Effects of environmental enrichment on gene expression in the brain

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An enriched environment is known to promote structural changes in the brain and to enhance learning and memory performance in rodents [Hebb, D. O. (1947) *Am. Psychol.* 2, 306–307]. To better understand the molecular mechanisms underlying these experience-dependent cognitive changes, we have used high-density oligonucleotide microarrays to analyze gene expression in the brain. Expression of a large number of genes changes in response to enrichment training, many of which can be linked to neuronal structure, synaptic plasticity, and transmission. A number of these genes may play important roles in modulating learning and memory capacity.

To test the role of environmental stimulation on animal learning and memory, Hebb developed a set of experiments to examine the behavioral consequences of exposing rats to enriched environments (1). Since then, a number of studies have reported that rodents reared in enriched environments show improved performance in various spatial maze and avoidance tasks (2–4). Consistent with these behavioral tests, exposure to an enriched environment has been shown to induce biochemical and structural changes in the cortex and other brain regions, including the hippocampal dentate gyrus and CA1 region (5). It has been postulated that these structural and biochemical changes may account for the long-term effects of enrichment, as rats enriched during youth perform better in learning and memory tasks at old age (6). To further characterize the effects of environmental enrichment in the brain, gene expression changes were analyzed in the cortex of mice after exposure to enriched environments for three hours to fourteen days. Our results demonstrate that enrichment training causes a significant change in the expression of genes whose products are involved in neuronal structure, plasticity, and neurotransmission.

Materials and Methods

Animals. Adult CBA/B6 hybrid mice (4 months) were randomly assigned to the group of animals kept in standard cages with water and food ad libitum (naive group, three mice) or to the group of mice trained in an enriched environment for up to 6 h daily (enriched group, three mice from each time point). The enriched environment consisted of two big, black plywood boxes ($1.5 \times 0.8 \times 0.8$ m high), in which various toys, wooden blocks, a spin wheel, and small houses were well arranged. In the box, food and water bottles were available for the animals. The mice were trained daily for 3 h in each of the two boxes whose items were changed or rearranged every half-day.

RNA Preparation. Cortices were dissected, pooled, and immediately frozen in liquid nitrogen or dry ice, and then stored at −80°C. Total RNA was isolated from tissue using RNA Extraction Kit (Amersham Pharmacia), which involved extraction by ultracentrifugation in a cesium trifluoroacetate gradient. Briefly, 60–120 mg of tissue was manually homogenized in 2 ml of prewarmed extraction buffer. The homogenate was centrifuged for 5 min at room temperature to remove cellular debris. The supernatant was transferred to a fresh sterile tube and then sheared by passing through a 23-gauge needle and syringe several times. This homogenate was layered over cesium trifluoroacetates, then centrifuged overnight at 125,000 × *g* at 15°C. Following centrifugation, the supernatant was aspirated, and the RNA pellet at the bottom of the tube was resuspended, followed by ethanol precipitation. RNA concentration was determined using spectrophotometer at 260 nm. Samples were stored at −80°C.

Gene Chip Analysis. Double-stranded DNA was synthesized from 5 μg of total RNA using the SuperScript Choice System (Life Technologies, Rockville, MD) and a primer containing poly(dT) and a T7 RNA polymerase promoter sequence (Genset). *In vitro* transcription using double-stranded cDNA as a template in the presence of biotinylated UTP and CTP was carried out using Enzo BioArray High Yield RNA Transcript Labeling Kit (Affymetrix and Enzo Diagnostics). Biotin-labeled cRNA was purified, fragmented, and hybridized to the arrays in 100 mM Mes, pH 7.4/1 M NaCl/20 mM EDTA/0.01% Tween 20. The arrays were washed and stained with streptavidin-phycoerythrin and then scanned with an Affymetrix GeneArray Scanner. Data were analyzed with the Affymetrix GeneChip Expression Analysis Software (version 3.1) as described (7). Labeled RNA samples were hybridized twice to two different arrays, and differences observed consistently in the replicates were analyzed further.

Results and Discussion

Early Gene Expression Changes of Mice in Enriched Environment. To examine gene expression changes occurring during an enrichment period, adult mice (4 months old) were exposed to an enriched environment for 3 h, 6 h, 2 days, or 14 days (enriched group). The enriched environment, which has been described previously (8), consists of a large container in which the floor was covered with bedding material and various items (toys, birdhouses, small castles with multiple floors, a running wheel, paper tunnels, etc.). Gene expression changes in the brains of these animals were compared with littermate controls housed under standard laboratory conditions (naïve group). Oligonucleotide array probes for more than 11,000 (13,069 probe sets) mouse genes were used to examine the gene expression patterns of these groups of mice. Labeled RNA prepared from pooled samples of each animal group was hybridized twice to different arrays. Gene expression level changes observed consistently in both arrays were analyzed.

Abbreviations: apoE, apolipoprotein E; NMDA, *N*-methyl-D-aspartate; RXR, retinoid X receptor.

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Table 1. Gene expression comparison of cortex from naïve mice and mice undergoing 3- and 6-h enriched environmental training

Accession no.	Gene name	3 h	6 h
DNA/RNA synthesis			
D63902	Estrogen-responsive finger protein	2.2	2.1
AA117835	Myelin gene expression factor	2.1	2.1
AA415044	Polyhomeotic, Hox transcription repressor	5	7
C80883	Hif 1alpha transcription factor	6	7
AA003541	Kinase, regulate DNA replication	3	5
X14805	DNA methyl-transferase	17	10
X66223	Retinoid X receptor α	−26	−16
Neuronal signaling			
AA270734	Calmodulin gene III	1.8	1.7
AB006361	Prostaglandin D synthetase	2.1	2.6
D17584	Neurokinin A	2.9	2.6
U27106	Clathrin AP-2	2.2	2.3
U60150	Synaptobrevin	1.9	NC
AA462551	MAP kinase 4K3	5	NC
D78645	Glucose-regulated protein 78	2.6	2
Neuronal growth/structure			
AF180805	Microfibril-associated glycoprotein-2	1.9	2.1
AA161790	RhoA gene	3	2.6
AA545177	Microfibrillar protein	3	3
AA416453	Integrin alpha-4	3	3
M93310	Brain specific metallothionein III	−2.5	−1.7
U95116	Lissencephaly-1 protein	−2.4	−2.1
M74773	Brain spectrin	−4	−2.1
U39904	Citron, rho/rac binding protein	−5	−12
AA017811	Neurogranin, calmodulin binding protein	NC	−1.6
Protease/cell death			
L22472	Bcl-2 associated protein, Bax	−2.9	−2.5
AA013993	Prolyl oligopeptidase	−2.6	−2.7
AA020512	Caspase-6	−3	−3
M55616	Protease 4	−3	−4
AA154337	Ribosome-associated DnaJ, molecular chaperone	NC	−2.7

Each RNA sample was hybridized twice to two different arrays and fold change values are averages of the duplicate measurements. Positive values indicate an increase in gene expression; negative values indicate a decrease, and no change marked NC.

The differential expression of genes after 3 and 6 h of exploration in the enriched environment likely reveals the early molecular events resulting from environmental stimulation. Our initial experiments focused on the cortex, a primary site responsible for enrichment-induced improvement of learning and memory performance (8). Only 78 of the 13,069 probe sets on the arrays were changed by more than 1.5-fold; 60 of the 78 genes (77%) were consistently altered at both 3- and 6-h time points (Table 1). Almost half (46%) of the environmentally responsive, differentially expressed genes code for proteins involved in macromolecule synthesis and processing, including transcription factors, translational regulatory enzymes, and enzymes involved in DNA, RNA, and protein processing. Among these, the highest level of induction is in DNA methyltransferase (over 10-fold increase), the major function of which is to maintain DNA methylation during DNA replication. It has been shown that DNA methyltransferase activity is critical for neuronal cell differentiation induced by nerve growth factor (9). In addition, nonproliferating neurons express high levels of DNA methyltransferase, suggesting it may perform functions other than in DNA replication (10), such as DNA mismatch repair in the adult brain (11). Many genes encoding transcription factors were also up-regulated (e.g., myelin gene expression factor and an estrogen-responsive finger protein), with the notable exception of retinoid X receptor (RXR) alpha, whose expression decreased dramatically (−26 and −16-fold at 3 and 6 h, respectively) (Table 1). It has been shown that expression of RXR alpha is also decreased by elevated intracellular calcium during T cell activation (12), suggesting that down-regulation of RXR alpha in neurons may also involve calcium signaling.

A group of genes encoding proteolytic proteins involved in signaling and apoptosis were also found to be differentially expressed after enrichment. For example, prolyl oligopeptidase, caspase-6, and protease 4 were down-regulated after 3 and 6 h of training. Prolyl oligopeptidase regulates the degradation of neuropeptides, such as vasopressin, substance P, and thyrotropin releasing hormone, which play important roles in neuronal signalling (13, 14). Caspase-6 is involved in apoptosis by the degradation of poly(ADP-ribose) polymerase and DNA topoisomerase I (15). Recently, it has been shown that caspase-6 and prolyloligopeptidase participate in the processing of amyloid precursor protein (13, 16, 17). In addition, inhibitors of prolyloligopeptidase have been reported to enhance cognition and are being explored for the treatment of Alzheimer's disease (18, 19). The Bcl-2 associated protein Bax was also down-regulated after training, again indicating a possible antiapoptotic effect of enrichment training (20).

Mice raised in the enriched environment also showed changes in the expression of genes involved in formation of new synapses and reorganization or strengthening of existing synapses. For example, the gene encoding integrin alpha-4, which is important for neuritogenesis and neuronal plasticity (21), is induced 3-fold

Table 2. Gene expression comparison of cortex from naïve mice and mice undergoing enriched environment training for 2 days and 14 days

Accession no.	Gene name	2 days	14 days
RNA/protein synthesis			
D32167	Zic, a zinc-finger protein	10	7
AA030563	General transcription factor BTF3	3	3
AF027963	X box binding protein-1 transcription factor	2.4	NC
D83999	RNA polymerase II subunit; mRPB31	20	NC
V00727	C-fos	−5	−2.5
Neuronal signaling			
D50621	PSD-95/SAP90A	7	2.3
M19380	Calmodulin	2.5	2.4
U06670	Apoliprotein-E receptor	2.3	2.2
ET63385	Connexin-30, a gap junction protein	4	2.8
X73985	Calretinin, neuronal calcium-binding protein	NC	2
D29763	SEZ-6, a brain-specific seizure-related protein	NC	3
AA086684	78 kDa glucose-regulated protein	3	−7
AA103475	cAMP-dependent protein kinase regulatory subunit	NC	−2.5
Neuronal growth/structure			
M72414	Microtubule-associated protein 4	2.8	2.6
AA174982	Coronin-1, actin-binding protein	2.1	2.2
M31131	N-cadherin, cell adhesion molecule	2.1	2.5
AA110732	Dynactin subunit p25	2.3	2.1
W75814	Defender against cell death 1 protein	2.1	2.1
U03184	Cortactin, filamentous-actin binding	14	NC
J04181	A-X actin	−3	−3
AA017811	Neurogranin	−3	−2
Protein processing			
U05333	Co-chaperonin ''cofactor A''	2.6	2.7
AA118268	26S proteasome, subunit p112	35	NC
AA274721	ClpP protease	27	NC
C75968	Ubiquitin specific protease 16	14	NC
AA259803	Aspartyl aminopeptidase	2.7	NC
AA512103	Aspartyl aminopeptidase like protein	16	NC
AA407689	Proteasome subunit C10-II	NC	3
C79022	Cytoplasmic aminopeptidase	NC	3

Each RNA sample was hybridized twice to two different arrays and fold change values are averages of the duplicate measurements.

after both 3 and 6 h of training. The expression of GTPase RhoA is also increased after training (3.2- and 3.6-fold after 3 and 6 h). In neurons, Rho proteins are involved in the induction of integrin-mediated events in surface adhesion and proliferation, thereby contributing to synapse formation and neuronal plasticity (22, 23). A cluster of genes encoding proteins involved in synaptic vesicle trafficking and neurotransmitter release, including synaptobrevin and clathrin-AP2, was up-regulated after 3 and 6 h of enrichment. Synaptobrevin is a synaptic vesicle-associated protein, whereas the clathrin–adaptor protein AP2 interacts with synaptic vesicle protein synaptotagmin and regulates vesicle exocytosis (24, 25). Changes in the expression of these genes clearly suggest that presynaptic processes are being modified by enriched experiences.

A number of genes whose products are associated with neuronal excitability also changed their expression levels after 3 and 6 h of enrichment. The seizure-related protein, the 78-kDa glucose-regulated protein, was up-regulated after enrichment training (2- and 2.6-fold after 3 and 6 h); expression of this 78-kDa protein also increased after 2 days of training (3-fold) but dramatically decreased after 14 days of training (−7-fold). These data indicate that the dynamic regulation of the 78-kDa protein could be intimately coupled with brain activity (29, 30). Similarly, neurokinin A, a neurotransmitter of the tachykinins family, is induced almost 3-fold after 3 and 6 h of training. This protein has been shown to play an important role in the control of neuronal excitability, seizure sensitivity, and apoptosis (31). The expression of the gene for Lissencephaly-l also decreased in the trained mice (−2.4- and −2.1-fold after 3 and 6 h, respectively). The Lissencephaly-1 gene encodes the lipoprotein-associated phospholipase A2 (Lp-PLA2), also known as platelet-activating factor acetyl-hydrolase. It has been shown that expression of the protein decreases following the initiation of seizure (26). Mutation of the gene in human results in a severe brain developmental disorder caused by abnormal neuronal migration (27). Because the platelet-activating factor plays a role in the retrograde signaling cascade involved in hippocampal LTP (28), down-regulation of the Lissencephaly-1 gene during enrichment may modify synaptic plasticity.

Finally, the prostaglandin D synthetase, which is responsible for prostaglandin D2 synthesis and retinoic acid transport in the brain (32), was up-regulated during the early phase of enrichment (2.1- and 2.6-fold at 3 and 6 h). Data have suggested that prostaglandin D synthetase plays a role in sleep regulation (47). Although prostaglandin D synthetase also functions as a retinoic acid transporter, the physiological role of this activity in the brain is still not clear.

Late Gene Expression Changes of Mice in Enriched Environment. The longer-term effects of enrichment were examined by comparing the gene expression profiles of the naïve mouse brain with those from mice exposed to an enriched environment for 2 or 14 days

(Table 2). Approximately 100 transcripts changed by at least 1.5-fold; most are different from those whose expression levels changed at the early stage of training, and most are involved in neuronal transmission and structural changes.

The mRNA level of the transcription factor X-box binding protein-1 (XBP-1) increased 2.4-fold after 2 days of training. It is interesting to note that XBP-1 interacts with cAMP-responsive elements of many genes and activates their expression (33). Because the cAMP pathway is essential for memory formation (34), our data suggest a putative role for the XBP-1 transcription factor in learning and memory mechanisms. Moreover, the cAMP-dependent protein kinase regulatory subunit was down-regulated 2.5-fold after 14 days of training. It has been shown that overexpression of this regulatory subunit in transgenic mice significantly impairs the late phase of hippocampal long-term potentiation (LTP) as well as long-term memory (39). From the studies in *Aplysia*, the down-regulation of this regulatory subunit is also required for long-term facilitation (42, 43).

A number of genes associated with NMDA receptor function were affected by enrichment. For example, the expression level of postsynaptic density 95 (PSD-95) increased after 2 days (7-fold) and 14 days (2.3-fold) of enrichment. PSD-95 participates in the anchoring of the NMDA receptor and interacts with neuronal NO synthase at the postsynaptic membrane and thereby plays a fundamental role in synaptic transmission and memory formation (35, 36). Mutant mice lacking PSD-95 show enhanced LTP but are severely impaired in spatial learning (37). The PSD-95 protein complex seems to be important in coupling the NMDA receptor to pathways that control synaptic plasticity and learning. Taken together, we postulate that up-regulation of PSD-95 during enrichment may play a role in facilitating learning and memory ability.

Environmental enrichment was also associated with changes in expression of molecules downstream of the NMDA receptor, including the up-regulation of calmodulin after 3 h (1.8-fold), 6 h (1.7-fold), 2 days (2.5-fold), and 14 days (2.4-fold) of training. Calmodulin has been suggested to modulate the interaction of the PSD-95 protein complex with NE-dlg/SAP102, a neuronal and endocrine tissue-specific membrane-associated guanylate kinase. Calmodulin also regulates clustering of neurotransmitter receptors at central synapses (38). Neurogranin, a neuron-specific calmodulin-binding postsynaptic protein regulated by RXR, decreased during enrichment. Neurogranin regulates calmodulin availability in axons and dendritic spines and has been shown to play a role in dendritic development as well as long-term potentiation and depression (40, 41). These results strongly suggest that NMDA function at the synapse level can be modified by environmental enrichment.

Again, a number of proteolytic proteins are up-regulated after long enrichment training, including ubiquitin-specific protease 16, which increased 14-fold after 2 days of training. ClpP protease (27-fold), aspartyl aminopeptidase (2.7-fold), aspartyl aminopeptidase-like protein (16-fold), and prolidase (8-fold) were also up-regulated after 2 days of enrichment. After 14 days of training, we also found that proteasome subunit C 10-II and cytoplasmic aminopeptidase were up-regulated 3-fold. These proteases are likely involved in the regulation of protein processing and signal transduction, which are activated by an enriched environment (44).

Longer-term enrichment training also altered the mRNA levels of many genes associated with structural changes that occur during neuronal growth. The cytoskeletal protein dynactin, which is involved in retrograde axonal transport and may play an important role in neuronal growth and synaptogenesis (45), is up-regulated after enriched environmental training. Cortactin, another actin-binding protein, was induced 14-fold after 2 days of training. This protein participates in synaptic formation and plasticity (46) by mediating the local assembly of the cytoskeleton and interacting with *N*-methyl-D-aspartate (NMDA) receptors and the postsynaptic density 95 (PSD95) protein complex.

We also found that apolipoprotein E (apoE) is increased after 2 days (2.3-fold) and 14 days (2.2-fold) of training. Mutation of apoE has been linked to Alzheimer's disease (47); age-related memory disorders are also associated with apoE signaling (48). ApoE receptor signaling is directly involved in the dephosphorylation of tau protein and may have neuroprotective activity (49). These results suggest that apoE signaling may play an important role in learning and memory.

Finally, the protein, defender against cell death 1 (DAD1), was up-regulated (2.1-fold) after enriched environmental training. DAD1 is a subunit of the oligosaccharyltransferase complex that initiates N-linked glycosylation. Mice harboring a disrupted dad1 gene exhibit abnormal cell death, and the homozygous deleted mice die soon after implantation (50), implicating DAD1 in the control of programmed cell death during development. Changes in the expression of DAD1, like caspase 6 and Bax, during enrichment may play an important antiapoptotic role.

In conclusion, environmental enrichment affects the expression levels of a number of genes involved in neuronal structure, synaptic signaling, and plasticity. Some of these genes are known to be associated with learning and memory. Others are linked to age-related memory deficits. We are currently characterizing the function of a number of these genes in transgenic animals.

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