchanged.

2002; Ying *et al.*, 2002; Gooney *et al.*, 2004). Expression profiling was performed in RNA samples from microdissected dentate gyrus collected 3 h after BDNF infusion. Pooled tissue from five BDNF-infused dentate gyrus were compared with pooled contralateral control dentate gyrus. Before starting the microarray experiments, we sought to validate these samples by examining expression of the immediate early genes *Arc* and *zif268*. Both genes are up-regulated in an all-or-none manner following NMDA receptor-dependent LTP. Using *in situ* hybridiation histochemistry we previously demonstrated robust up-regulation of *Arc*, but not of *zif268*, mRNA during BDNF-LTP (Ying *et al.*, 2002). In the present study, threshold real-time PCR showed a similar selective elevation of *Arc* mRNA in BDNF-treated dentate gyrus. Increases in *Arc* and *zif268* gene expression were 56.5-fold and 1.14-fold, respectively (Fig. 1B). End-point PCR analysis confirmed the increase in *Arc* mRNA, while showing unaltered expression of *zif268* and two standard control genes, *tubulin* and *enolase* (Fig. 1C). The specific induction of *Arc* served as a positive control, validating the pooled samples for further microarray experiments.

mRNA expression profiling and PCR validation of candidate regulated genes

The goal of the present study was to identify genes that are strongly co-upregulated with *Arc*. Of 13 000 genes represented on the array, approximately 97 were up-regulated more than two-fold in the BDNF-treated dentate gyrus in both the forward and the reverse (dye swap) experiments. Table 2 details 14 genes that were up-regulated more than four-fold. Five of these cDNA sequences showed similarity only to expressed sequence tags in GenBank. Of the remaining nine cDNAs coding for known genes, five were selected for further characterization: neuronal activity-regulated pentraxin (*Narp*), *neuritin*, ADP-ribosylation factor-like protein-4 (*ARL4L*), transforming growth factor early gene-1 (*TIEG1*) and calcium/calmodulin kinase-related peptide (*CARP*). As an initial confirmation of the expression profiling results, real-time PCR was performed on the same pooled samples used in the microarray experiments. All five genes were strongly up-regulated to levels ranging between four- and 18-fold above control (Fig. 2).

Next we sought to validate independently the expression of these genes in a separate series of experiments in which six rats received infusion of BDNF or cytochrome c (Cyt C), a protein control similar to BDNF in molecular weight and charge (Fig. 3). BDNF infusion led to a significant 51% increase in the fEPSP slope whereas Cyt C infusion had no effect on synaptic efficacy during 3 h of post-infusion recording (Fig. 3A), in agreement with earlier reports (Messaoudi *et al.*, 2002; Ying *et al.*, 2002; Gooney *et al.*, 2004). Real-time PCR analysis revealed significant up-regulation of all five transcripts in dentate gyrus samples from BDNF-LTP-treated rats ($P < 0.05$, $n = 5$). The magnitude of mRNA elevation ranged from four- to 18-fold. By contrast, mRNA levels in Cyt C-treated animals remained at control levels for all genes (Fig. 3B). Taken together, these PCR findings strongly validate the results from the microarray screen and couple the five candidate genes to BDNF-LTP. Finally, we sought to examine gene product expression by Western blotting. For this purpose we focused on NARP, for which well-characterized antibodies were available. Consistent with the gene expression data, NARP protein expression was significantly elevated following BDNF-LTP induction, but not after Cyt C treatment (Fig. 3C and D; $P < 0.05$).

Up-regulation of BDNF-LTP-regulated genes in dentate granule cells

Next we sought to determine the cellular distribution of the BDNF-LTP-regulated genes by non-radioactive *in situ* hybridization histochemistry. As shown in Fig. 4A, mRNA levels for all five genes were up-regulated within the granule cell layer of the BDNF-infused dentate gyrus. No changes were seen in the molecular layer, indicating an absence of significant mRNA trafficking to granule cell dendrites. The basal distribution of four of the candidate genes (*Narp*, *neuritin*, *TIEG1*, *CARP*) in brain tissue matched previous anatomical descriptions (Naeve *et al.*, 1997; Hevroni *et al.*, 1998; Reti *et al.*, 2002).

ADP-ribosylation factors (ARFs) and ARF-like proteins (ARLs) belong to the Ras superfamily of small GTP-binding proteins. ARFs are important in membrane trafficking events, but the function of the ARL GTPase family is little understood (Pasqualato *et al.*, 2002; Burd *et al.*, 2004). *ARL4L* mRNA was expressed at low levels in cellular layers of the hippocampus. Following BDNF-LTP induction, strong up-regulation of *ARL4L* was observed specifically in granule cell somata. Semi-thin sections (10 μm thick) were examined at high magnification in order to evaluate the subcellular staining pattern of the three most strongly expressed mRNAs (*Narp*,

TABLE 2. mRNAs up-regulated following BDNF-LTP induction in the rat dentate gyrus *in vivo* (four-fold cut-off)

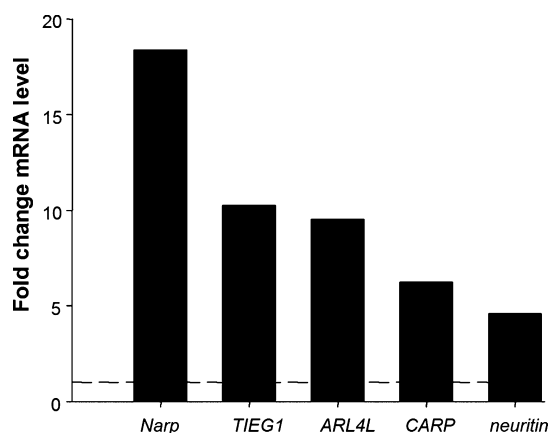| mRNA i.d.	Accession no.	Gene	Fold increase
Transcription			
UI-R-A0-bb-h-04-0-UI	NP_112397	TGFβ-inducible early growth response (*TIEG1*)	12.1
UI-R-C0-jr-e-12-0-UI	AF009330.1	Enhancer-of split and hairy-related protein 2 (*SHARP-2*)	10.5
UI-R-E0-dm-d-05-0-UI	XM_216837	Thyroid hormone receptor associated protein 5 (*Thrap5*)	6.1
Neurite outgrowth			
UI-R-C1-js-g-08-0-UI	P97738	Neuronal activity-regulated pentraxin (*NARP*)	17.1
UI-R-A1-ev-b-01-0-UI	NM_053346	*Neuritin*	4.9
Signaling			
UI-R-C0-jd-c-08-0-UI	XM_220933	ADP-ribosylation factor-like protein-4 (*ARL4L*)	8.1
Others			
UI-R-E1-gh-d-08-0-UI	AF030089	Calcium/calmodulin kinase-related peptide (*carp*)	11.3
UI-R-C1-kv-e-01-0-UI	XM_230899	Nedd4 WW-binding protein 4	8.0
UI-R-E1-gb-b-03-0-UI	XM_214836	Fibrillarin	6.1
Expressed sequence tags with no similarity to characterized proteins			
UI-R-C0-hi-d-07-0-UI	BF561272		11.5
UI-R-C0-hu-b-07-0-UI	AA997849		7.8
UI-R-A0-aw-c-08-0-UI	BF549853		6.9
UI-R-A0-an-c-07-0-UI	AA819615		5.6
UI-R-BT0-qg-c-11-0-UI	AI146042		4.5

FIG. 2. Real-time PCR confirmation of BDNF-LTP regulated genes in samples used for microarray expression profiling. Threshold real-time PCR was performed in the same pooled dentate gyrus samples used for expression profiling. Values are normalized to *tubulin* mRNA and expressed as the fold change between BDNF-infused and control dentate gyrus. All values are means of triplicate measurements.

neuritin and *ARL4L*) (Fig. 4B). *Narp* and *neuritin* mRNA labeling was restricted to the cytoplasmic margin of the cell body. By contrast, *ARL4L* showed a unique pattern of staining characterized by one or two darkly stained puncta. This punctate staining pattern of *ARL4L* mRNA was not detected in granule cells of non-treated tissue.

Induction of the BDNF-regulated genes during HFS-induced LTP

If these genes are part of a core transcription program underlying synaptic plasticity, one would expect to see up-regulation of one or more of these genes following HFS-LTP. Confirming this prediction, *in situ* hybridization revealed up-regulation of all five genes in dentate granule cells 30 min after HFS-LTP induction. The strongest increases at this time point were observed for *Narp*, *neuritin* and *ARL4L*
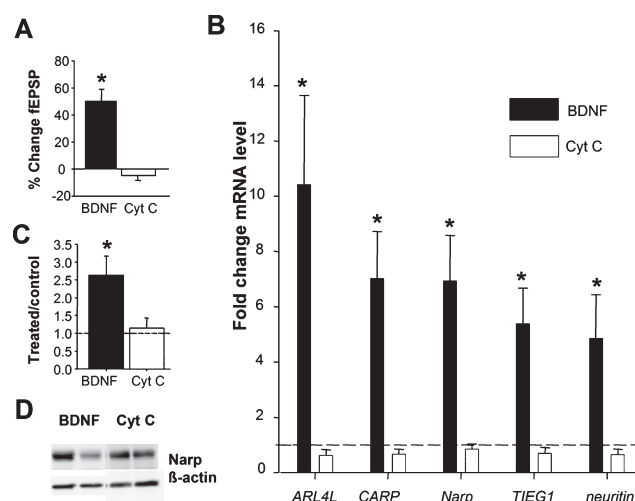

FIG. 3. Independent confirmation of BDNF-LTP-regulated genes by real-time PCR. (A) Bar graphs shows mean changes in fEPSP slope recorded 3 h after BDNF ($n = 6$) or Cyt C ($n = 6$) infusion. (B) Threshold real-time PCR was performed in samples from individual dentate gyrus. Values are mean (+ SEM) fold change in mRNA levels relative to contralateral control (*$P < 0.05$). (C) Group mean + SEM changes in Narp protein immunoreactivity levels in dentate gyrus homogenate samples. Optical density values are expressed as the ratio treated/control. Narp protein expression was elevated following BDNF-LTP treatment, but not after Cyt C infusion ($n = 3$ for both groups; *$P < 0.05$). (D) Representative Narp Western blots and β-actin loading control.

(Fig. 5). Again, high-magnification images of thin sections revealed punctate nuclear staining of *ARL4L* mRNA in granule cell somata (data not shown).

BDNF-LTP is associated with up-regulation of VGF and c-fos, but not of rab3a

Next we examined expression of several genes of specific interest in the context of BDNF modulation that were not represented on the microarray. BDNF incubation of immature hippocampal neurons in
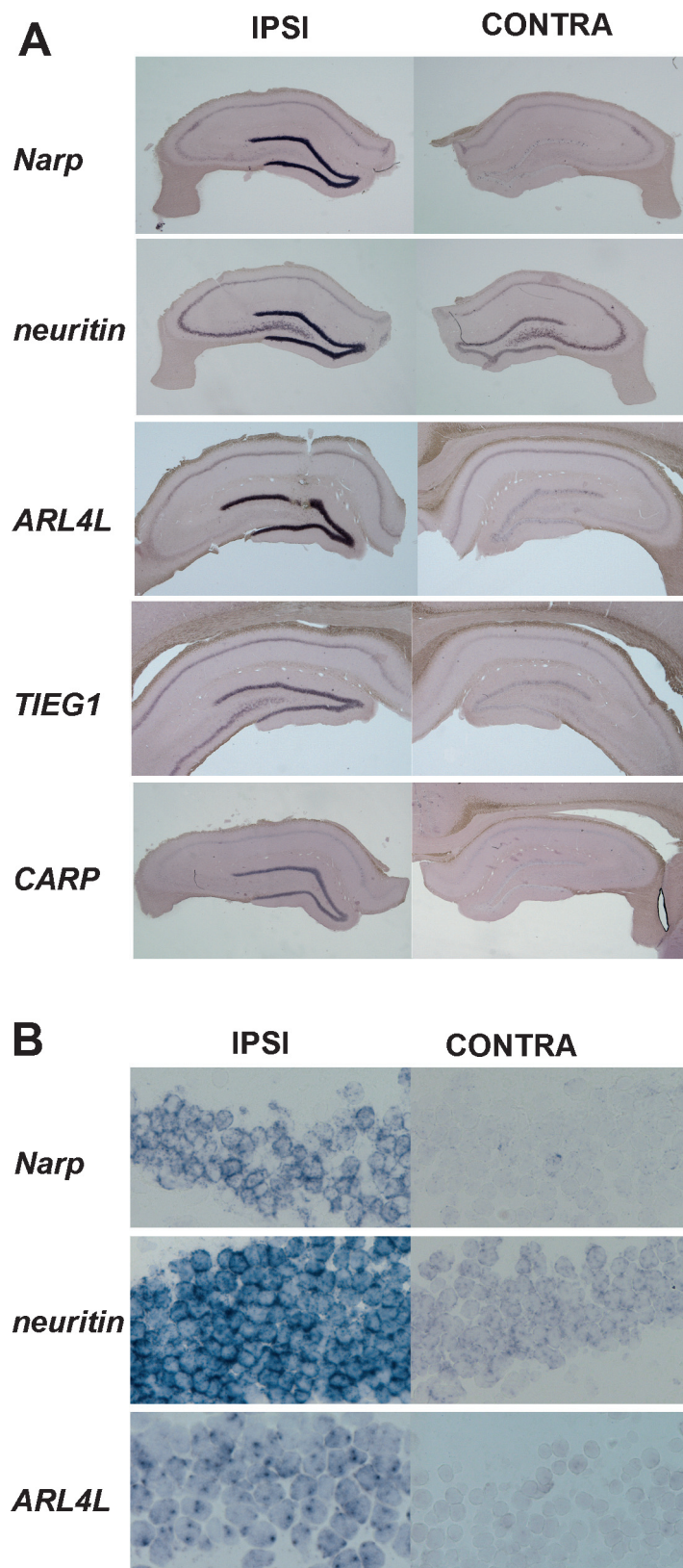
FIG. 4. Localization of novel BDNF-LTP-regulated genes by *in situ* hybridization histochemistry. (A) Representative staining of digoxigenin-labeled cRNA probes for *NARP*, *neuritin*, *ARL4L*, *TIEG1* and *CARP* in ipsilateral (IPSI) and contralateral (CONTRA) hippocampus 3 h after BDNF infusion. All mRNAs were strongly up-regulated in the ipsilateral granule cell layer. Similar results were obtained in three experiments. (B) Subcellular mRNA labeling pattern in granule cell somata. High-magnification images of *in situ* hybridization labeling in 10-μm-thick coronal sections. Note the unique puntate pattern of *ARL4L* labeling, compared with the cytoplasmic distribution of *Narp* and *neuritin*.
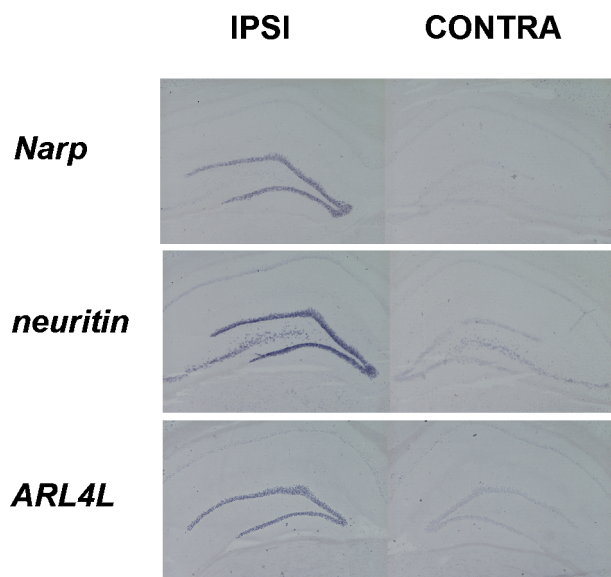
FIG. 5. Up-regulation of BDNF-regulated genes following HFS-LTP induction. (A) Representative staining of digoxigenin-labeled cRNA probes for *NARP*, *neuritin* and *ARL4L* in ipsilateral (IPSI) and contralateral (CONTRA) hippocampus 30 min after HFS. All mRNAs were strongly up-regulated in the ipsilateral granule cell layer. Similar results were obtained in three experiments.
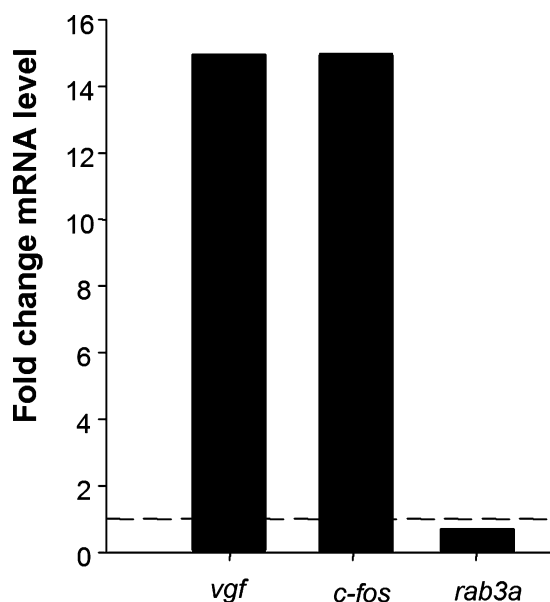

FIG. 6. BDNF-LTP in the adult dentate gyrus is associated with up-regulation of *VGF* and *c-fos*, but not *Rab3a*, mRNA expression. Threshold real-time PCR was performed in the same pooled dentate gyrus ($n = 6$) samples used for expression profiling. Values are normalized to *tubulin* mRNA and expressed as fold change between BDNF-infused and control dentate gyrus. All values are means of triplicate measurements.

cell culture results in a transient potentiation of synaptic transmission associated with induction of *VGF* (non-acronymic), *c-fos* and *Rab3a* mRNA (Thakker-Varia *et al.*, 2001; Alder *et al.*, 2003). Here we investigated whether these genes are up-regulated in the context of persistent transcription-dependent BDNF-LTP in the adult brain. BDNF-LTP was associated with a 14-fold increase in *VGF* and *c-fos* mRNA levels, whereas *Rab3* mRNA expression remained at control levels (Fig. 6).

Up-regulation of total and exon 3-specific BDNF expression during BDNF-LTP

We considered that BDNF may regulate its own transcription either as part of a transcriptional program underlying synaptic plasticity, or simply to replenish depleted stores of BDNF protein. The rat *BDNF* gene consists of one 3′ exon that encodes the BDNF protein and four different promoter-specific 5′ exons (Timmusk *et al.*, 1993). Alternative promoter usage and differential splicing generates four unique transcripts coding for the same protein. The promoters are differentially regulated by signaling pathways that may allow context-specific transcription and cellular localization of *BDNF* transcripts (Shieh *et al.*, 1998; Tao *et al.*, 1998). Here we examined the expression of *BDNF* using specific probes for exon III, a highly calcium-responsive promoter, and exon V, which recognizes all *BDNF* transcripts. Real-time PCR showed up-regulation of both exon III- and exon V-containing *BDNF* transcripts following BDNF-LTP, but not after Cyt C infusion (Fig. 7A). *In situ* hybridization revealed elevated expression of exon III and exon V *BDNF* mRNA specific to the granule cell layer (Fig. 7B).

Discussion

Novel BDNF-LTP coupled genes identified by microarray expression profiling

Using microarray expression profiling we have shown that *Narp*, *neuritin*, *CARP*, *TIEG1* and *ARL4L* are co-upregulated with *Arc* following BDNF-LTP. Up-regulation of these transcripts was confirmed by real-time PCR analysis in the samples used on the microarray as well as by PCR analysis in an independent series of BDNF-LTP experiments. The fact that infusion of Cyt C had no effect on synaptic efficacy or gene expression rules out possible non-specific effects related to electrode insertion, pathway stimulation or protein infusion. *In situ* hybridization further demonstrated up-regulation of these genes in dentate granule cells following both BDNF-LTP and classical HFS-LTP. Enhanced expression of Narp was also shown at the protein level.

Formation of stable HFS-LTP is associated with insertion of glutamate receptors at post-synaptic membranes, an increase in post-synaptic density diameter and structural remodeling of spines (Geinisman, 2000; Weeks *et al.*, 2001; Harris *et al.*, 2003). These changes are all intimately connected with regulation of actin dynamics (Lisman & Zhabotinsky, 2001; Zhou *et al.*, 2001; Fukazawa *et al.*, 2003; Matsuzaki *et al.*, 2004; Okamoto *et al.*, 2004; Zito *et al.*, 2004; Oertner & Matus, 2005). Persistent LTP is thought to occur when small spines are converted to large mushroom-shaped spines through a mechanism dependent on actin polymerization (Matsuzaki *et al.*, 2004). Recent work employing *Arc* antisense application at multiple time points during HFS-LTP maintenance has identified a critical window of sustained Arc synthesis that is necessary for stable LTP formation and actin polymerization at synapses. Furthermore, Arc synthesis is necessary for the induction and time-dependent consolidation of BDNF-LTP (Bramham & Messaoudi, 2005; Soule *et al.*, 2005). Thus, a process of synaptic consolidation involving Arc synthesis, actin polymerization and synapse expansion is emerging. The present results identify a panel of genes that are simultaneously up-regulated with Arc. These genes are prime candidates for loss-of-function studies on consolidation of synaptic plasticity and memory, particularly in the context of co-ordinate mechanisms requiring Arc synthesis. With the exception of *ARL4L*, all five genes are known to be activity-induced genes. *Narp* and *TIEG1* are also known to be induced following HFS-LTP (Tsui *et al.*, 1996; Hevroni *et al.*, 1998).
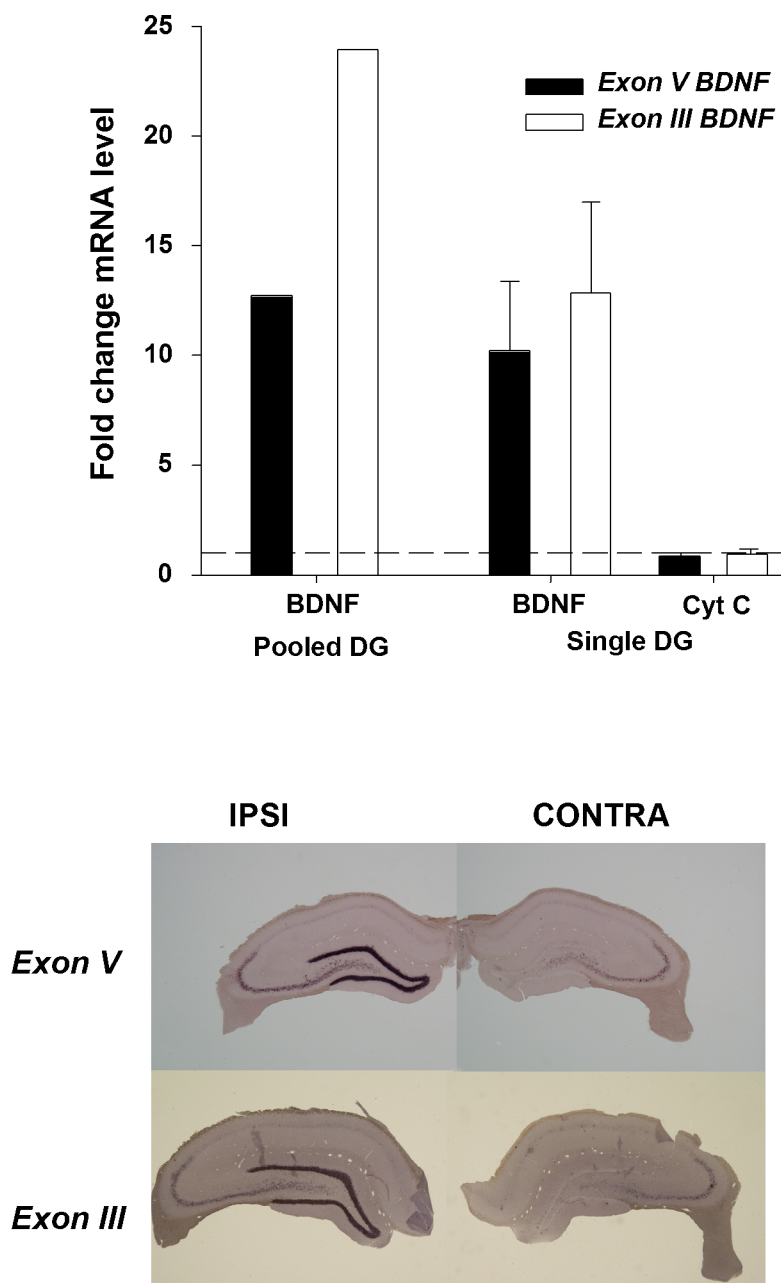
FIG. 7. BDNF-LTP induction is associated with up-regulation of exon III promoter-specific *BDNF* gene expression. (A) Fold changes in exon V (total) and exon III promoter-specific *BDNF* mRNA levels. Measurements from pooled samples were performed in triplicate and averaged. Measurements from single dentate gyrus are based on an independent series of animals which received BDNF or Cyt C infusion (values are means + SEM). (B) Representative staining of digoxigenin-labeled cRNA probes for exon V- and exon III-specific *BDNF* mRNA in the ipsilateral (IPSI) and contralateral (CONTRA) hippocampus. Marked up-regulation was observed in the ipsilateral granule cell layer. Similar results were obtained in three experiments.

Neuritin and Narp play important roles in synapse formation and maturation during development. Neuritin, also known as Candidate Plasticity Gene 15, is a small extracellular protein that attaches to the membrane through a glycosylphosphatidylinositol anchor. During development, neuritin promotes dendritic and axonal arbor growth as well as synapse maturation (Naeve *et al.*, 1997; Nedivi *et al.*, 1998, 2001; Cantallops *et al.*, 2000; Di Giovanni *et al.*, 2005; Javaherian & Cline, 2005). In early postnatal life neuritin is induced by sensory stimuli such as light in the visual cortex and by intracerebroventicular injection of BDNF (Naeve *et al.*, 1997; Corriveau *et al.*, 1999). Narp, a member of the pentraxin family of secreted calcium-dependent lectins, was originally identified following a screen for seizure-regulated genes (Tsui *et al.*, 1996). Narp is enriched at excitatory synapses and is expressed on axon terminals and dendrites. Like other pentraxins, secreted Narp forms multimeric complexes. Narp com-plexes through its N-terminal coiled-coil domain with neuronal pentraxin 1, while the C-terminal domain associates with AMPA-type glutamate receptors. Narp induces clustering of AMPA receptors and plays a critical role in synaptogenesis of excitatory synapses (O'Brien *et al.*, 1999, 2002; Xu *et al.*, 2003).

ARFs and ARLs belong to the Ras superfamily of small GTP-binding proteins. ARF proteins have well-established functions in membrane trafficking pathways (Jacobs *et al.*, 1999; Pasqualato *et al.*, 2002). The role of ARL GTPases is only beginning to emerge and their functions appear highly diverse and distinct from traditional ARFs (Burd *et al.*, 2004). ARL4L (also known as ARF4L) is a member of a subgroup of ARL proteins characterized by the presence of a nuclear localization signal and rapid nucleotide exchange activity (Jacobs *et al.*, 1999). ARL GTPases have been suggested to serve as adaptors for cargoes lacking nuclear

localization signals. Work on ARL4L-mutated proteins also suggests an important role in vesicle and protein trafficking between endosomes and the plasma membrane (Katayama *et al.*, 1998, 2004). Recently, a member of this gene family, ARL6, has been identified as the gene underlying Bardet–Biedl syndrome, a pleiotropic disorder that includes a neuronal component characterized by learning disability (Chiang *et al.*, 2004; Fan *et al.*, 2004). The present study specifically couples *ARL4L* to long-term synaptic plasticity. Interestingly, *ARL4L* mRNA was expressed in distinct nuclear punta. These puncta resemble nuclear structures such as PcG domains and coiled bodies. PcG domains are suggested to represent gene-silencing complexes and coiled bodies are involved in snRNP maturation (Lamond & Earnshaw, 1998; Matera, 2003). Whether nuclear *ARL4L* transcripts are associated with these or other nuclear structures requires further investigation.

TIEG1 encodes a Krüppel-like zinc-finger transcriptional repressor belonging to the Sp1 family of transcription factors (Subramaniam *et al.*, 1995; Suske, 1999). TIEG1 is an IEG effector of TGF-β and is induced in brain following kainic acid treatment and LTP induction (Subramaniam *et al.*, 1995; Hevroni *et al.*, 1998). *TIEG1* overexpression mimics TGF-β function in several cell types. In *Drosophila*, TGF-β also activates retrograde synaptic signaling pathways to regulate homeostatically transmitter release and synaptic density at nerve–muscle synapses (Sanyal *et al.*, 2004). In *Aplysia*, TGF-β promotes long-term facilitation (Zhang *et al.*, 1997). The present data suggest that TGF-β-TIEG1 signaling is modulated in the context of BDNF-induced synaptic plasticity.

CARP, also known as Ania-4, encodes a 55-amino acid IEG peptide (Berke *et al.*, 1998). CARP and the related doublecortin CaMK-like kinase, DCAMKL, are alternative splice products from the same gene (Vreugdenhil *et al.*, 1999, 2001). CARP lacks catalytic activity and is structurally similar to the autoinhibitory domain of CaMKIV. CARP, but not DCAMKL, are induced following kainic acid seizures. The role of CARP is unclear but it has been suggested to modulate CaMK activity and doublecortin function.

The four genes not selected for independent validation at this time are Nedd4 WW-binding protein-4 (N4WBP4), thyroid hormone receptor-associated protein (Thrap5), fibrillarin and enhancer of Split and HAiry Related Protein 2 (Sharp-2). Although these genes need to be confirmed, they are likely to reflect true modulations as five similarly modulated genes were all confirmed. N4WBP4 interacts with the WW domain of Nedd4, a ubiquitin protein ligase implicated in nervous system development and axon guidance (Jolliffe *et al.*, 2000; Wilkin *et al.*, 2004; Bakkers *et al.*, 2005). Thrap5 is part of a multimeric Trap-mediator complex that co-purifies with the thyroid hormone receptor. This Trap-mediator complex is thought to serve as a co-activator for a broad range of nuclear hormone receptors and other transcriptional activators (Ito *et al.*, 1999). Fibrillarin (also known as as nucleolar protein 1) is a component of a nucleolar small ribonucleoprotein particle thought to participate in processing of preribosomal RNA (Turley *et al.*, 1993). Sharp-2 is a member of a novel group of basic helix–loop–helix (bHLH) proteins distantly related to *Drosophila enhancer-of-split* and *hairy* proteins (Rossner *et al.*, 1997). bHLH proteins are involved in the determination of progenitor cells and regulation of neuronal differentiation at the level of transcription. In contrast to other bHLH proteins, SHARP expression commences at the end of embryonic development, thus marking differentiated neurons. SHARP-2 is induced with rapid kinetics following kainic acid-induced seizures and is up-regulated by NGF treatment in PC12 cells (Rossner *et al.*, 1997). This is the first study coupling these genes to BDNF treatment and synaptic plasticity in the adult brain.

VGF, but not Rab3a, is induced during BDNF-LTP: comparison with findings from hippocampal cell culture

The modulation of gene expression by BDNF has previously been studied in hippocampal neuronal cell cultures (Thakker-Varia *et al.*, 2001; Alder *et al.*, 2003). The present work in the adult brain shows intriguing similarities and differences with the findings from cell culture studies. Incubation of immature hippocampal neurons in BDNF elicits potentiation of excitatory synapses that lasts 10–15 min. This transient BDNF-induced potentiation is associated with enhanced expression of the secretory peptide VGF and is mimicked by VGF application. VGF is also induced following HFS-LTP and eye-blink associative learning (Snyder *et al.*, 1998; Alder *et al.*, 2003). The present data showing enhanced expression of VGF during BDNF-LTP further couple this peptide to synaptic plasticity. In cell culture, expression of the presynaptic trafficking protein Rab3a is induced by BDNF and necessary for the presynaptic component of the potentiation (Alder *et al.*, 2005). Although BDNF-LTP in the adult dentate gyrus is associated with a lasting increase in evoked glutamate release (Gooney *et al.*, 2004), we did not observe enhanced expression of *Rab3a*. However, it is possible that Rab3a regulation occurs in the cell bodies of neurons in the entorhinal cortex that give rise to the perforant path. Indeed, recent work shows that BDNF infusion into the dentate gyrus leads to cAMP response element binding protein activation in the entorhinal cortex, suggesting activation of a retrograde signaling pathway (Gooney *et al.*, 2004). Such regulation of Rab3a in the entorhinal cortex would not be detected in microdissected dentate gyrus.

Autoregulation of BDNF transcription in synaptic plasticity

BDNF is capable of stimulating its own release through mobilization of intracellular calcium stores via TrkB-coupled phospholipase C (Canossa *et al.*, 1997). Such a regenerative mechanism may underlie the prolonged secretion of BDNF that follows HFS-LTP induction (Aicardi *et al.*, 2004; Gooney *et al.*, 2004; Bramham & Messaoudi, 2005). BDNF gene expression is enhanced following HFS-LTP induction (Patterson *et al.*, 1992; Castren *et al.*, 1993; Bramham *et al.*, 1996; Lee *et al.*, 2005), and this increase lasts at least 24 h in the dentate gyrus of freely moving rats (Bramham *et al.*, 1996). The present results show that BDNF can stimulate its own transcription in the context of long-term synaptic plasticity and that a major component of this response is attributable to the highly calcium-responsive exon III promoter (Tao *et al.*, 1998). The key question of whether new BDNF transcription actually contributes to synaptic strengthening remains to be addressed. It is just as likely that BDNF transcription serves only to replenish depleted stores of secreted BDNF protein.

Conclusions

This microarray screen identified and validated a panel of genes that are co-upregulated with *Arc* during BDNF-induced LTP. These genes are candidate effectors of BDNF-controlled consolidation in synaptic plasticity and memory (Lee *et al.*, 2004; Barco *et al.*, 2005; Bramham & Messaoudi, 2005). Consistent with a process of synaptic growth and remodeling, *Arc* is co-upregulated with several genes (*Narp*, *neuritin*, *TIEG1*, *N4WBP4*) involved in synaptogenesis, axon guidance and glutamate receptor clustering. Although it is important to pursue the function of these robust modulations, more studies are needed to map out smaller modulations of gene expression on a genome-wide scale over time. Finally, it should be pointed out that BDNF signaling has recently been implicated in unipolar depression and the action of antidepressant

drugs (Castren, 2004). Indeed, brief infusion of BDNF into the rat dentate gyrus using a protocol not unlike the one used here has behavioral antidepressant-like effects (Shirayama *et al.*, 2002). The identification of BDNF-regulated genes in the adult dentate gyrus may therefore be of importance for a range of long-term adaptive responses.

Acknowledgements

Abbreviations

ARFs, ADP-ribosylation factors; ARLs, ARF-like proteins; BDNF, brain-derived neurotrophic factor; bHLH, basic helix–loop–helix; Cyt C, cytochrome C; fEPSP, field excitatory post-synaptic potential; HFS, high-frequency stimulation; LTP, long-term potentiation.

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