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Neurotrophic signaling molecules associated with cholinergic damage in young and aged ratsEnvironmental enrichment as potential therapeutic agent

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Abstract

The aim of this study was to determine the neurobiological bases of behavioral deficits associated with cholinergic damage and the potential of long-term environmental enrichment as a therapeutic agent. Rats were submitted to intra-structures injection of 192 IgG-saporin and then behaviorally tested 1 month and 1 year post-lesion in a nonmatching-to-position task. The gene expression changes were assessed by cDNA macroarray technology using the GE array Q series designed to profile the expression of neurotrophic signaling molecules. Results showed that (1) cholinergic injury modulated the expression of genes such as brain-derived neurotrophin factor but also genes associated with inflammatory response, neuron apoptosis, regulation of angiogenesis, and synaptic plasticity, (2) aging is associated with regulation of glial proliferation and apoptosis, and (3) long-term enriched environment housing enhanced behavioral performance in lesioned and non-lesioned rats and upregulated gene expression. This therapeutic role of the enriched environment seemed to be associated with a suppression of expression of genes involved in apoptosis, glial cell differentiation, and cell cycle, but also with an enhanced expression of a subset of genes involved in signal transduction.

Keywords: 192 IgG-saporin; bdnf; GE array; Immunohistochemistry; T-maze

1. Introduction

The cholinergic basal forebrain is a neuronal system known to be implicated in cognitive processes like memory and attention (Baxter and Chiba, 1999; Sarter et al., 2003; Wenk, 1997). Many studies have dealt with the cholinergic system in aging (Gallagher and Colombo, 1995; Sarter and Bruno, 1998) and in related pathologies like Alzheimer's disease (AD) (Casu et al., 2002; Dickinson-Anson et al., 2003; Zhang, 2004). Many years ago the cholinergic hypothesis of aging and AD emerged from human and animal data (Gallagher and Colombo, 1995; Muir, 1997), establishing a link between the cognitive decline observed in aged healthy persons and in AD patients and alterations of the cholinergic basal forebrain (Bartus, 2000; Shinotoh et al., 2000; Yan and Feng, 2004). Anatomically, the cholinergic basal forebrain includes cells in the nucleus basalis of Meynert (called basalis magnocellularis nucleus (NBM) in animals) and cells in the complex medial septum (MS)/diagonal band of Broca (DBB). These cells project to the entire cortical mantle and the amygdala and to the hippocampus, respectively (Fibiger, 1982).

In animals, the cholinergic system has been largely studied through experiments involving brain lesions, which showed a wide range of cognitive deficits. The immunotoxin 192 IgG-saporin is considered a valid tool to study the consequences of specific cholinergic loss in the basal forebrain (Paban et al., 2005a; Perry et al., 2001). The functional consequences of administration of the immunotoxin 192 IgG-saporin have

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been reviewed (McGaughy et al., 2000; Rossner et al., 1995; Wrenn and Wiley, 1998). The cognitive deficits consecutive to cholinergic lesion depend on many parameters like injection type (intracerebroventricular or intra-strutures), lesion extent (MS/DBB lesion, NBM lesion, or global lesion), and task demand (Parent and Baxter, 2004).

We have previously shown that the behavioral effects of 192 IgG-saporin-induced cholinergic lesion depended on the time at which testing is conducted after surgery (Paban et al., 2005a). In particular, we demonstrated that at short post-lesion time – less than 1 month – no deficit was observed whereas at longer post-lesion times – from 1 month and up to 1 year – rats showed memory impairments. Although the behavioral effects of cholinergic immunolesion are well established, there are only few data on underlying molecular events. We have previously shown that the expression of molecules such as the highly polysialylated neural cell adhesion molecule (PSA-NCAM) was upregulated 1 month and 1 year after surgery, suggesting that remodeling occurred in cholinergic-lesioned rats (Chambon et al., 2007).

The aim of the present study was to determine the neurobiological bases of behavioral deficits associated with cholinergic damage in young and middle-aged rats. Attention was focused on neuroplasticity-related molecules (Johansson, 2004). Indeed, several studies have indicated that brain injuries are associated with altered expression of a number of genes encoding neurotrophins, growth factors, and signaling molecules (Lessmann et al., 2003; McAllister, 2000; Patz and Wahle, 2004). Thus, we used a technology allowing expression-profiling analyses of several genes, that is, cDNA arrays. This technique presents the advantage of evaluating many genes at the same time and is now used in many fields of research including aging (Fraser et al., 2005; Lee et al., 2000; McCarroll et al., 2004; Prolla, 2002) and models of pathologies (Cheng et al., 2007; Wei et al., 1999). We used a cDNA macroarray GE Array Q series (MM18) including 96 gene fragments for studying neurotrophic signaling gene expression after cholinergic injury at 1 month and 1 year post-lesion times. Because of the central importance of brain-derived neurotrophic factor (bdnf) as a neurotrophic molecule, its expression was analyzed at a protein level by immunohistochemistry. Interestingly, we had previously shown that, during entry to senescence (around 15 months old), housing in an enriched environment had beneficial effects on cognitive performance (Paban et al., 2005a). Indeed, 192 IgG-saporin-lesioned rats performed better when housed in an enriched environment relative to animals housed in standard cage conditions, raising the question of the use of enriched environment as therapeutic agent. The molecular events underlying these beneficial effects are not fully understood (Cotman and Berchtold, 2002). Thus, to gain insight into this question, we compared the gene expression profile of lesioned rats housed in enriched environment to the one of standard-lesioned rats. In particular, the goal was to identify new molecular and cellular pathways that can be targeted, opening new directions for treatment strategies.

2. Materials and methods

2.1. Animals and experimental design

Male Wistar rats (Charles River, France) were used in the experiments. Animals were housed in standard conditions, i.e., in groups of 2–3 rats per cage or in enriched environment, i.e., in groups of 10 rats in a cage of approximately 2 square meters equipped with a rearrangeable set of plastic tubes, a running wheel, nesting material, and toys (Paban et al., 2005a). Rats were reared under 12 h light/12 h dark conditions with *ad libitum* access to food and water, except when food-deprived during behavioral training. Every effort was made to minimize animal suffering and to reduce the number of rats used. All experiments conformed to international guidelines on the ethical use of animals.

Sixty-one rats were used. Thirty were injected with 192 IgG-saporin (SAP) and thirty-one with phosphate buffer saline solution only (PBS). Rats were 3 months old at the time of the surgery and were tested behaviorally 1 month after surgery – groups 1-month (PBS, $N = 10$; SAP, $N = 10$) or 1 year – groups 1-year (PBS, $N = 11$; SAP, $N = 9$). Rats were housed in a standard environment during all this period. Two other groups of rats were used (PBS, $N = 10$; SAP, $N = 11$). As for group 1-year, these animals were tested 1 year after surgery but were housed in an enriched environment during the entire experimental period (from surgery to behavioral testing); the rats were then 15 months old at the beginning of the behavioral session. Rats were killed immediately after the behavioral testing by anesthesia with isoflurane gas and decapitated. The brains were immediately cut on ice with RNase-free instruments through the interhemispheric sulcus: the right hemisphere was used for RNA isolation and subsequent macroarray/PCR processings and the left hemisphere for immunohistochemistry.

2.2. Cholinergic lesion surgery

Animals were anesthetized with an intramuscular injection of a solution of Imalgen 500 (ketamine, 62.5 mg/kg), Rompun (xylazine, 3.17 mg/kg) and Calmivet (acepromazine maleate, 0.62 mg/kg) and placed in a stereotaxic instrument. The immunotoxin 192 IgG-saporin (Chemicon, France) or its vehicle solution (phosphate-buffered solution) was slowly injected bilaterally into the medial septum (MS) and the nucleus basalis magnocellularis (NBM) at the following coordinates: from the interaural point, AP −8.5 mm, ML ±0.4 mm, DV +3.4 mm for the MS, and AP −7.5 mm, ML ±2.5 mm, DV +2.5 mm for the NBM. The following doses of 192 IgG-saporin were used: MS (37.5 ng/side), and NBM (75 ng/side) to allow for maximal ChAT depletion. The toxin or the vehicle was injected with a 10 μl Hamilton microsyringe in a volume of 0.5 μl, allowing 5 min for diffusion before the cannula was retracted as previously described (Chambon et al., 2007; Paban et al., 2005a,b).

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2.3. Nonmatching-to-position task

The animals were trained in a nonmatching-to-position task in a T-maze apparatus, as described in detail (Paban et al., 2005a,b). Briefly, the rats were gradually food-deprived to 85% of the free-feeding level. The learning consisted of 8 daily trials until the criterion for acquisition was met. Each trial consisted of paired runs: one forced and one choice run. On the forced run, the side arm was closed by the guillotine door and the other door was open with food pellets in the cup. This forced the animal to enter a pre-selected arm and then allowed it to eat the food. Immediately after the forced run, the rat was placed again at the start box for a choice run. At this time, no guillotine door was lowered, and the rat was allowed to choose between the two arms. The criterion for scoring an arm visit consisted of the rat placing a hind leg in one of the arms. No backtracking was permitted. When the rat entered the arm opposite to the one rewarded in the forced run (nonmatching), a correct response was recorded, and it was allowed to eat the pellets before returning to its cage. If not, it was confined to the incorrect arm for approximately 20 s and then returned to its cage. The rats were tested in groups of 3 or 4 with each one given one trial per turn, so that the intertrial interval was about 4–6 min. Acquisition was defined as 7 correct trials on 2 consecutive days.

2.4. RNA isolation and macroarray processing

Four rats per group were used. Medial septum, hippocampus, and frontal and entorhinal cortices from the right hemisphere were removed. Ten to fifteen micrograms of tissue were taken from each region, pooled, and mixed in 1 ml of Trizol reagent. A phase separation was carried out on the homogenate to separate the different molecular components: RNA, DNA, and proteins. Total RNA was isolated from the aqueous phase by the Trizol reagent protocol. Its integrity and quality were checked using 1% agarose gel electrophoresis, and its concentration was measured by spectrophotometry ($A = 260$ nm). For each rat (one chip per rat), total RNA was reversed transcribed, radioactively marked with ^{32}P, and hybridized to the GE Array Q series MM18 gene array (Super Array Bioscience Corporation, France) according to the protocol provided by the manufacturer. This kit is designed to profile the expression of 96 neurotrophic signaling molecules involved in growth, differentiation, survival, and apoptosis. They are classified in 7 subsets of genes: neurotrophins and receptors (Subset 1, S1), neuropeptides and receptors (S2), growth factors involved in neuronal growth and differentiation (S3), chemokine receptors (S4), cytokines and receptors (S5), signaling molecules indicative of downstream pathways (S6), and genes involved in neuronal apoptosis in response to neurotrophic factors (S7). The radioactive signal was detected after exposure to X-ray film (Kodak, France). X-ray films were then scanned and analyzed with the quantification software Quantity One (Biorad, France). All raw signal intensities were corrected for background by subtracting the signal intensity of a blank and were normalized to the mean of housekeeping genes (beta-actin, GAPDH, cyclophilin A, and ribosomal protein L13a).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed on selected genes using RNA from the same animals as for macroarray analysis. Four genes chosen from the array data analysis were examined. It seems of interest to select genes with a highly differential expression and genes that showed no expression modification in order to show that the macroarray method yields reliable results. Based on our cDNA macroarray results, bdnf and orexine receptor 2 (hcrt2) were chosen as genes showing a significant and high expression change in 192 IgG-saporin-treated rats whatever the post-lesion time. Neurotrophic tyrosine kinase receptor type 3 (ntrk3) and fibroblast growth factor 9 (fgf9) were chosen as genes showing no significant change between lesioned and non-lesioned rats. For every RNA preparation, a negative control was run in parallel, omitting the RNA sample. A 1-μg RNA sample was used for one-step RT-PCR analysis kit (Qiagen) according to the manufacturer's instructions, using a Gene Amp PCR system 9700 (Applied Biosystems), in 50 μl reactions containing 10 μM of each of the gene specific primers for bdnf, hcrtr2, ntrk3, fgf9, puc18 (for negative control), and beta-actin (for positive control). The following oligonucleotide primers were used: for bdnf, 5′-ATGAAGGCTGCGCCCATGAAAGAAA-3′ and 5′-TCCTTATGAACCGCCAGCCAATTCTC-3′; for hcrtr2, 5′-TGCGGTACCTGTGGAGGGAATACCTA-3′ and 5′-GCGCCACGTGCTCTGAGAGTTTTGATA-3′; for ntrk3, 5′-TCGGGAATTGAGACTGGAGCAGAACT-3′ and 5′-AGCATCCAGCGATGAAGGTGTAGTGA-3′; for fgf9, 5′-CCACCTGGGTCAGTCCGAAGCA-3′ and 5′-GGTACTTTGTCAGGGTCCACTGGTCTA-3′; or puc18, 5′-TTCACACCGCATATGGTGCACTCTCA-3′ and 5′-TGTTGCCATTGCTACAGGCATCGT-3′; for actin, 5′-TGACCCTGAAGTACCCCATTGAACACG-3′ and 5′-ATACCCAGGAAGGAAGGCTGGAAGAGA-3′. Amplified PCR products were subjected to 1% agarose gel electrophoresis and stained with ethidium bromide. Gel images were captured with an Imager (Bio-1D, Fisher Bioblock Scientifics SAA) and the fluorescent densities of the PCR products were evaluated through comparison with different concentrations of the corresponding DNA ladder.

2.6. Immunohistochemistry

Immunohistochemistry was used to determine whether gene expression identified by macroarray was associated with the synthesis of its protein. Because the macroarray study showed a characteristic change in bdnf messenger RNA no matter what the experimental conditions were (see

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Section 3), we investigated bdnf at the protein level by immunohistochemical analysis. The left hemispheres (4 per group of rats) were frozen in isopentane solution and cut into 40-μm serial coronal sections. The sections were fixed in 4% paraformaldehyde for 3 h and then washed 3 times for 10 min in 0.3% H_2O_2 and 0.05 Tris-buffered saline (TBS) solution, and then incubated for 48 h at 4 °C with a rabbit polyclonal antibody to bdnf (Santa Cruz Biotechnology, CA, 1:1000 dilution in TBS 0.3% Triton X-100). After this procedure, sections were washed 3 times for 10 min in 0.3% H_2O_2 and 0.05 Tris-buffered saline (TBS) solution, incubated for 2 h in a biotinylated rabbit anti-goat IgG (Chemicon International, CA; 1:500 dilution in TBS 0.3% Triton X-100), washed 3 times for 10 min in TBS, incubated in avidin–biotin complex (1 h), and then immersed in 0.25% diaminobenzidine, 0.01% H_2O_2 solution, mounted on gelatinized slides, dehydrated in alcohol, and coverslipped with DePex for examination by light microscopy. A quantitative analysis of bdnf immunoreactivity was performed via computer-assisted image analysis using a Leitz Aristoplan light microscope equipped with a Nikon high-resolution digital camera (756 × 581 pixels) interfaced to a PC computer, Image software (Lucia, Nikon) for capturing and processing the images. The zones of interest were acquired with a 10× objective lens. Measurement consisted of computing the area of the labelled cells. This was performed using a grey-level method which consisted of adjusting a threshold brightness value. Thus, only labelled cells with a grey value above this cutoff were taken into account. Bdnf immunoreactivity was quantified at the level of the medial septum, hippocampus, frontal and entorhinal cortices.

Immunohistochemistry of choline acetyltransferase (ChAT) was done to investigate the immunotoxicity of 192 IgG-saporin. The immunochemical staining for ChAT was carried out as described above, in which the primary antibody was a goat polyclonal antibody against ChAT protein (Chemicon, France, 1:500 dilution in TBS 0.3% Triton X-100) and the secondary antibody was a biotinylated goat anti-rabbit IgG (Vector, France, 1:200 dilution in TBS 0.3% Triton X-100). Also, parallel sections were stained with cresyl violet to determine the extent of nonspecific damage due to the needle tracks, as described previously (Paban et al., 2005a,b).

2.7. Statistical analysis

For the behavioral data, analysis of variance (ANOVA) was used to study the main effects of Group and Post-Lesion Time and their interaction. The effects of the enriched environment were analyzed using ANOVA to determine the main effects of Group and Environment and their interaction. Further post hoc comparisons were made using the Newman–Keuls *t*-test. Differences were considered significant when $P < 0.05$.

For immunohistochemistery data, bdnf protein expression between PBS and SAP groups was assessed using Mann–Whitney *U* test. Differences were considered significant when $P < 0.05$.

For the gene expression macroarray analysis, the Mann–Whitney *U* test was used for paired comparison in each experimental condition. Genes of interest were those satisfying the following two criteria: (1) the difference in *P* value is less than 0.05, (2) the fold change is >1.5. These genes were selected for further analyses. In particular, the expression data of these selected genes were used to calculate Pearson correlations between every pair-wise combination of genes of interest. A hierarchical clustering analysis was then performed using Euclidian distance and Ward linkage to visualize clusters of highly correlated genes that might be involved in some common regulation pathway. Functional profiling of differentially expressed genes was performed with OntoExpress (http://vortex.cs.wayne.edu/projects.htm) (Khatri et al., 2002; Draghici et al., 2003) and DAVID websites (http://david.abcc.ncifcrf.gov/ease/ease.jsp) (Hosack et al., 2003), which allowed us to thoroughly characterize sets of functionally related genes based on Gene-Ontology categories.

3. Results

3.1. Behavioral analysis

The results are shown in Fig. 1. ANOVA on the performance of rats tested at 1 month and 1 year post-lesion time and housed in standard environment revealed a Group effect (F (1, 36) = 17.85, $P < 0.0002$), a Post-Lesion Time effect (F (1, 36) = 43.39, $P < 0.0001$), but no Group X Post-Lesion Time interaction (F (1, 36) = 0.22, $P = 0.64$), indicating that 192 IgG-saporin (SAP)-lesioned rats had worse performance than control rats (PBS), irrespective of the post-lesion times at which animals were tested, 1 month vs. 1 year. Note that the number of days to meet the criterion increased with the age of rats. Thus, groups 1 year (15 months old), PBS as well as SAP, needed more time to reach the criterion than groups 1 month (4 months old).

ANOVA on the performance of rats housed in standard and enriched environment and tested at 1 year post-lesion time yielded a Group effect (F (1, 37) = 17.49, $P < 0.0002$), an Environment effect (F (1, 37) = 118.05, $P < 0.0001$), but no Group × Environment interaction (F (1, 37) = 3.22, $P = 0.08$), showing that PBS and SAP rats displayed better performance when housed in an enriched environment. Student's *t*-test analysis on the performance of PBS and SAP rats housed in enriched environment yielded a Group effect ($P < 0.05$), indicating that lesioned rats needed more time to reach the criterion than control rats.

3.2. Macroarray analysis

The results are shown in Fig. 2 and Tables 1–3. One month after 192 IgG-saporin cholinergic immunolesion, among the

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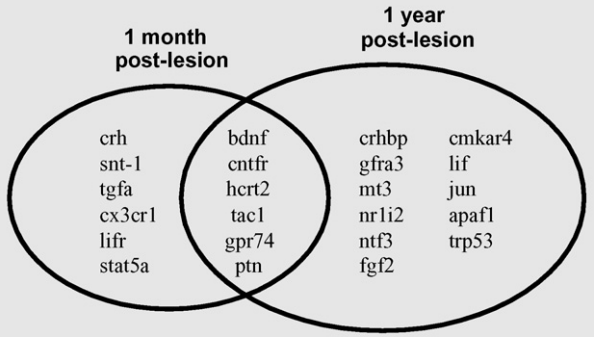
Table 1
Gene expression changes in 192 IgG-saporin-lesioned rats at 1 month (A) and 1 year (B) post-lesion. Only genes showing significantly ($P < 0.05$) different changes greater than 1.5-fold are reported. Gene symbols, bank accession numbers, and names are provided. S1, neurotrophins and receptors; S2, neuropeptides and receptors; S3, growth factors involved in neuronal growth and differentiation; S4, chemokine receptors; S5, cytokines and receptors; S6, signaling molecules indicative of downstream pathways; S7 genes involved in neuronal apoptosis in response to neurotrophic factors. Positive fold change values indicate an increase in gene expression in SAP-treated rats. (C) 2-way Venn diagram indicating the gene expression profiles following cholinergic injury at 1 month vs. 1 year post-lesion. The common genes, i.e., genes affected by the lesioning no matter what the post-lesion time was, are noted in the center.

Gene symbol	Gene Bank No.	Gene name	Subset	*P*-value	Fold change
A					
bdnf	NM_007540	Brain-derived neurotrophic factor	S1	<0.02	4.89
cntfr	NM_016673	Ciliary neurotrophic factor receptor	S1	<0.02	2.80
crh	BE993430	Corticotropin releasing hormone	S1	<0.02	1.98
hcrtr2	AF394597	Orexine receptor 2	S1	<0.02	3.07
snt-1	BB661937	Suc-1 associated neurotrophic factor target	S1	<0.04	1.56
tac1	NM_00931	Tachykinin 1	S1	<0.02	3.27
gpr74	AF236084	G-protein-coupled recptor 74	S2	<0.02	1.54
ptn	D90225	Pleiotrophin	S3	<0.02	3.55
tgfa	U65016	Transforming growth factor alpha	S3	<0.02	2.29
cx3cr1	AF102269	CX3 CR1	S4	<0.02	1.59
lifr	NM_013584	Leukaemia inhibitory factor receptor	S5	<0.02	1.62
stat5a	NM_011488	Signal transducer and activator of transcription	S6	<0.02	1.57
B					
bdnf	NM_007540	Brain-derived neurotrophic factor	S1	<0.02	5.84
cntfr	NM_016673	Ciliary neurotrophic factor receptor	S1	<0.04	1.55
crhbp	BE656478	Corticotropin-releasing factor binding protein	S1	<0.02	3.52
gfra3	NM_010280	Glial-derived neurotrophic factor receptor alpha 3	S1	<0.02	3.03
hcrtr2	AF394597	Orexine receptor 2	S1	<0.02	1.59
mt3	NM_013603	Metallothionein 3	S1	<0.02	3.81
nr1i2	AF031814	Pregnane X receptor	S1	<0.02	2.52
ntf3	NM_008742	Neurotrophin 3	S1	<0.04	2.26
tac1	NM_00931	Tachykinin 1	S1	<0.02	2.89
gpr74	AF236084	G-protein-coupled recptor 74	S2	<0.02	1.96
fgf2	M30644	Fibroblast growth factor 2	S3	<0.04	4.56
ptn	D90225	Pleiotrophin	S3	<0.02	2.71
cmkar4	AB000803	CXCR4	S4	<0.02	3.17
lif	NM_008501	Leukaemia inhibitory factor	S5	<0.02	1.96
jun	J04115	Jun oncogene	S6	<0.02	3.22
apaf1	NM_009684	Apoptotic protease activating factor 1	S7	<0.02	3.76
trp53	K01700	Transformation related protein 53	S7	<0.02	1.99

C



96 genes that were represented in the GE Array Q series MM18, 12 genes showed significantly altered expression levels and all were upregulated. Twenty-six transcripts changed more than 1.5-fold between control (PBS) and 192 IgG-saporin (SAP)-treated rats housed in standard environment (Fig. 2A and B and Table 1A). Only genes showing significantly ($P < 0.05$) different changes, greater than 1.5-fold, were reported in tables and figures and considered for further analyses. When we took into account the functional classification in 7 subsets as described above, these genes belong mainly to S1. Note that the highest upregulation was seen in bdnf with a 4.89-fold increase in mRNA levels in SAP vs. PBS rats. Further analysis using hierarchical clustering showed that the 12 genes could be split into 3 well-identified clusters. The functional profiles of each of these clusters were obtained using extensive literature

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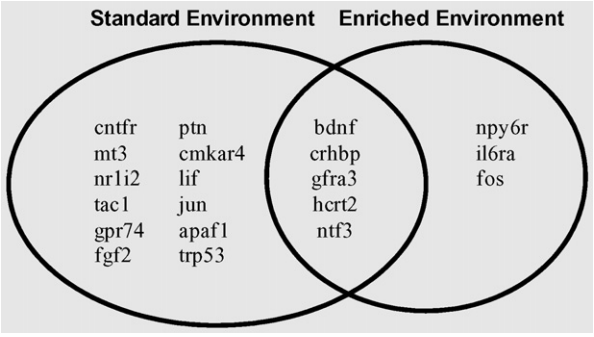
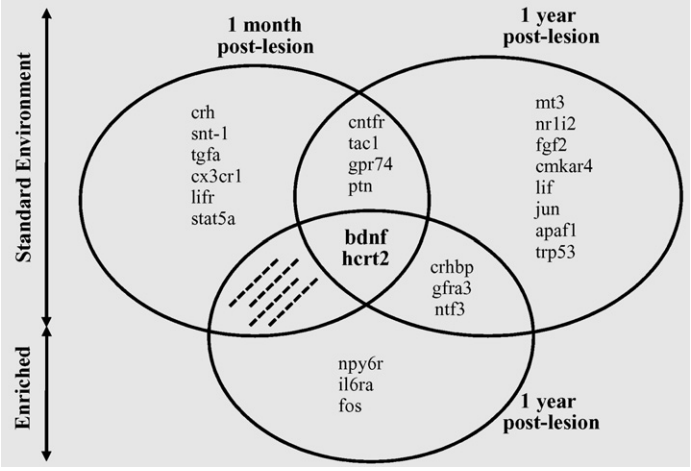
Table 2
Gene expression changes following long-term enriched environment housing in 192 IgG-saporin-immunolesioned rats (A, PBS vs. SAP-lesioned rats housed in enriched environment and tested 1 year post-lesion). Only genes showing significantly ($P < 0.05$) different changes greater than 1.5-fold are reported. Gene symbols, bank accession numbers, and names are provided. S1, neurotrophins and receptors; S2, neuropeptides and receptors; S3, growth factors involved in neuronal growth and differentiation; S4, chemokine receptors; S5, cytokines and receptors; S6, signaling molecules indicative of downstream pathways; S7 genes involved in neuronal apoptosis in response to neurotrophic factors. Positive fold change values indicate an increase in gene expression in SAP-treated rats. (B) 2-way Venn diagram indicating the gene expression profiles following cholinergic injury when rats were housed in standard vs. enriched environment. The common genes, i.e., genes affected by the cholinergic injury no matter what the housing condition was are noted in the center. (C) 3-way Venn diagram indicating the gene expression profiles after cholinergic injury at 1 month and 1 year post-lesion of rats housed in standard or enriched environment.

Gene symbol	Gene Bank No.	Gene name	Subset	*P*-value	Fold change
A					
bdnf	NM_007540	Brain-derived neurotrophic factor	S1	<0.02	4.97
crhbp	BE656478	Corticotropin-releasing factor binding protein	S1	<0.02	2.75
gfra3	NM_010280	Glial-derived neurotrophic factor receptor alpha 3	S1	<0.02	1.58
hcrtr2	AF394597	Orexine receptor 2	S1	<0.02	3.27
ntf3	NM_008742	Neurotrophin 3	S1	<0.02	2.01
npy6r	NM_010935	Neuropeptide Y receptor Y6	S2	<0.02	1.84
il6ra	X53802	Interleukin 6 receptor alpha	S5	<0.02	1.59
fos	V00727	c-fos oncogene	S6	<0.04	1.51

B



C



searches in PubMed and Onto-Express and DAVID websites.

- Cluster 1 included crh, gpr74, snt1, and lifr genes and would be involved in biological processes such as inflammatory processes and positive regulation of cell adhesion.
- Cluster 2 included cx3cr1 and stat5a genes and would be involved in biological processes such as positive regulation of inflammatory response, regulation of cell adhesion, growth, and proliferation.
- Cluster 3 included bdnf, cntfr, hcrt2, tac1, ptn, and tgfa genes and would be involved in biological processes such as regulation of cellular plasticity, dendrite development, cell proliferation, and neuron recognition.

The full names of all transcripts are given in tables. To avoid redundancy, only abbreviations are used.

One year after cholinergic immunolesion, 17 transcripts demonstrated a significant expression change between control and 192 IgG-saporin-treated rats housed in standard

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Table 3
Gene expression changes during aging. (A) Gene expression changes in 4- vs. 15-month-old PBS control rats housed in standard condition. (B) Gene expression changes in 15-month-old control rats housed in standard vs. enriched environment. Only genes showing significantly ($P < 0.05$) different changes greater than 1.5-fold are reported. Gene symbols, bank accession numbers, and names are provided. S1, neurotrophins and receptors; S2, neuropeptides and receptors; S3, growth factors involved in neuronal growth and differentiation; S4, chemokine receptors; S5, cytokines and receptors; S6, signaling molecules indicative of downstream pathways; S7 genes involved in neuronal apoptosis in response to neurotrophic factors. Positive fold change values indicate an increase in gene expression and negative values indicate a decrease in 15-month-old control rats housed in standard condition (A) and in 15-month-old control rats housed in enriched environment (B).

Gene symbol	Gene Bank No.	Gene name	Subset	*P*-value	Fold change
A					
gmfb	NM_022023	Glial maturation factor beta	S1	<0.02	2.16
hcrtr2	AF394597	Orexine receptor 2	S1	<0.04	−2.58
nrg4	NM_032002	Neuregulin 4	S1	<0.02	−3.1
nmbr	NM_008703	Neuromedin B receptor	S2	<0.04	−2.75
fgfr1	M33760	Fibroblast growth factor 1	S3	<0.02	1.79
tgfa	U65016	Transforming growth factor alpha	S3	<0.02	1.73
tgfb1	M13177	Transforming growth factor beta 1	S3	<0.02	−2.99
il1b	M15131	Interleukin 1-beta	S5	<0.04	−2.79
jun	J04115	Jun oncogene	S6	<0.02	2.11
myc	X01023	c-myc gene	S6	<0.02	−3.48
stat6	NM_009284	Signal transducer and activator of transcription 6	S6	<0.04	1.53
apaf1	NM_009684	Apoptotic protease activating factor 1	S7	<0.04	3.06
B					
bdnf	NM_007540	Brain-derived neurotrophic factor	S1	<0.02	5.14
gmfb	NM_022023	Glial maturation factor beta	S1	<0.02	3.16
gdnf	NM_010275	Glial-derived neurotrophic factor	S1	<0.04	1.57
gfra3	NM_010280	Glial-derived neurotrophic factor receptor alpha 3	S1	<0.02	1.60
mt3	NM_013603	Metallothionein 3	S1	<0.02	2.88
ngfb	NM_013609	Nerve growth factor beta	S1	<0.02	1.94
pspn	NM_008954	Persephin	S1	<0.04	1.59
tac1	NM_00931	Tachykinin 1	S1	<0.02	2.33
gpr74	AF236084	G-protein-coupled recptor 74	S2	<0.04	1.56
fgf2	M30644	Basic fibroblast growth factor	S3	<0.02	2.10
ptn	D90225	Pleiotrophin	S3	<0.02	1.67
cmkar4	AB000803	CXCR4	S4	<0.02	1.83
myc	X01023	c-myc gene	S6	<0.04	1.58
stat5a	NM_011488	Signal transducer and activator of transcription	S6	<0.04	1.62
trp53	K01700	Transformation related protein 53	S7	<0.04	1.57

environment; all were upregulated. Thirty-one genes changed more than 1.5-fold (Fig. 2C and D, and Table 1B). Most of them belonged to S1. The bdnf gene (S1) showed the highest fold change, followed by the fgf2 gene (S3). Hierarchical clustering analysis showed that the 17 genes selected could be grouped into 3 clusters.

- Cluster 1 included bdnf, crhbp, ntf3, tac1, gpr74, ptn, cmkar4, lif, apaf, and trp53 genes and would be involved in biological processes such as regulation of apoptosis.
- Cluster 2 included gfra3, hcrt2, mt3, nr1i2, and fgf2 genes and would be involved in biological processes such as positive regulation of cell differentiation, regulation of neurogenesis, and angiogenesis.
- Cluster 3 included cntfr and jun genes and would be involved in biological processes such as regulation of cell cycle and positive regulation of cell proliferation.

Interestingly, 6 genes were found in common, i.e., their expressions were altered by cholinergic damage no matter what the post-lesion time was 1 month or 1 year (Table 1C). The 6 genes were bdnf, cntfr, hcrtr2, tac1, gpr74, and ptn and all were upregulated. Most of them belong to subset 1.

Similar analyses were done to evaluate gene expression changes following cholinergic damage when rats were housed in enriched environment. Among the 96 genes represented on the GE Array, 8 genes demonstrated a significant expression change between PBS and SAP rats housed in enriched environment; all were upregulated. Seventeen genes showed a fold change > 1.5 (Fig. 2E and F, and Table 2A). These genes belong mainly to S1, including bdnf gene, which showed the highest fold change, then S2, S5, and S6. Hierarchical clustering results indicated that the 8 genes could be grouped into 3 clusters.

- Cluster 1 included npy6r and fos genes and would be involved in biological processes such as signal transduction, axon guidance, and synaptic plasticity.
- Cluster 2 included gfra3 and il6ra genes and would be involved in biological processes such as axon guidance and synaptic plasticity.
- Cluster 3 included bdnf, crhbp, hcrt2, and ntf3 genes and would be involved in biological processes such as

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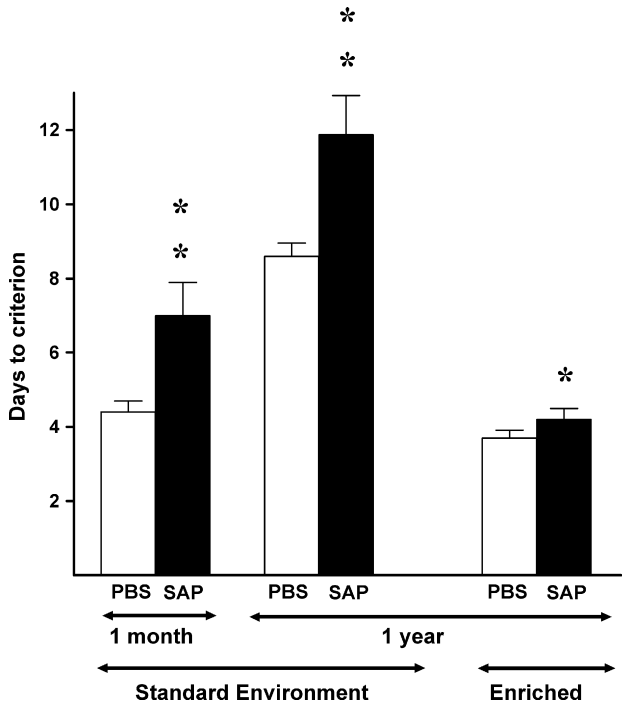

Fig. 1. Performance of control (PBS) and 192 IgG-saporin-lesioned (SAP) rats in the nonmatching-to-position task at 1 month and 1 year post-lesion and enriched environmental housing. The histograms represent the average (mean ± S.E.M.) numbers of days required to reach the criterion. Lesioned rats had worse performance than control rats no matter what the time after surgery was. In enriched environment, lesioned and non-lesioned rats displayed better performance. However, SAP-enriched rats required more days than PBS rats to reach the criterion. $*P<0.05$; $**P<0.01$ (vs. PBS group).

axon guidance, dendrite development, positive regulation of neuron differentiation, regulation of synaptic plasticity, and neuron apoptosis.

Note that 5 genes were found in common, i.e., their expressions were affected by the cholinergic injury no matter what the housing condition was—standard or enriched environment (Table 2B). There were 3 genes whose expressions were upregulated in lesioned rats but only if they were housed in enriched environment. There were 12 genes whose expressions were upregulated in lesioned rats but only if they were housed in standard environment. In other words, the expression of these 12 genes was repressed by housing in enriched environment.

Interestingly, a 3-way Venn diagram including the gene expression profiles after cholinergic injury at 1 month and 1 year post-lesion times of rats housed in standard or enriched environment revealed 6 groups of genes (Table 2C): (1) bdnf and hcrt2 genes were common to all groups, regardless of lesioning or age; (2) crhbp, gfra3, and ntf3 genes were common to middle-aged rats, regardless of housing condition; (3) cntfr, tac1, gpr74, and ptn genes were common to lesioned rats, regardless of the post-lesion time; (4) crh, snt-1, tgfa, cx3cr1, lifr, and stat5a genes were unique to 1 month post-lesion-standard condition group; (5) mt3, nr1i2, tgf2, cmkar4, lif, jun, apaf1, and trp53 genes were unique to 1 year post-lesion-standard condition group; and (6) npyr6, il6ra, and fos genes were unique to 1 year post-lesion-enriched condition group.

Comparing groups 1 month (4 months old) and 1 year (15 months old) of PBS control rats allowed us to evaluate the gene expression changes during aging. Among the 96 genes represented on the GE Array, 12 showed a significant differential expression and 29 genes showed a fold change > 1.5. Five genes were downregulated and 6 were upregulated (Fig. 2A and C and Table 3A). The myc gene (belonging to S6) showed the highest fold change. Hierarchical clustering identified two clusters.

- Cluster 1 included nmbr, fgfr1, tgfb1, jun, and apaf1 genes and would be involved in biological processes such as regulation of astrocytes/glial proliferation and growth.
- Cluster 2 included gmfb, hcrt2, nrg4, tgfa, il1b, myc, and sta6 genes and would be involved in biological processes

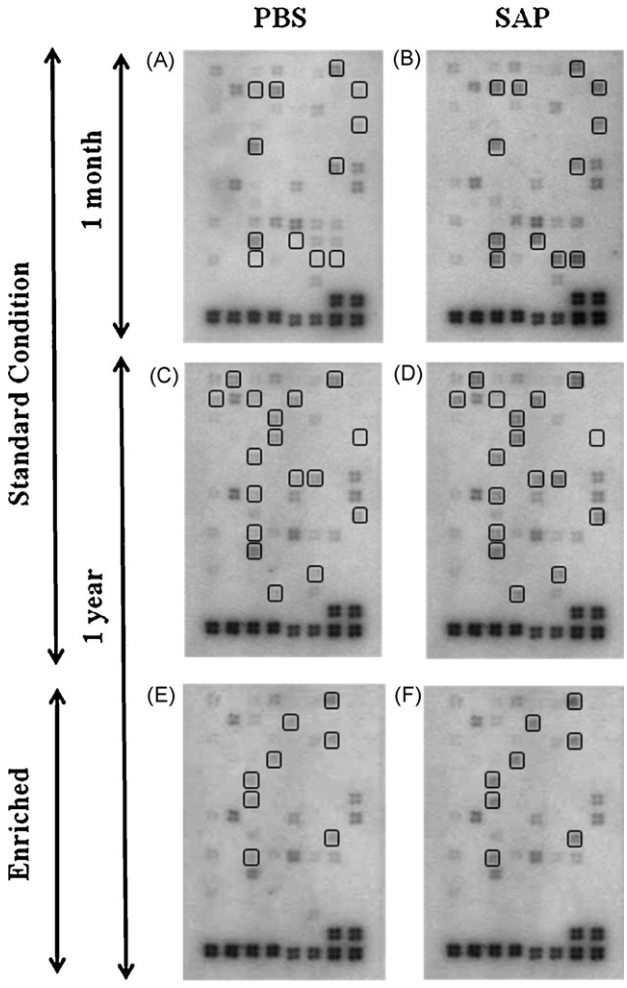

Fig. 2. Gene expression patterns were determined by GE Array Q series MM18 designed to profile the expression of 96 neurotrophic signaling molecules involved in growth, differentiation, survival, and apoptosis. Squared spots indicate the position of the differentially expressed genes in control (PBS) and 192 IgG-saporin-lesioned (SAP) rats at 1 month (A and B) and 1 year (C and D) after surgery. Rats were housed in standard (A–D) or enriched environment (E and F).

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such as positive regulation of oligodendrocytes.

Non-lesioned rats 15 months old housed in enriched environment had gene expression changes. Fifteen transcripts demonstrated a statistically significant expression change between PBS rats housed in standard condition and PBS rats housed in enriched environment; all were upregulated. Thirty-five genes changed more than 1.5-fold (Fig. 2C and E, and Table 3B). Most of them belonged to S1. Bdnf gene was the most upregulated. Further analysis using hierarchical clustering showed that the 15 genes selected could be split into three clusters.

- Cluster 1 included bdnf, mt3, and pspn genes and would be involved in biological processes such as positive regulation of neuron differentiation, regulation of synaptic plasticity, and negative regulation of neuron apoptosis.
- Cluster 2 included gdnf, gfra3, gpr74, ptn, cmkar4, stat5a, and trp53 genes and would be involved in biological processes such as regulation of cell cycle, neurotransmitter secretion, and regulation of apoptosis.
- Cluster 3 included gmfb, ngfb, tac1, fgf2, and myc genes and would be involved in biological processes such as glial cell differentiation, positive regulation of cell proliferation, and angiogenesis.

3.3. RT-PCR analysis

To validate data obtained with macroarray, we used semi-quantitative RT-PCR, using the same RNA samples as for macroarrays. The results, illustrated in Fig. 3, confirmed the upregulation of bdnf and hcrt2 observed in the GE Array analysis. 192 IgG-saporin-treated rats showed a marked increase in bdnf and hcrt2 messenger RNA expression no matter what the experimental condition was, i.e., post-lesion time or housing condition. Genes such as ntrk3 and fgf9 that were not identified by macroarray were also not revealed by RT-PCR. So, one may argue that, for these two genes, the corresponding mRNA amounts were not sufficient to be detected by this molecular technology.

3.4. Immunohistochemical analysis

To complement molecular experiments, we carried out work on the expression of bdnf protein in various brain regions, including the areas where cholinergic cells somata are localized and the cholinergic target projection areas. Bdnf immunoreactivity showed higher labeling in SAP-lesioned rats than in PBS rats and that did not depend on the time rats were killed (1 month vs. 1 year) or on housing condition (standard vs. enriched environment) (Fig. 4). This increase in bdnf staining in SAP-treated rats was about 34% in the medial septum, 43% in the hippocampus, 24% in the frontal cortex, and 35% in the entorhinal cortex. The results are illustrated in Fig. 4A, B, and C for rats housed in standard condition and killed 1 year after surgery. At the level of the basal forebrain cholinergic cells, i.e. the medial septum (Fig. 4A and D),

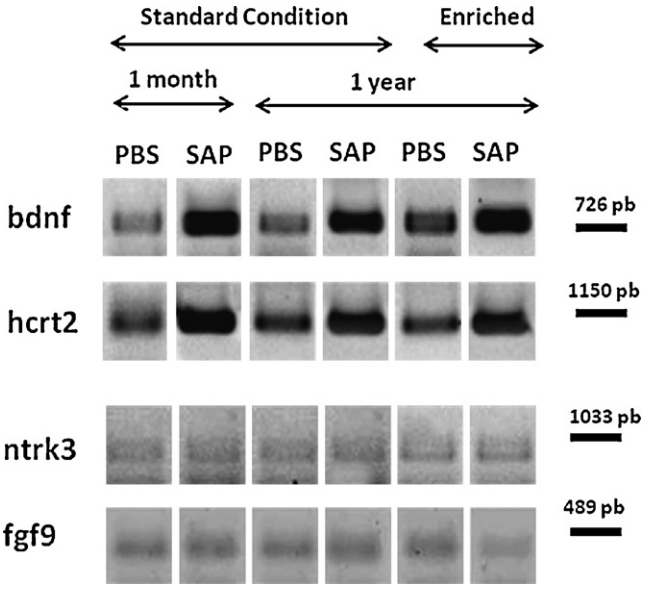

Fig. 3. Representative semi-quantitative RT-PCR performed on brain-derived neurotrophic factor (bdnf), orexin receptor 2 (hcrt2), neurotrophic tyrosine kinase receptor type 3 (ntrk3), and fibroblast growth factor 9 (fgf9) genes in control (PBS) and 192 IgG-saporin (SAP)-treated rats 1 month and 1 year post-lesion. Rats were housed in standard or enriched environment. Note that for each rat, 10–15 mg of medial septum, hippocampus, and frontal and entorhinal cortices were pooled. The results indicated that SAP-treated rats had a marked increase in bdnf and hcrt2 messenger RNA expression no matter what the experimental condition was, confirming data obtained from GE Array analysis. Ntrk3 and fgf9 genes that were not identified by macroarray were also not revealed by RT-PCR.

lesioned rats exhibited elevated bdnf protein expression. In the dorsal hippocampus (Fig. 4B and D), marked immunostaining was observed in the CA1 field of 192 IgG-saporin rats. In the frontal cortex (Fig. 4C and D) and entorhinal cortex (data not shown), a clear immunoreactive signal for bdnf was observed in lesioned rats but not in PBS rats.

No ChAT-immunoreactive cells were observed in the lesioned areas (MS/DBB and NBM) of immunolesioned rats. The results are illustrated in Fig. 5 for rats killed 1 month after surgery. The extent of the lesions appeared to be equivalent across experimental groups. After staining with cresyl violet, there were no apparent histological differences in the structures of the PBS and SAP-lesioned rats (Fig. 5C and F).

4. Discussion

This is the first study to examine gene expression changes in rat brain after 192 IgG-saporin immunolesion of cholinergic basal forebrain through cDNA macroarray analysis. Results show that cholinergic neuronal loss modulates the expression of a number of genes associated with neurotrophins, in particular bdnf, but also genes associated with inflammatory response, neuron apoptosis, regulation of angiogenesis, and synaptic plasticity. Also, aging was shown to be associated with regulation of glial proliferation and

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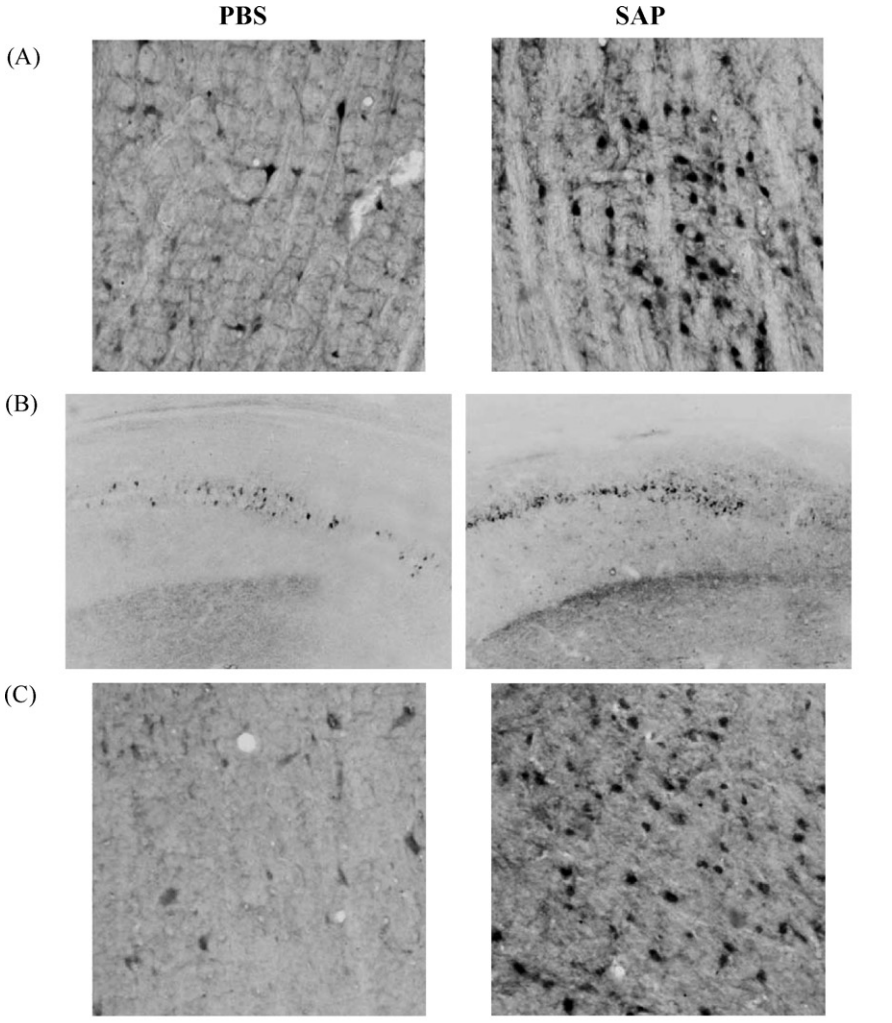

	PBS	SAP	PBS	SAP	PBS	SAP
Medial Septum	17,8 ± 1,1	47,1 ± 2,7*	19,4 ± 0,9	58,4 ± 1,2 *	18,7 ± 1,2	59,8 ± 2,7 *
Hippocampus	24,9 ± 2,1	62,4 ± 2,6 *	30,1 ± 1,5	68,7 ± 1,9 *	28,7 ± 1,9	64,9 ± 3,1 *
Frontal Cortex	14,2 ± 0,4	58,7 ± 2,8	13,5 ± 0,9	64,5 ± 3,1 *	13,9 ± 0,5	49,5 ± 1,7 *
Entorhinal Cortex	16,2 ± 0,6	43,7 ± 1,9*	18,5 ± 1,5	61,0 ± 1,1 *	19,7 ± 1,1	54,2 ± 2,1 *

Fig. 4. Expression of bdnf in medial septum (A), dorsal hippocampus (B), and frontal cortex (C) revealed by immunohistochemistry. Representative sections from control (PBS) and 192 IgG-saporin (SAP)-treated rats at 1 year post-lesion. A marked increase in the expression of bdnf immunoreactivity was found following cholinergic damage. Similar results were obtained in the other experimental conditions (post-lesion time of 1 month and housing environment). (A and C) Magnification 25×; (B) Magnification 10×. (D) Number of positive bdnf cells in various brain regions of control (PBS) and 192 IgG-saporin-lesioned (SAP) rats at 1 month and 1 year post-lesion housed in standard or enriched environment. *$P < 0.05$ (vs. PBS group).

apoptosis. Lastly, we show that long-term enriched environment housing upregulates gene expression and enhances behavioral performance in lesioned and non-lesioned rats. This therapeutic role of the enriched environment seemed to be associated with a suppression of expression of genes involved in apoptosis, glial cell differentiation, and cell cycle, but also with an enhanced expression of a subset of genes involved in signal transduction through transcription regulator or neurotransmitter receptor activity.

Consistent with results from our laboratory in the same experimental conditions (Paban et al., 2005a,b), rats with cholinergic damage after 192 IgG-saporin injections had memory deficits in the nonmatching-to-position task. At these post-lesion times of 1 month and 1 year, rats required

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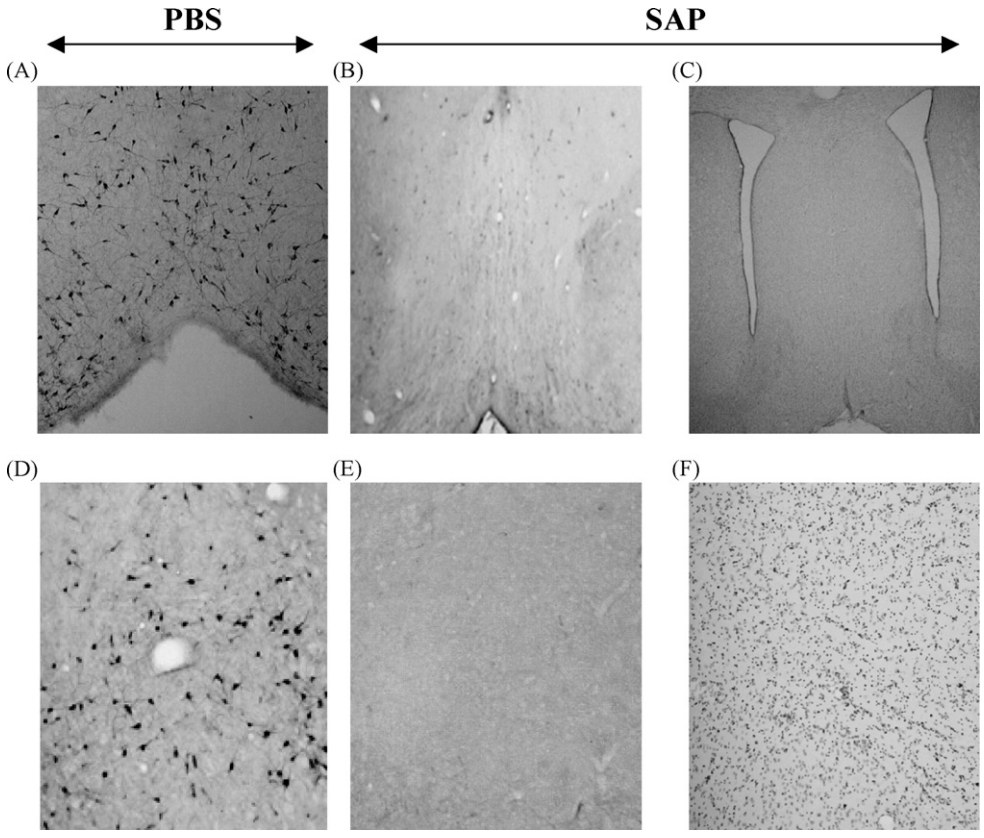

Fig. 5. Representative photomicrographs of ChAT-immunostained (A, B, D, and E) and cresyl violet-stained sections (C and F) in medial septum (A–C) and nucleus basalis magnocellularis (D–F) of control (PBS, A and D) and 192 IgG-saporin (SAP, B, C, E, and F)-treated rats at 1 month post-lesion. No ChAT immunoreactivity was observed throughout the lesioned areas, as compared with control rats. (A–C) Magnification 2.5×; (D–F) Magnification 25×.

more time to reach the criterion than did the control rats. These results corroborate and add to a growing body of data emphasizing the behavioral effects of the post-operative testing time. Indeed, Perry et al. (2001) and we (Paban et al., 2005a) previously reported deficits at these post-lesion times. Note that at 1 year post-lesion, rats were 15 months old and that consequently, in addition to the lesion effect, a deleterious effect due to age takes place progressively. Environmental enrichment reduced the age-related impairment. It also reduced the deleterious effect of the lesion, as previously demonstrated in the laboratory (Paban et al., 2005a). Correlatively, in accordance with previous data from the laboratory (Paban et al., 2005a), the present study showed by immunohistochemistry that choline acetyltransferase (ChAT) immunoreative cells had totally disappeared 1 month and 1 year after intra-structure injection of 192 IgG-saporin. Following intra-structure injection, numerous data from studies using routes and doses of 192 IgG-saporin comparable to the ones used in here have demonstrated the selectivity of the immunotoxin for producing lesions of the cholinergic basal forebrain with no specific loss of parvalbumin-, neuropeptide Y-, NADPH-diaphorase, or glutamate decarboxylase-immunoreactive neurons (Baxter, 2001; Torres et al., 1994; Wenk, 1997). No changes in the levels of monoamines and their metabolites in basal forebrain cholinergic projections have been detected after 192 IgG-saporin lesions (Pizzo et al., 2002; Walsh et al., 1996). In addition, the depletion in hippocampal and cortical ChAT activity has been paralleled by equivalent reductions in the acetylcholine synthesis rate and release and in the high-affinity choline uptake following intraventricular 192 IgG-saporin injection (Rossner et al., 1995; Waite and Chen, 2001). Thus, the immunotoxin is currently considered a valid tool for selectively eliminating cholinergic pathways.

4.1. Gene expression changes following cholinergic injury in behaviorally trained rats

cDNA macroarray served to investigate the molecular effects of cholinergic injury in behaviorally trained rats. Following lesion, gene expression changes concerned especially the subset of neurotrophins. This is hardly surprising since alterations in neurotrophin protein synthesis and/or mRNA level have been well established in various lesioned-animal models (Chen et al., 2008; De March et al., 2008; Hattiangady et al., 2006; Imai et al., 2007; Macias, 2008; Wu et al., 2006). In the present study, the most relevant molecule belonging to this subset was bdnf. Both cDNA macroarray and RT-PCR methods have shown that bdnf was upregulated in lesioned rats. Interestingly, our immunohistochemistry data

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showed that cholinergic-lesioned rats exhibited an elevation of bdnf protein expression in various brain regions, including the medial septum, the dorsal hippocampus, and the frontal and entorhinal cortices. Bdnf is known to play various roles in the central nervous system, including promoting survival, maintenance, and growth of distinct neuronal populations (Labelle and Leclerc, 2000). In particular, bdnf is involved in the maintenance of forebrain cholinergic neurons (Gómez-Palacio-Schjetnan and Escobar, 2008; Morse et al., 1993; Pencea et al., 2001; Taupin and Gage, 2002; Xuan et al., 2008). Furthermore, bdnf plays a crucial role in cognition, learning, and memory formation by modulating synaptic plasticity (Schindowski et al., 2008; Todd et al., 2007). Hsieh et al. (2003), to our knowledge, are the only team to have applied cDNA microarray technology to investigate gene expression changes after cholinergic damage. They used a cDNA collection containing 9600 sequence-verified human cDNA clones spotted on a nylon membrane to explore the gene expression profile in the hippocampus of scopolamine-treated rats. Animals were killed 30 min after intracisternal injection. In spite of some experimental differences (animal models, structures, and cDNA array technology), their results add to ours, indicating an upregulation of genes associated with the signaling pathways of muscarinic receptors (e.g., overexpression of inositol 1,4,5-triphosphate receptor) and with Alzheimer's disease (e.g., upregulation of amyloid, protein tau) in treated rats.

Interestingly, we have shown that gene expression changes following cholinergic immunolesion depended on the post-lesion time. At 1 month post-lesion, 12 genes showed differential expression. To extract information from gene expression and discover more complex relationships, we used extensive literature searches in PubMed and then relied on the GO database. In particular, we used Onto-Express which is able to identify the most relevant biological processes from lists of selected genes (http://vortex.cs.wayne.edu/projects.htm) (Draghici et al., 2003; Khatri et al., 2002) and DAVID websites (http://david.abcc.ncifcrf.gov/ease/ease.jsp) (Dennis et al., 2003). Thus, the biological processes the most represented by the cluster including cx3cr1 and stat5a genes, but also by the cluster including lifr (Covey and Levison, 2007; März et al., 2002), crh (Bayatti and Behl, 2005; Hanstein et al., 2008), or gpr74, would be positive regulation of inflammatory response, cell adhesion, and growth. In contrast, the cluster including genes such as bdnf (Kohara et al., 2003; Tolwani et al., 2002), cntfr (Lin et al., 1998; Sleeman et al., 2000), ptn (Iseki et al., 2002), or tac1 genes would be involved in a common way in cellular plasticity, dendrite development, and, notably, neuron recognition. At 1 year post-lesion, 17 genes showed differential expression. The bdnf gene as well as its protein product still showed a high level of expression. It belonged to a cluster that also includes trp53, ntf3, ptn, and notably, apaf1. The biological profiling of this cluster indicated it is involved in regulation of neuron apoptosis, in particular (Ferrer and Planas, 2003; Heaton et al., 2002; Liot et al., 2004; Ma et al., 2002; Troy et al., 2002; Peria et al., 2007). The second cluster including fgf2 (Jin et al., 2005; Kiprianova et al., 2004), gfra3 (Wang et al., 2002; Wang et al., 2004), nr1i2 (Langmade et al., 2006), mt3 (Carrasco et al., 2003), and lif (Holmberg and Patterson, 2006) genes for instance would be implicated in glial cell differentiation, negative regulation of neurogenesis, and interestingly in positive regulation of angiogenesis. The two highly correlated genes – cntfr and jun genes – identified in the third cluster would be involved in a common cellular pathway associated with regenerative program/cell body response to injury (Dhandapani et al., 2003). When one considers all of the six genes (bdnf, cntfr, hcrtr2, tac1, gpr74, and ptn) affected by the cholinergic injury no matter what the post-lesion time was (1 month or 1 year), it is interesting to note that there were all up-regulated, suggesting that cholinergic depletion in rats behaviorally tested elicited stimulation of cerebral biological processes. More precisely, these genes seem to be involved in common biological processes such as regulation of neuron differentiation and synaptic plasticity. In particular, they stimulate axon target recognition and dendrite development (Bayer et al., 2004; Bevan et al., 2008; Carter et al., 2002; Fukumitsu et al., 2006; Hienola et al., 2004; Ozog et al., 2007; Sheridan and Adler, 2006; Smith and Pang, 2005). Altogether, cholinergic injury induced gene expression changes that seem to be associated with synthesis of potent survival factors for neurons, which may be important for reducing tissue destruction following inflammatory attacks, and also associated with structural remodeling of the neuronal network, which appeared to start as soon as 1 month after surgery and be maintained up to 1 year. Interestingly, it appears that neurotrophins were not able to compensate the behavioral deficits since lesioned rats performed badly relative to controls; genes associated with neuronal death and inflammatory response might account for the behavioral deficits-induced cholinergic injury. Further pharmacological approaches should give more insight into the question.

Lesioned rats housed in enriched environment and behaviorally tested 1 year post-lesion had gene expression changes that affected notably bdnf, crhbp, hcrt2, and ntf3 genes. Biological profiling of this cluster indicated that these genes were associated with axon guidance and dendrite development. Interestingly, for rats submitted to 192 IgG-saporin injections into the NBM, Mandolesi et al. (2008) showed that housing in enriched environment enhanced the number of dendritic spines in the parietal neurons. Also, biological profiling suggested the involvement of cellular pathways associated with regulation of apoptosis through ntf3 gene. Bates et al. (2002) reported that ntf3, classically known as a neuronal survival factor, could also promote cell death during acute stroke. The two other clusters, npy6r - fos genes and gfra3 - il6ra genes, were associated with processes of regulation of synaptic plasticity. Altogether, we have shown that enriched environment upregulated gene expression and enhanced behavioral performance in cholinergic lesioned rats. This therapeutic role of the enriched environment seemed to have a two-way effect: (1)

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inducing the expression of certain genes, such as npy6r, il6ra, and fos genes, i.e., genes associated with signal transduction through transcription regulator or neurotransmitter receptor activity (Bensadoun et al., 2001; Zhang et al., 2002) and (2) preventing the expression of other genes, e.g., cntfr, tac1, gpr74, apaf1, i.e., genes involved in regulation of apoptosis, glial cell differentiation, and cell cycle (Carrasco et al., 2003; Heaton et al., 2002; Langmade et al., 2006; Peria et al., 2007). However, any interpretation of such data calls for further studies. In particular, pharmacological approaches should provide more insight. Interestingly, the 3-way Venn diagram indicating the gene expression profiles after cholinergic injury at 1 month and 1 year post-lesion times of rats housed in standard or enriched environment revealed that bdnf and hcrt2 genes were common to all groups, regardless of lesioning or age. The crucial role of bdnf in synaptic plasticity has been discussed above. Hcrt receptor 2 is highly expressed in medial septum- diagonal band of Broca. It mediates the action of the hcrt peptides (orexins also known as hypocretins). Hcrt has a strong and direct excitatory effect on the release of neurotransmitters such as glutamate and acetylcholine. It has also been involved in motivated behaviors through an action on attentional and sensory systems (Siegel, 2004; Wu et al., 2004). Sutcliffe and De Lecea (2000) discussed the complex role of the hcrt peptides, notably in some aspects of energy metabolism, cardiovascular function, hormone homeostasis, and sleep-wake behaviors.

4.2. Gene expression changes during aging in behaviorally trained rats

Gene expression changes in non-lesioned middle-aged rats (15 months old) and behaviorally trained consisted of upregulation of gmfb, fgfr1, stat6, and apaf1 for instance and downregulation of hcrtr2, nrg4, il1b, and myc genes. These genes could be split into two clusters of highly correlated genes. Biological profiling suggested an involvement in regulation of astrocytes/glial proliferation and also oligodendrocytes (Iseki et al., 2002; Isono et al., 2003; Lesné et al., 2002; Matsumura et al., 2003; Zeis et al., 2008). Our data also suggested that aging would be associated with apoptosis through activation of genes such as apaf1 (Cregan et al., 2002) and myc oncogene (Morrish et al., 2003). The present study extends our knowledge on gene expression-profiling during aging. Indeed, literature data indicate that rodent aging was associated mainly with altered expression of genes related to inflammation, protein processing, oxidative stress, lipid/protein metabolism, neurite/axon growth, and cytoskeletal/extracellular assembly (Blalock et al., 2003; Cheng et al., 2007; Prolla, 2002; Rowe et al., 2007; Weindruch et al., 2002). The use of different statistical techniques and gene microarray technologies makes it difficult to directly compare studies. Moreover, the brain regions, species, strain, and ages all differed in these studies. Interestingly, in spite of these experimental differences some genes showed a similar expression pattern: myc, which plays roles in cell proliferation, differentiation, and death and tgfa, which is an endogenous, mitogenic ligand that can promote changes in astrocytes/glial proliferation and survival.

These rats (non-lesioned, 15 months old) housed in enriched environment had gene expression changes that affected pspn, bdnf, and mt3 genes. These genes belong to the same cluster, suggesting that they could act together as modulators of excitotoxicity in the central nervous system, with pronounced neuroprotective activity (Golden et al., 2003). Biological profiling of the two other clusters suggested the involvement of processes associated with regulation of angiogenesis (Presta et al., 2005; Zhang et al., 2008), neurotransmitter secretion (Huh et al., 2008), and cellular plasticity. These results add to a growing body of data showing the beneficial effect of environmental enrichment on the brain of old rodents when enrichment is applied during a long period or late in life (Branchi et al., 2004; Cotman and Berchtold, 2002; Ronnback et al., 2005; Van Praag et al., 2000).

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