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Biochemical and Biophysical Research Communications 322 (2004) 210–216
BBRC
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Regulated transcripts in the hippocampus following transections of the entorhinal afferents

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Received 20 June 2004
Available online 7 August 2004

Abstract

Based on the data from a cDNA microarray experiment which was carried out to screen the differential expressed genes in the rat hippocampus 10 days after removal of the entorhinal afferents, we confirmed the increase of expression of eight transcripts encoding protein osteonectin, thymosin-β4, gelsolin, MHC I, MHC II, β2-microglobulin, and interferon-γ receptor using Northern blot. In situ hybridization revealed that the up-regulation of all these 8 transcripts localized specifically in the denervated target areas, the hippocampal stratum lacunosum-moleculare, and the dentate outer molecular layer. The results suggest that these molecules may have roles in the plasticity events in the hippocampus after entorhinal deafferentation.

Keywords: Deafferentation; Plasticity; Northern blot; In situ hybridization

Entorhino-hippocampal system is a well-established model for the analysis of target-specific axonal sprouting and structural re-organization of adult brain tissues in response to injury. Entorhinal fibers originating from stellate neurons in layer II and pyramidal cells in layer III of the entorhinal cortex (EC) project to their targets, the hippocampal stratum lacunosum-moleculare (SLM) and the dentate outer molecular layer (OML) via the perforant pathway (PP) and the alvear pathway [1,2]. After entorhinal deafferentation, the intact fibers within the denervated hippocampus, including the crossed entorhinal fibers, commissural/associational fibers, and septo-hippocampal fibers, undergo reactive collateral sprouting and terminal proliferation which finally form new synapses with post-synaptic partners [3–7]. An outstanding feature of the plasticity events is that the sprouting of remaining axons is restricted within their original target zones [8]. Analogous to this layer-specific sprouting of axons in the hippocampus, the axons from the embryonic EC transplanted into the entorhinally deafferented adult hippocampus were found to specifically grow within the denervated host SLM and OML [9]. Recently, a stripe assay reveals that cell membranes obtained from either the entorhinally denervated adult hippocampus or the neonatal hippocampus are attractive substratum for growing axons from neonatal EC explants, while those obtained from the normal adult hippocampus are not [10]. These in vitro and in vivo experiments strongly suggest that following deafferentation, the adult hippocampus re-expresses outgrowth-promoting and/or guidance molecules for EC axons and other proper afferent fibers, which exist in the developing but not normal adult hippocampus. It has been documented that a variety of molecules belonging to distinct functional categories, including immediate-early gene products [11], cytoskeletal proteins [12,13],

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.07.099

neurotrophic factors [14,15], cell adhesion molecules [16,17], and extracellular matrix proteins [18,19], are differentially regulated in the hippocampus after injury, but none of these changes are sufficient to convincingly account for the layer-specific plasticity in the denervated hippocampus [20].

Based on the data from a cDNA microarray experiment, which was performed to screen the differentially expressed genes in the denervated hippocampus, we identified eight new transcripts with up-regulated expression using Northern blot and in situ hybridization in the present study. The functional significance of these molecules in the plasticity in the denervated hippocampus was subsequently discussed.

Materials and methods

Animals and surgery. Adult female Sprague–Dawley rats (body weight 220–250 g) and ICR mice (20–25 g) housed under standard laboratory conditions were used in the present study. All procedures for animals were performed in agreement with the SIBS Guide for the Care and Use of Laboratory Animals and approved by Animal Care and Use Committee, Shanghai Institutes for Biological Sciences. Rats were used for cDNA microarray screening and Northern blot analysis and mice for in situ hybridization. The procedure for performing transections of the entorhinal afferents has been previously described elsewhere [21].

cDNA microarray analysis. Microarray experiment was performed on Incyte Genomics rat central nervous system (CNS) microarray that contains more than 8000 cDNA elements [represent 6234 sequence verified genes and expressed sequence tags (ESTs)]. At 10 days post-lesion (dpl), the entorhinally deafferented rats as well as the normal controls were decapitated under deep anesthesia and the brains were quickly removed. The hippocampus was carefully dissected, snap-frozen in liquid nitrogen and the total RNA was isolated. The pooled hippocampal total RNAs from 10 lesioned and 10 intact animals were directly sent to Incyte Genomics for further experiment. All the following experimental procedures, including poly(A)$^+$ RNA purification, probe labeling, hybridization, and data analysis, were conducted by Incyte Genomics. Briefly, poly(A)$^+$ RNAs purified, respectively, from both pooled total RNAs were reverse-transcribed to cDNAs with Cy5 and Cy3 primers, respectively. The two fluorescence-labeled cDNA samples were then combined to use as probes to hybridize the same microarray. After hybridizing and washing, the microarray was scanned using a two-color laser scanning fluorometer, and the signal intensities were color-coded. Data analysis was performed at Incyte company using GermTools (Incyte's proprietary software) and the results were exported to MS excel 2000 for further analysis. (Incyte microarray business has been incorporated into Agilent Technologies; for technical information concerning microarray experiments, refer to http://www.chem.agilent.com/Scripts/PCol.asp?lPage=494.)

Northern blot analysis. Total RNA was extracted from the frozen denervated 10dpl and normal rat hippocampus ($n = 3$ for each group) using acid guanidinium–isothiocyanate–phenol method [22], electrophoresed (20 μg/lane) on a 1% formaldehyde agarose gel, blotted onto nylon membrane, cross-linked by ultraviolet fixation, and hybridized as described elsewhere [23]. DNA probes for target transcripts were synthesized from RT-PCR amplified cDNAs using the random primed DNA labeling kit (Roche). The GenBank accession number, PCR primers, and annealing temperature for each target cDNA are listed in Table 1. The sequences of all PCR amplified cDNAs have been verified by DNA sequencing after being cloned into pGEM-T Easy vectors (Promega). To control the total RNA amount loaded, GAPDH transcript was used as an internal standard in each Northern blot.

In situ hybridization histochemistry. At 10 days following surgery, the unilaterally deafferented mice ($n = 5$) were anesthetized deeply with overdose pentobarbitone sodium and trancardially perfused with ice-cold fixative of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. The brain was dissected out, post-fixed for 4 h at 4 °C, incubated in 30% sucrose to sink, and cut coronally at 30 μm on a cryostat. In situ hybridization was carried out for the genes whose differential expression had been verified by Northern blot. PCR amplified cDNAs were cloned into pGEM-T Easy vector (Promega), linearized, and digoxigenin-labeled antisense and sense riboprobes were synthesized using in vitro transcription kit (Roche). The procedure of in situ hybridization for free-floating section was described elsewhere [23]. The in situ hybridization for each transcript was repeatedly performed on at least three animals with unilateral entorhinal deafferentation.

The staining for acetylcholinesterase (AChE) was used to examine whether entorhinal deafferentation is correct [3], and only the animals with complete transections of the unilateral entorhinal afferents were used for further processing.

Table 1
The primers and conditions of PCR products

Gene name	Accession No.	Primer pair	Annealing T_m (°C)
MHC I	U38972	5′-gatgtcaccctgaggtgctg-3′ 5′-ggcatgtgtaattctgctcc-3′	55
MHC II	Y00480	5′-actgtgaggtggatcactg-3′ 5′- cagtgatccacctcacagtc-3′	56
β2-Microglobulin	Y00441	5′-ttgccattcgaaactccc-3′ 5′-tggtccagatgattcagagc-3′	54
Interferon-γ receptor	AF201901	5′-tgattgtccacatatttcac-3′ 5′-tccgtcctcgtatttcaccc-3′	55
Profilin	X96967	5′-ctgcaagacggggaatttac-3′ 5′-tggcaccaataagggaaatg-3′	55
Thymosin-β4	M34043	5′-cagcaaccatgtctgacaaa-3′ 5′-tcattccaccatctccca-3′	56
Gelsolin	J04953	5′-tgatgcctatgtcatcctgaag-3′ 5′-atttgttgctgccagagcc-3′	57
Osteonectin	M20692	5′-ggaagttacagacacaggta-3′ 5′-gacatcgaagaaaatggggag-3′	55

Results

From more than 8000 cDNA elements that actually represent 6234 unique genes and ESTs, the microarray screening experiment identified 152 genes/ESTs with 1.5- to 2.8-fold change in expression in the hippocampus 10 days following injury, in which the most differentially regulated transcript was glial filament acidic protein (GFAP) whose expression was increased to 2.8-fold of control. Based on their known function, these candidate transcript-encoded proteins could be roughly classified into the following categories: immunity-associated molecules, cellular environmental response molecules (stress, acute inflammation, and wound healing), cytoskeleton and associated proteins, protein turnover and maintenance molecules, cell membrane transporters and ion channels, extracellular matrix molecules, growth factors/cytokines and their receptors, molecules involved in metabolism, signal transduction components, cell adhesion and recognition molecules, and proteins involved in transcription/translation and cell secretion/trafficking (Table 2).

Since a large portion of screened transcripts in microarray experiment encode immunity and cytoskeleton-associated proteins, Northern blot analysis was carried out to test the differential expression of a set of transcripts located in these two categories. From 12 tested candidate transcripts, we verified that 8 of them, MHC I, MHC II, β2-microglobulin (β2-M), interferon-γ receptor (IFN-γR), thymosin-β4, gelsolin, profilin, and osteonectin, were up-regulated in the denervated hippocampus, respectively (Fig. 1).

To further confirm the expression patterns of the selected genes, we performed in situ hybridization to determine the tissue distribution of the 8 up-regulated transcripts in the animals with successful unilateral entorhinal transections demonstrated by AChE staining (data not shown). As shown in Fig. 2, in situ hybridization experiments clearly demonstrated that all these eight transcripts are up-regulated in the hippocampus ipsilateral to the injury compared to the contralateral tissue. Interestingly, although these transcripts distributed thoughout the sub-regions of the hippocampus, the up-regulation specifically occurs within the denervated target zones, the hippocampal SLM and dentate OML (Fig. 2). The parallel control experiment showed that the hybridization with sense probes for all transcripts was completely devoid of any signal (data not shown).

Discussion

In the present study, we examined the global gene expression regulation in the hippocampus following entorhinal deafferentation by using Incyte CNS cDNA

Table 2
Differentially regulated genes identified by cDNA microarray experiment in the denervated rat hippocampus[a]

Gene name	Fold[b]
Immunity	
Rat liver α2-macroglobulin	2.2
Rat MHC class II-associated invariant chain	2.2
Rat MHC class I RT1.O type −149 pseudogene	1.9
Rat MHC class I RT1.Au heavy chain precursor	1.9
Rat RT1.B-1 chain	1.9
Rat mature MHC class Ib α-chain	1.9
Rat β2-microglobulin	1.9
Rat MHC class Ia A2b antigen	1.7
Rat MHC class II-α chain RT1.D α	1.7
Mouse TL antigen	1.5
Rat MHC class II antigen RT1.B-1 β-chain	1.5
Mouse MHC class III region	1.5
Rat interferon-γ receptor	1.5
Rat integral membrane protein (RT1.D)	1.5
Mouse ASWS1 antibody heavy chain	1.5
Rat IgH chain VJ region	−1.7
Rat gene for CD1 antigen	−1.6
Rat Ig γ-chain variable region	−1.5
Mouse interleukin 2 receptor	−1.5
Environmental response	
Rat GPI-anchored ceruloplasmin	1.8
Rat transthyretin	−2.5
Rat cyclooxygenase-2	−2.0
Mouse ecotropic viral intetration site 2	−1.9
cysteine-rich secretory protein-1	−1.8
Rat heat shock protein 22 (Hsp22)	−1.8
Rat platelet phospholipase A2	−1.7
Rat cytochrome P450 IVA2	−1.6
Mouse serum amyloid A (SAA) 3 protein	−1.6
Rat pancreatitis-associated protein II	−1.6
Rat retinoblastoma-associated protein	−1.5
Breast cancer resistance protein 1, BCRP1	−1.5
Extracellular matrix	
Rat lumican	−1.9
Mouse col8a1	−1.7
Mouse α-1(XVIII) collagen (COL18A1)	−1.7
Mouse type XV collagen	−1.6
Rat mRNA for laminin γ 1	−1.5
Cytoskeleton-associated	
Rat glial fibrillary acidic protein (GFAP)	2.8
Rat profilin	2.5
Rat desmin	1.5
Rat thymosin-β4	1.5
Mouse gelsolin	1.5
Rat α-tropomyosin 2	−2.0
Mouse cofilin isoform	−1.8
Mouse microtubule-associated protein 44	−1.6
Rat utrophin	−1.6
Mouse periplakin	−1.6
Cofactor D	−1.6
Rat actin bundling protein (dematn)	−1.5
Rat troponin I	−1.5
Protein turnover/maintenance	
Rat cathepsin S	1.6
Rat cystatin C	1.6
Rat cathepsin B	1.5

(*continued on next page*)

Table 2 (*continued*)

Gene name	Fold[b]
Rat cathepsin H	1.5
SPI-1 serine protease inhibitor	−2.0
Rat plasminogen activator inhibitor 2	−1.9
Rat aminopeptidase A	−1.7
Mouse F-box protein FBA	−1.6
Rat pancreatic cationic trypsinogen	−1.5
Rat chymotrypsin B	−1.5
α-1 Antitrypsin	−1.5
Rat mast cell protease-3 precursor	−1.5
Membrane transporter/channel	
Putative anion transporter 1	1.5
Mouse opsin	−2.3
Mouse Ran-binding protein 2	−1.7
Rat sodium/glucose transporter	−1.7
Mouse cystine/glutamate transporter	−1.6
Mouse Na^+/myo-inositol cotransporter	−1.5
ABC transporter protein	−1.5
Rat potassium channel-Kv1	−1.5
Rat potassium channel protein	−1.5
Rat glutamate receptor subunit (GluR6)	−1.5
Mouse ARL-6 interacting protein-2	−1.5
Growth factors/cytokines and their receptors	
Rat osteoactivin	2.4
Rat CSF-1 receptor	1.5
PDGF β-receptor	−1.6
Rat c-kit receptor tyrosine kinase	−1.6
Rat plasma kallikrein	−1.6
Mouse mast cell growth factor (Mgf)	−1.5
Rat nerve growth factor-inducible protein	−1.5
Rat HGF activator	−1.5
Rat γ-tachykinin	−1.5
Carbohydrate/lipid/energy metabolism	
Rat apolipoprotein E (ApoE)	1.7
Rat salivary protein 1	−1.9
Rat ubiquitous mitochondrial creatine kinase	−1.7
Rat lanosterol 14-α-demethylase	−1.7
Rat liver stearyl-CoA desaturase	−1.6
Rat arylacetamide deacetylase	−1.5
Vacuolar adenosine triphosphatase subunit C	−1.5
Rat apolipoprotein B (apoB)	−1.5
Rat senescence marker protein 2B	−1.5
Transcription factor/nucleic acid modification	
DNase I	−2.2
Cricetulus griseus TRIP protein	−1.7
Rat nuclear receptor (RNR-1)	−1.7
Mouse Mit1/Lb9	−1.6
Mouse enhancer of polycomb (Epc1)	−1.6
Rat bHLH transcription factor Mist1	−1.5
M96A protein	−1.5
Signal transduction	
Rat G-protein β 5 subunit	1.6
Rat Na-K-Cl cotransporter (Nkcc1)	−1.7
Purinergic receptor P2Y5	−1.7
Mouse c-yes	−1.7
Rat TM6P1	−1.6
Small G protein	−1.6
Adhesion/cell recognition	
Mouse osteonectin	1.5
Rat N-cadherin	−2.0
Rat RoBo-1	−1.7
Rat protein S	−1.6
Mouse MUC18 glycoprotein	−1.5
Secretion/trafficking	
Rat ARF-like 3	1.5
Vesicle-associated membrane protein	−1.6
Mouse caveolin-2	−1.6
Mouse SEC23B protein	−1.5
Undefined	
KIAA0027 protein	1.5
Unnamed protein product	−2.0
KIAA0586 protein	−1.9
Mouse chromosome2 clone RP23-291P1	−1.7
Unnamed protein product	−1.6
Hypothetical protein	−1.6
Mouse brain cDNA, clone MNCb-4285	−1.5
Mouse clone RP23-117o7	−1.5
dJ206D15.3	−1.5
Mouse Kiaa0188	−1.5
Unknown gene product	−1.5
BM-017	−1.5

[a] The ESTs were not included.
[b] Minus sign means down-regulation of transcripts.

microarray. The microarray data are likely relatively reliable judged by its high coincidence with the assessment of Northern blot and in situ hybridization, and the identification of genes that have been reported differentially regulated in the denervated hippocampus such as GFAP [24], ApoD and ApoE [25], cathepsin S and cathepsin B [26], and cystatin C [23].

Of the candidate genes that are differentially regulated, eight were confirmed by Northern blot and in situ hybridization. The up-regulation of immune-associated molecules MHC I, MHC II, β2-M, and IFN-γ receptor is indicative of immune activities in the denervated hippocampus. Interestingly, recent studies revealed that immune molecule MHC participates in the neural plasticity processes in addition to their documented functions. In a screening for molecules involved in activity-dependent retina-lateral geniculate nuclei projections, MHC I was unexpectedly found to be differentially regulated in the lateral geniculate nuclei [27]. Furthermore, in MHC I-deficient mice, LTD disappears, while LTP is enhanced [28]. The β2-M knockout mice has the phenotype similar to that of MHC I-deficient mice [28]. These results suggest that the elevation of MHC antigen expression would be of functional significance in the plasticity events in the denervated hippocampus. It is thus interesting to use the MHC antigen-knockout mice as a model to test whether the structural re-organization process in the hippocampus is affected following entorhinal denervation. Since IFN-γ is a powerful inducer of MHC antigen expression

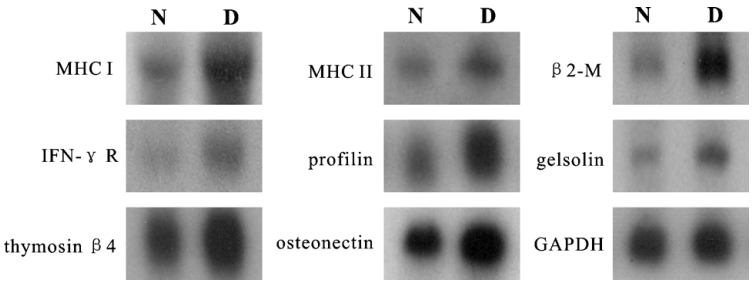

Fig. 1. Northern blot confirmation of the regulation of a subset of transcripts. The expression of transcripts for MHC I, MHC II, β2-microglobulin (β2-M), interferon-γ receptor (IFN-γR), profilin, thymosin-β4, gelsolin, and osteonectin is increased in the denervated hippocampus (D) compared with the normal control (N). GAPDH was used as internal standard to determine the equal RNA loading. Experiments are repeated three times for each transcript.

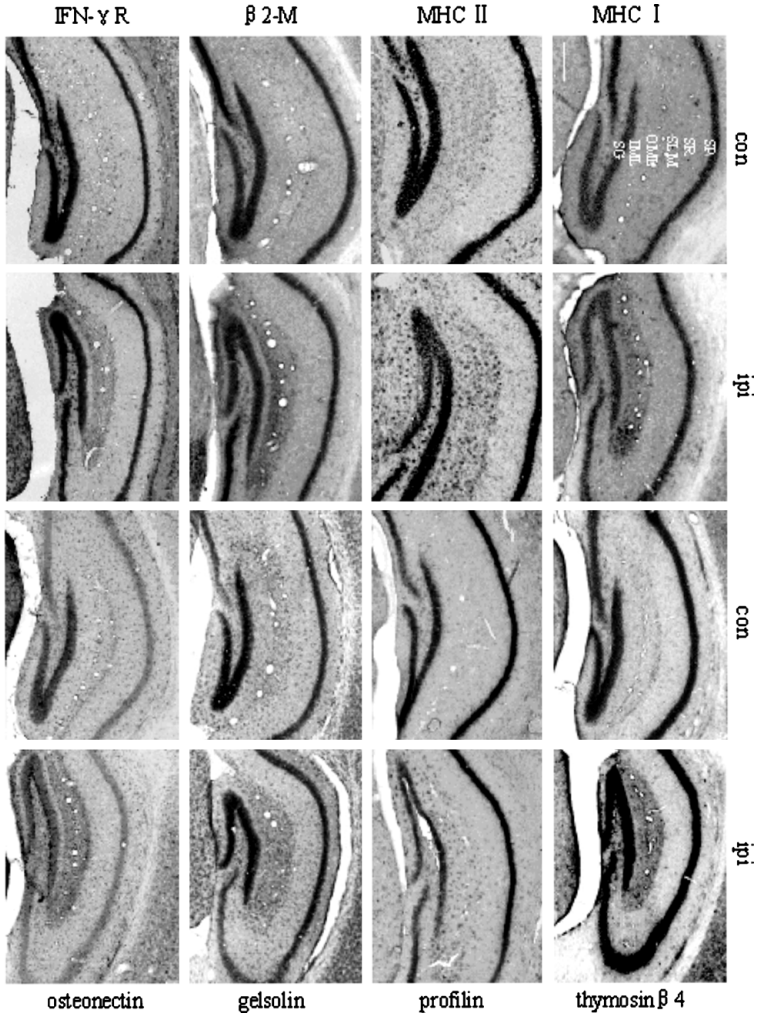

Fig. 2. In situ hybridization analysis of differentially expressed genes. Showed are representative results for MHC I, MHC II, β2-microglobulin (β2-M), interferon-γ receptor (IFN-γR), profilin, thymosin-β4, gelsolin, and osteonectin, revealing that these transcripts are up-regulated in the ipsilateral (ipi, right side) hippocampus compared with that in contralateral (con, left side) to the lesion. Note that the elevation specifically occurs within the entorhinally denervated layers, the SLM and OML. SP, stratum pyramidale; SR, stratum radiatum; SLM, stratum lacunosum-moleculare; OML, outer molecular layer; IML, inner molecular layer; and SG, stratum granulare. Scale bar, 200 μm.

in glial cells [29], the observed up-regulation of IFN-γ receptor may very well explain the elevation of MHC transcripts in the hippocampus. The extracellular matrix glycoprotein osteonectin is an anti-adhesion molecule expressed by astrocytes and shows a prominent binding affinity to other matrix proteins such as laminin and collagen as well as growth factors such as FGF and PDGF [30–32]. Different from other matrix proteins, osteonec-

tin plays a role in promoting cell migration and proliferation rather than maintaining cell structure [30–33]. It is therefore plausible to speculate that osteonectin is involved in axon growth based on its biochemical properties and the up-regulation in the denervated hippocampus. Thymosin-β4, profilin, and gelsolin are three closely related proteins that function in dynamic remodeling of actin cytoskeleton in living cells, in which the former two are actin-monomer (G-actin) sequestering proteins and act as G-protein pool for actin filament assembly while the latter is an actin filament (F-actin) severing and capping protein in a Ca^{2+}-dependent manner during actin filament disassembly [34,35]. It is known that regulation of actin cytoskeleton is involved in various physiological activities including cell motility (such as migration, neurite elongation, and guidance), cell survival, cell division, and growth. Both β-thymosin and profilin have been shown to function in modulating neurite outgrowth and axon guidance during development and regeneration [36,37]. Similarly, it has been revealed that gelsolin overexpression enhances neurite outgrowth in transfected PC12 cells [38]. These data suggest that the actin cytoskeleton interaction proteins thymosin-β4, profilin, and gelsolin may directly participate in remodeling of dendrites of hippocampal pyramidal and dentate granule cells as well as axonal sprouting of hippocampal afferents that occur following denervation. Also, our findings are consistent with the studies that showed the expression changes of additional cytoskeleton and associated proteins in the denervated hippocampus [18]. Furthermore, immediate up-regulation of thymosin-β4 in glial cells with a time course as early as 1 h after injury has been observed in various types of brain injury including ischemia, kainite-induced injury, and degeneration [39–41], suggesting that up-regulation of thymosin-β4 transcript in the denervated hippocampus may occur more earlier than the time-point examined in this study.

Finally, it is worthy to point out that the expression changes of all these eight transcripts were found locating specifically within the denervated target layers, the hippocampal SLM and the dentate OML, which are in abundance of glial cells in the adult brain. This observation suggests, as many studies have shown, that the activated glial cells might be of extreme importance for the repair of the CNS tissue after injury. The next task in the laboratory would be to systematically examine the expression patterns of these molecules in time-scale in the murine hippocampus following entorhinal deafferentation, as well as using in vitro and/or in vivo experiments such as tissue culture, transfection, and antisense oligonucleotide techniques, to test whether and how these molecules correlate to the plasticity events, especially the layer-specific axonal sprouting, in the adult hippocampus following entorhinal denervation.

Acknowledgments

This work was supported by the National Basic Research Program (G1999054005) and National Natural Science Foundation (39930090) of China.

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