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Global view of transcriptome in the brains of aged NR2B transgenic mice

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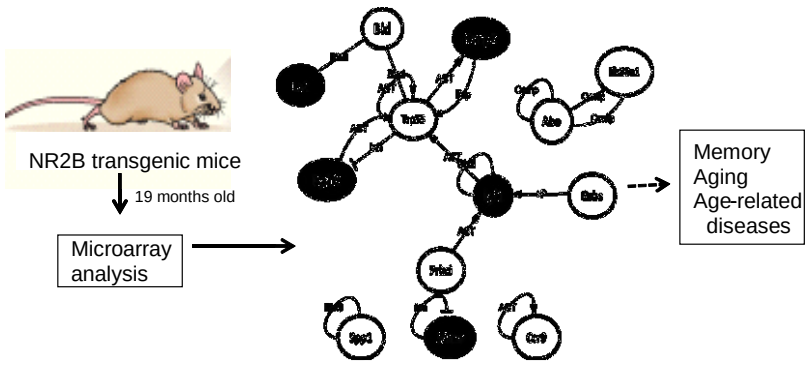
Graphical Abstract



Abstract

NR2B subunits are involved in regulating aging, in particular, age-related learning and memory deficits. We examined 19-month-old NR2B transgenic mice and their littermate controls. First, we detected expression of the NR2B subunit gene, *Grin2b*, in the neocortex of transgenic mice using real-time PCR. Next, we used microarrays to examine differences in neocortical gene expression. Pathway and signal-net analyses identified multiple pathways altered in the transgenic mice, including the P53, Jak-STAT, Wnt, and Notch pathways, as well as regulation of the actin cytoskeleton and neuroactive ligand-receptor interactions. Further signal-net analysis highlighted the P53 and insulin-like growth factor pathways as key regulatory pathways. Our results provide new insight into understanding the molecular mechanisms of NR2B regulated age-related memory storage, normal organismal aging and age-related disease.

Key Words

neural regeneration; memory; NR2B transgenic mice; aging; gene expression; P53; insulin-like growth factor; grants-supported paper; neuroregeneration

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INTRODUCTION

Aging has long been known to have detrimental effects upon nervous system function, and many brain functional processes including synaptic plasticity, long-term potentiation (LTP), learning and memory are altered by aging. The forebrain areas vulnerable to aging include the cortex and hippocampus.

The N-methyl-D-aspartic acid (NMDA) receptor is a heteromer consisting of NR1 and at least one of either NR2 or NR3 subunits[1-2]. Early in postnatal development, the NR2B subunit is predominant, but as the postnatal brain develops into an adult brain, the NR2B:NR2A ratio gradually decreases[3-4], leading to a gradual decrease in NMDA current duration and an increase in the synaptic plasticity induction threshold[5-6]. NMDA receptor dysfunction in the brain of aged animals has been shown[7-8], with age-related reductions in NMDA receptor subunit expression found in multiple brain areas[8-9]. This reduction is reversed in the forebrain of *NR2B* transgenic (Tg) mice[5]. In Tg mice, NR2B is overexpressed mainly in the forebrain, resulting in enhanced synaptic plasticity and improved learning and memory function in young adult mice[5, 10]. Interestingly, in rats, forebrain NR2B overexpression also causes memory function enhancement[6], suggesting *NR2B* gene function in memory formation is universal.

It is important that NR2B-containing NMDA receptor not only plays a pivotal in various memory processes, but it is also involved in regulating aging, especially age-related learning and memory deficits. Among the NMDA receptor subunits, the NR2B subunit shows the greatest age-related decline in mRNA and protein levels across species, in both the cerebral cortex and specific hippocampal regions, in aged animals[11]. Moreover, in aged rodents, there is a significant correlation between the decline in NR2B subunit expression and impaired memory function[12-13]. Furthermore, using antisense oligonucleotides to specifically knock down the hippocampal NR2B subunit in young rats, Clayton *et al* [14] found that NR2B antisense treatment diminished NMDA receptor responses, abolished NMDA-dependent LTP and impaired spatial learning in the Morris water maze task, thereby mimicking age-related changes in young rats. Interestingly, a paper from our group also confirmed the hypothesis that NR2B upregulation is beneficial for memory improvement in aged mice. Cao *et al* [15] showed that aged NR2B Tg mice display enhanced novel object recognition memory, fear memory and spatial memory. These studies also indicate that age-related reductions in expression of the NR2B subunit gene, *Grin2b*, likely contribute to memory impairment in aged animals.

Nevertheless, the molecular mechanisms underlying NR2B effects in aged brain remain to be explored. To better understand NR2B mechanisms in aging, we first used microarrays to profile gene expression changes in the neocortex of 19-month-old NR2B Tg mice. Second, we performed pathway and signal-net analyses of differentially expressed genes. Third, using real-time PCR and western blot assays, we validated our microarray data. Identification and characterization of differential gene expression profiles in the neocortex of aged Tg mice will provide important insights into how NR2B regulation works in organismal aging processes and aid identification of new therapeutic targets for age-related disorders such as memory impairments and neurodegenerative diseases.

RESULTS

Enhanced expression of the NR2B subunit gene, *Grin2b*, in aged NR2B transgenic mice

To confirm elevated *Grin2b* gene expression in the neocortex of 19-month-old Tg mice, we isolated neocortical RNA from both Tg mice and WT littermates. Using real-time PCR, we found a significant increase in *Grin2b* mRNA levels in aged Tg mice ($P < 0.01$; Figure 1).

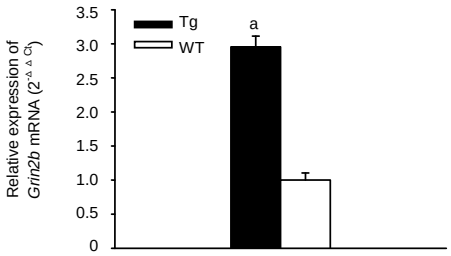

Figure 1 Relative expression levels of the NR2B subunit gene, *Grin2b*, in the neocortex of aged transgenic (Tg) and wild-type (WT) mice.

Grin2b expression was analyzed by real-time PCR. Error bars indicate SEM. Data were analyzed using the Student's *t*-test. $^{a}P < 0.01$, *vs*. WT mice. Five mice were used in each group, and the experiment was repeated three times.

Gene expression changes in the neocortex of aged Tg mice

The results of our microarray analysis showed that 457 genes were changed (> 1.5-fold) their expression levels in the neocortex of aged mice (data not shown). Examination of the function of these genes, found that a number of ion channel and hormone/growth factor genes were regulated, as well as genes involved in neurotransmission, signal transduction, structure/cytoskeleton, development, apoptosis and transcription (Table 1).

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for differentially expressed genes showed that the genes were involved in many pathways including p53, Jak-STAT, Wnt and Notch, as well as MAPK and calcium signaling pathways. KEGG pathway analysis also showed the genes were associated with neuroactive ligand-receptor interactions, cytokine-cytokine receptor interactions, cell communication, glycosphingolipid biosynthesis-lactoseries, focal adhesions, complement and coagulation cascades, extracellular matrix-receptor interactions, long-term depression, bladder cancer, glycine/serine/threonine metabolism, regulation of actin cytoskeleton, basal cell carcinoma, glycan structures-biosynthesis, glioma, melanoma, prostate cancer, apoptosis, natural killer cell mediated cytotoxicity, and tight junction and cell adhesion molecules (Figure 2).

Signal-net analysis

Using the KEGG database, we performed signal-net analysis to construct networks of differentially expressed genes. Key genes identified (Figure 3) include apoptosis-related, for example Bid and p53 that participate in the P53 signaling pathway, and insulin-like growth factor 1 (*Igf1*) that is involved in the IGF signaling pathway.

Validation of microarray data by real-time PCR

To validate the genes identified by microarray, we selected four genes (*Ppp2r5e*, *Grid2*, *Ttr* and *Egr2*) for real-time PCR confirmation. We found *Ppp2r5e*, *Grid2* and *Egr2* were increased 1.71, 1.23, and 1.78-fold, respectively, in the cortex of aged Tg mice, while *Ttr* was decreased 0.47-fold. These expression changes were consistent with our microarray analysis (Figure 4).

Expression changes of P53 and *Igf1* in the neocortex of aged Tg mice

We also examined P53 protein and *Igf1* mRNA expression in the neocortex of aged Tg mice. Western blot analysis showed significantly reduced P53 levels (Figure 5A, B), while real-time PCR showed increased *Igf1* mRNA levels (Figure 5C), in Tg mice compared to WT littermates.

DISCUSSION

Aging leads to changes in brain morphology and function, which are related with an increased risk for neurological and psychiatric disorders. To investigate the molecular mechanisms of NR2B overexpression during aging, and identify the effect of NR2B on age-related transcriptional changes in the brain, we analyzed gene expression profiles in the neocortex of aged (19-month-old) Tg mice. We found expression of 457 genes in the neocortex were changed > 1.5-fold. Ion channel genes and hormone/growth factor genes, as well as those involved in signal transduction, neurotransmission, cytoskeletal structure, development, apoptosis and transcription, were significantly altered in aged NR2B Tg mice. KEGG pathway analysis showed multiple pathways were involved, while signal-net analysis identified the P53 and IGF pathways as key regulatory pathways. Our results provide new insight into understanding the molecular mechanisms of NR2B regulated age-related memory storage, normal organismal aging and age-related diseases.

In aged Tg mice, many ion channel genes showed altered expression levels. The cholinergic receptor gene, *Chrna4*, has previously been implicated in working memory[16] and age-related memory[17], and was upregulated in Tg mice, as was another cholinergic receptor subunit gene, *Chrna9*. In addition, we found the gamma-aminobutyric acid (GABA) receptor epsilon subunit, *Gabre*, and rho 2 subunit, *Gabrr2*, were also increased. GABA(A) mRNA shows age-related changes[18], and recently, two potentiators of GABA(A) alpha 5, were shown to improve hippocampus-depen- dent memory in aged rats[19].

Table 1 Fold changes of selected genes in the neocortex of aged NR2B transgenic mice during aging

Functional classification	Accession No.	Gene name	Fold change (Tg mice *vs.* WT mice)
Ion channel and neurotransmission	NM_008429	Potassium inwardly-rectifying channel, subfamily J, member 9 (*Kcnj9*)	0.66
	NM_007474	aquaporin 8 (*Aqp8*)	1.54
	NM_008861	Polycystic kidney disease 2 (*Pkd2*)	1.78
	AK010496	Cholinergic receptor, nicotinic, alpha polypeptide 9 (*Chrna9*)	1.95
	NM_017369	Gamma-aminobutyric acid (GABA-A) receptor, subunit epsilon (Gabre)	1.99
	NM_008076	Gamma-aminobutyric acid (GABA-A) receptor, subunit rho 2 (*Gabrr2*)	1.66
	AF492840	Cholinergic receptor, nicotinic, beta polypeptide 4 (*Chrnb4*)	1.63
	AK045348	Glutamate receptor, ionotropic, delta 2 (*Grid2*)	1.55
	ENSMUST00000053880	Glutamate receptor, ionotropic, NMDA2B (*NR2B*)	1.89
	AK035644	Solute carrier family 18 (vesicular monoamine), member 2 （*Slc18a2*)	1.66
	BC022720	Glutamate receptor, metabotropic 6 (*Grm6*)	0.65
	NM_021889	synaptotagmin 9 (*Syt9*)	0.62
	NM_028924	Membrane targeting (tandem) C2 domain containing 1 (*Mtac2d1*)	0.61
	D29797	Syntaxin 3A(*Stx3*)	0.63
Signal transduction	NM_007788	Casein kinase II, alpha 1 related sequence 4 (*Csnk2a1-rs4*)	1.62
	AK019899	P21-activated kinase 2 (*Pak-2*)	1.53
	BC026444	WD repeat domain 62 (*Wdr62*)	2.09
	NM_010831	SNF1-like kinase (*Snf1lk*)	1.60
	AK081588	Neurotrophic tyrosine kinase, receptor, type 1(*Ntrk1*)	1.56
	NM_008874	Phospholipase C, beta 3 (*Plcb3*)	1.58
	AK016942	Protein phosphatase 1F (PP2C domain containing)(*Ppm1f*)	1.73
	ENSMUST00000019153	Synaptojanin ec_3.1.3.36 synaptic inositol 1 4 5 trisphosphate 5 phosphatase	1.58
	AK014554	Serine/threonine protein phosphatase 2a, b subunit, b56 epsilon isoform (*Ppp2r5e*)	1.87
	AF022802	Phospholipase C beta-2 (*Plcb2*)	0.64
	NM_008857	Protein kinase C, lambda (*Prkcl*)	0.51
	AI627018	Protein phosphatase 1, catalytic subunit, alpha isoform (*Ppp1ca*)	0.52
	NM_011153	G substrate (*Gsbs*)	0.65
	NM_010276	GTP binding protein (*Gem*)	1.76
	AI854138	Ral exchange factor (*Angptl2*)	1.54
Structure/cytoskeleton	NM_010662	Keratin complex 1, acidic, gene 13 (*Krt1-13*)	1.72
	L00919	Erythrocyte protein band 4.1 (*Epb4.1*)	1.75
	NM_016879	Keratin complex 2, basic, gene 18 (*Krt2-18*)	1.54
	NM_010060	Dynein, axon, heavy chain 11 (*Dnahc11*)	1.66
	NM_010473	Histidine rich calcium binding protein (*Hrc*)	1.61
	NM_010213	Four and a half LIM domains 3 (*Fhl3*)	2.01
Transcription	AJ318416	N-WASP protein (*Wasl*)	1.94
	NM_133665	Myocyte enhancer factor 2D (*Mef2d*)	1.76
	AF193435	HMG box transcription factor (*Sox14*)	1.63
	NM_013633	POU domain, class 5, transcription factor 1 (*Pou5f1*)	1.65
	X06746	Early response gene 2 (*Egr2*)	1.67
	NM_011565	TEA domain family member 2 (*Tead2*)	1.50
	NM_010127	POU domain, class 6, transcription factor 1 (*Pou6f1*)	2.05
	NM_021307	Zinc finger protein 112 (*Zfp112*)	2.14
	NM_010866	Myogenic differentiation 1 (*Myod1*)	0.45
	NM_010351	Goosecoid (*Gsc*)	0.65
	BC022605	cAMP responsive element binding protein 3-like 4 (*Creb314*)	0.63
Hormone and growth factor	NM_008009	Fibroblast growth factor binding protein 1 (*Fgfbp1*)	2.04
	AK047380	Fibroblast growth factor inducible 15(*Fin15*)	2.52
	NM_008010	Fibroblast growth factor receptor 3 (*Fgfr3*)	1.95
	NM_010512	Insulin-like growth factor 1 (*Igf1*)	1.77
	NM_011164	Prolactin (*Prl*)	0.14
	NM_008117	Growth hormone (*Gh*)	0.49
	NM_013566	Integrin beta 7 (*Itgb7*)	1.52
	BU920841	Vasoactive intestinal peptide receptor 2 (*Vipr2*)	1.52
	NM_008050	Nuclear receptor subfamily 5, group A, member 1 (*Nr5a1*)	1.56
	NM_016708	Neuropeptide Y receptor Y5 (*Npy5r*)	0.64
	NM_021332	Glucagon-like peptide 1 receptor (*Glp1r*)	0.63

Table 1 Continued

Functional classification	Accession No.	Gene name	Fold change (Tg mice *vs.* WT mice)
Development	NM_025312	Sclerostin domain containing 1c (*Sostdc1*)	0.57
	NM_013657	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C (*Sema3c*)	0.58
	AB031037	Eomesodermin homolog (*Eomes*)	1.62
	NM_007553	Bone morphogenetic protein 2 (*Bmp2*)	1.79
	NM_010949	Numb gene homolog (Drosophila) (*Numb*)	1.62
	NM_080428	F-box and WD-40 domain protein 7, archipelago homolog (Drosophila) (*Fbxw7*)	0.66
	NM_033041	Hairy and enhancer of split 7 (Drosophila) (*Hes7*)	1.74
	NM_010915	Nerve growth factor, alpha (*Ngfa*)	0.48
Apoptosis	NM_021310	Junction-mediating and regulatory protein (*Jmy*)	1.64
	NM_011641	Transformation related protein 63 (*Trp63*)	1.74
	NM_011640	Transformation related protein 53 (*Trp53*)	0.63
	NM_019573	WW domain-containing oxidoreductase (*Wwox*)	0.61
	NM_007544	BH3 interacting domain death agonist (*Bid*)	0.46
Others	AK003088	Carboxypeptidase A1 (*Cpa1*)	1.51
	NM_008604	Membrane metallo endopeptidase (*Mme*)	2.06
	AK050381	Trophinin associated protein (*Troap*)	1.82
	NM_009246	Serine protease inhibitor 1–4 (*Spi1–4*)	0.65
	NM_009252	Serine protease inhibitor 2–2 (*Spi2–2*)	0.54
	NM_013697	Transthyretin (*Ttr*)	0.50
	BU520753	cDNA, 5 end (*Eif2c2*)	0.62

Gene expression changed > 1.5-fold. Fold change < 0.666 7. Tg: Transgenic; WT: wild-type.

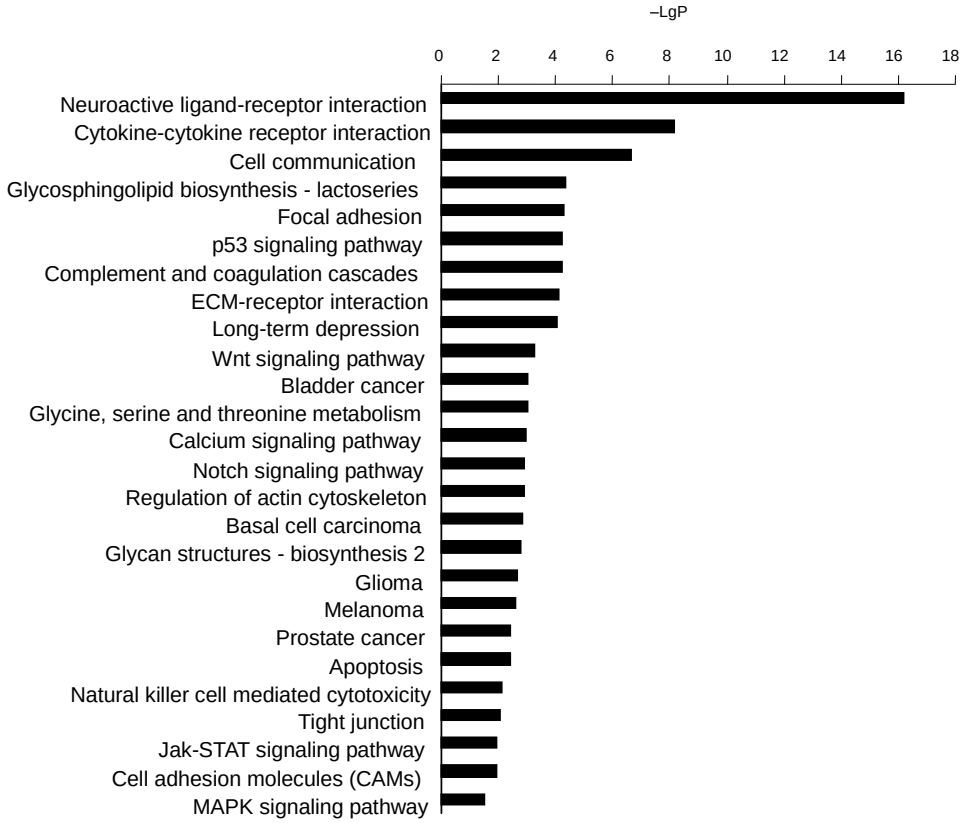

Figure 2 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differentially expressed genes in aged transgenic (Tg) mice.

P values < 0.05 and false discovery rates < 0.05 were used as thresholds to select significant KEGG pathways. The horizontal axis denotes the LgP (log(*P* value)) of significant pathways, with LgP indicating the logarithm of each *P*-value to the base 10. The significance of specific pathways in Tg mice compared to WT is denoted by –LgP.

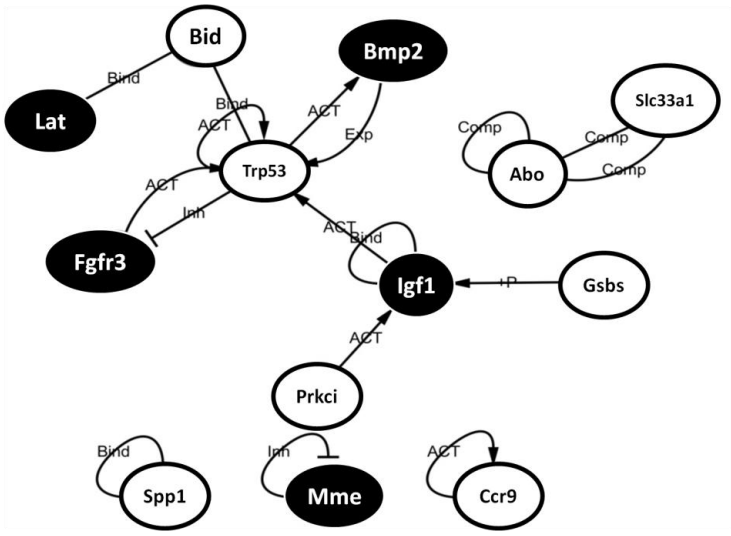

Figure 3 Network of differentially expressed genes in aged transgenic (Tg) mice.

Black and white nodes correspond to genes showing significant up- and downregulation, respectively, in the microarray data. Lines and arrowheads represent the interaction direction. Bind: The two proteins bind together to form a complex; ACT and Inh: a protein activates or inhibits another protein, respectively; +P: a protein transfers an ATP or GTP phosphate group to another protein, thereby activating that protein; Exp: a protein activates expression of another gene; Comp: two proteins, as two mutually adjacent metabolic enzymes, metabolize the same compound.

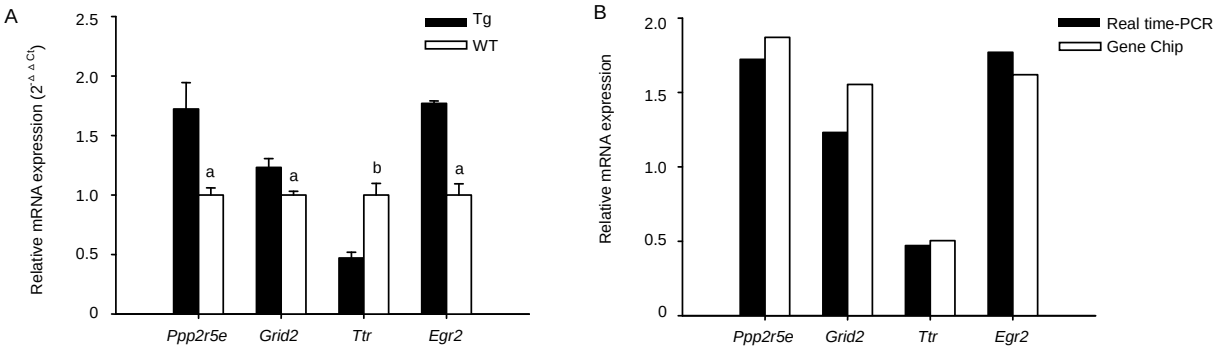

Figure 4 Relative mRNA expression of *Ppp2r5e*, *Grid2*, *Ttr* and *Egr2* in the neocortex of aged transgenic (Tg) mice.

(A) Relative expression levels of *Ppp2r5e*, *Grid2*, *Ttr* and *Egr2* in the neocortex of aged Tg and wild-type (WT) mice, analyzed using real-time PCR. Error bars indicate SEM. Data are analyzed using the Student's *t*-test. $^{a}P < 0.05$, $^{b}P < 0.01$, *vs*. Tg mice.

(B) Comparison of relative gene expression obtained by real-time PCR and microarray.

This raises the interesting possibility that modification of GABA receptor-mediated inhibition of cortical circuit excitability is involved in age-related memory formation and aging related functional changes in the brain.

Imbalances in protein kinases and phosphatases have been reported to be involved in age-related impairments of synaptic transmission[20]. Interestingly, we also found two protein phosphatases (*Ppp2r5e* and *Ppm1f*) were significantly upregulated, and a protein kinase (*Prkcl*) downregulated. This suggests that differential regulation of kinase and phosphatase activities is an important mechanism mediated by NR2B overexpression in aged mice. Conversely, *Ppp2r5e* upregulation was also observed in the hippocampus of 3-month old Tg mice[21], suggesting this may be a common regulatory mechanism in both young and aged animals.

Many studies have demonstrated that as a critical component in developmental regulation, the Notch pathway is also involved in learning and memory processes[22-24]. We found the Notch pathway-related genes, *Fbxw7*, *Numb* and *Hes7*, had altered expression in the cortex of aged Tg mice.

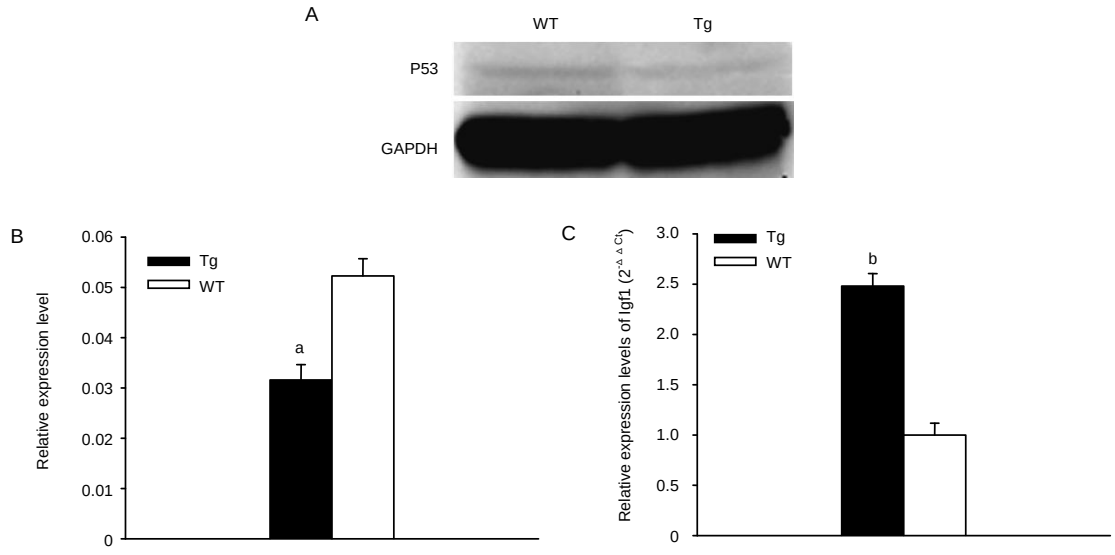

Figure 5 P53 and insulin-like growth factor 1 (*Igf1*) expression in the neocortex of aged transgenic (Tg) mice.

(A) Western blot assay of P53 expression in wild-type (WT) and Tg mice.

(B) Quantification of P53 protein levels was performed by western blot analysis. P53 levels were normalized to GAPDH.

(C) Relative *Igf1* mRNA levels were measured using real-time PCR. Relative expression was calculated as a ratio of *Igf1* to *Gapdh* expression in the same sample.

Error bars indicate SEM. Data are analyzed using the Student's *t*-test. $^{a}P < 0.05$, $^{b}P < 0.01$, *vs*. WT. There were five mice in each group, and the experiment was repeated three times.

Although the precise roles of Notch-related genes in memory have not been determined, our findings suggest that these genes may contribute to the enhancement of memory in aged Tg mice. Accumulating evidence shows an association between neurogenesis reductions and aging-related memory impairments and deficits in certain disorders, including Alzheimer's disease[25]. In this study, another developmentally related gene, *BMP2*, which has been implicated in neocortical neurogenesis[26], was upregulated in aged Tg mice, suggesting BMP2 signaling is involved in age-related memory processes by regulating neurogenesis.

Pathway analysis and further signal-net analysis identified the IGF-1 and P53 signaling pathways as altered in the neocortex of aged Tg mice. IGF-1 has been implicated in regulation of several aspects in CNS, including brain metabolism, neural modulation, neural growth and differentiation. A number of studies suggest that IGF-1 plays an important role in cognitive decline, both in aged humans and rodents. Transcriptome analysis of prefrontal cortex found expression of *IGF1* mRNA was lower in the frontal cortex of aged human subjects compared with young[27]. There may be a close temporal association between reduction of IGF-1 expression and memory impairments[28]. Replacement of IGF-1 in the brain results in improvement of working memory and novel recognition ability in aged rats[29]. Moreover, IGF-1 administration also ameliorates the age-related decrease in hippocampal neurogenesis[30]. These studies suggest there is an association between IGF-1 reduction and cognitive dysfunction during aging. In addition, other studies have found IGF-1 is also involved in anti-apoptotic actions and neuroprotective effects[30].

As a transcription factor, P53 is mainly involved in regulating of cellular effects including apoptosis, cell cycle arrest, and senescence in response to stress. A number of studies suggest P53 also plays a crucial role in regulation of aging and longevity in different species[31]. Recently, a study directly linked p53 to the IGF-1 pathway[32]. Overexpression of P44, a short isoform of p53, hyperactivated the P53 pathway and altered P53-dependent gene expression in mice and consequentially promote IGF-1 signaling, which may account for premature aging of p44 transgenic mice. Moreover, P53 can downregulate the insulin/IGF-1 pathways through transcriptional induction of its target genes, including IGF-BP3 and phosphatase and tensin homologs[31]. It is worth noting that P53 regulates aging and longevity in a context-dependent manner. In certain contexts P53 accelerates the aging process, while in others, P53 extends the life

span[31]. Our study found IGF-1 upregulation and P53 downregulation by NR2B overexpression in aged mice. This suggests that NR2B regulates learning and memory as well as various other physiological and pathological processes during aging, probably through regulation of P53 and IGF-1 signaling. Interestingly, we observed that IGF-1, but not P53, also changed expression in the neocortex of 3-month old Tg mice (unpublished data). It is possible that the IGF signaling pathway is regulated in both young and aged Tg mice, while the P53 pathway is specific to aged Tg mice. Further studies are required to explore the interactions between these two pathways.

In summary, our gene expression profiling results found that many genes involved in neurotransmission, signal transduction, structure/cytoskeleton, transcription, hormone and growth factor, development, and apoptosis may contribute to memory enhancement in aged NR2B Tg mice. Our study also suggests that NR2B overexpression affects many different pathways during aging, especially P53 and IGF-1 signaling. Altered gene expression and different signaling pathways may be associated with memory improvement in aged NR2B Tg mice.

MATERIALS AND METHODS

Design

A bioinformatics study.

Time and setting

This study was performed at the Key Laboratory of Brain Functional Genomics, MOE & STCSM, Institute of Cognitive Neuroscience, East China Normal University, Shanghai, China from June 2005 to January 2011.

Materials

Tg mice and their WT littermate controls were bred from previously generated lines[5]. Male Tg and WT mice aged 19 months were used for microarray experiment. All mice were housed at 20–26°C in 40–70% humidity with a 12-hour light/dark cycle, and allowed free access to food and water. All experimental procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[33].

Methods

Microarray analysis

Microarray analysis was performed as previously described[21, 34]. Briefly, after the decapitation of aged Tg and WT mice, brains were dissected on ice and immediately removed. In each group, whole cortices from five mice were pooled for RNA isolation. Using the Total RNA Isolation Mini Kit, total RNA was extracted (Agilent, Wilmington, DE, USA) and then cDNA synthesized. *In vitro* transcription was performed using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent) in the presence of Cy3- and Cy5-CTP (PerkinElmer, Santa Clara, CA, USA). Fluorescent labeled cRNA was used for oligo microarray hybridization. Hybridization solution was prepared using the *In Situ* Hybridization Kit Plus (Agilent). Agilent's 22K Mouse Oligo Microarray was used for hybridization. The hybridization reaction was carried out in a hybridization incubator for 17 hours at 60°C. The microarray scanner system (Agilent) and feature extraction software were used for data analysis (scan resolution 10 μm, PMT 100%). The LOWESS normalization algorithm was used to standardize microarray data. Log ratio values, equal to the ratio of Cy5 to Cy3 processed signals, were calculated and converted to fold changes. Genes with expression fold changes ≥ 1.5 or ≤ 0.66 (flag = 0, signal-to-noise > 2.6 and *P* value log ratio < 0.05) were selected for further cluster and functional classification analyses.

Pathway analysis

Pathway analysis was used to identify significantly different regulatory pathways, using the KEGG, Biocarta and Reatome bioinformatic databases, as previously described[21]. Two-sided Fisher's exact tests and 2 tests were used to determine statistical significance of pathways. False discovery rates were calculated to correct *P* values. *P* values < 0.05 and false discovery rates < 0.05 were used as thresholds to select significant KEGG pathways.

Signal-net analysis

We used the KEGG database to build networks of genes according to the relationships among the genes, proteins and compounds in the database, as previously described[35-36]. Signal-net analysis is not limited to one pathway and this method can be used to screen the upstream and downstream proteins of one specific protein in the entire KEGG database. To reflect the interactions between genes, connecting lines were drawn. Nodes represent genes, and lines and arrowheads represent the interaction direction.

Confirmation of microarray results by real-time PCR

Real-time PCR was performed as previously described[21]. Briefly, using Trizol (Invitrogen, Carlsbad, CA, USA) and M-MLV reverse transcriptase (Invitrogen), total RNA was extracted from frozen neocortex and cDNA

were generated. The cDNA samples were used as templates for SYBR Green Q-PCR. Primers were designed using Primer Express software (ABI, Vernon, CA, USA) and synthesized.

The primers are as follows:

Primer	Sequence
Grin2b	Forward: 5′-ACT AAC TAT CAA TGA AGA ACG GT-3′
	Reverse: 5′-CCA GAC CCC AGA GTA ACC A-3′
Ppp2r5e	Forward: 5′-AGG TAT GCG TAC CAC CAA GT-3′
	Reverse: 5′-GCA AGC AAG GTC TAC ACT ATC A-3′
Grid2	Forward: 5′-TGG AAG CAA AGT CGT GGT CA-3′
	Reverse: 5′-TGC CAT CAA CAA ACG TCA CA-3′
Ttr	Forward: 5′-ATG GTC AAA GTC CTG GAT GCT G-3′
	Reverse: 5′-CTG CGA TGG TGT AGT GGC-3′
Igf1	Forward: 5′-GCT CCG GAA GCA ACA CTC A-3′
	Reverse: 5′-GCT ATG GCT CCA GCA TTC G-3′
Egr2	Forward: 5′-TTT GAC CAG ATG AAC GGA GTG-3′
	Reverse: 5′-GCG ATA AGA ATG CTG AAG GA-3′

Detection of P53 protein expression by western blot assay

Neocortices from 19-month-old Tg and WT mice (three mice per group) were dissected and homogenized on ice, in lysis buffer (Beyotime, Haimen, Jiangsu Province, China) with 1:100 volume of phenylmethyl sulfonylfluoride. Samples were centrifuged at 14 000 × *g* for 15 minutes to remove debris. Total protein concentrations of the lysates were determined using the bicinchoninic acid assay with bovine serum albumin as the standard. Lysates were then separated by 10% sodium dodecyl sulphate-polyacry-lamide mini-gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA) at 100 V for 60 minutes using the Mill Blot-SDS system (Bio-Rad). After blocking in 10% skimmed milk powder prepared in Tris-buffered saline with Tween-20 at room temperature for 60 minutes, membranes were incubated in 5% bovine serum albumin with mouse anti-p53 monoclonal antibody (1:500; Beyotime) and mouse anti-GAPDH monoclonal antibody (1:5 000; KangChen, Shanghai, China), at room temperature for 1 hour. After two washes in Tris-buffered saline with Tween-20, membranes were incubated in goat anti-mouse IgG-horseradish peroxidase (1:5 000; KangChen) at room temperature for 45 minutes. After three washes, membranes were developed using an enhanced chemiluminescence kit with a 20 minute exposure. Protein levels were quantified using Quantity One software (Bio-Rad) and normalized against GAPDH.

Statistical analysis

Data are presented as mean ± SEM. Statistical significance was determined using Student's *t*-tests and SigmaPlot software (Systat Software Inc., Chicago, IL, USA). *P* values < 0.05 were considered statistically significant.

Research background: NMDA receptor dysfunction in the brain of aged animals has been demonstrated. In addition, there is significant correlation between the decline in NR2B subunit expression and impaired memory function in aged rodents.

Research frontiers: Age-related reductions in NR2B subunit expression are likely associated with memory impairments during aging. However, most studies have focused on behavior and electrophysiology, and the underlying molecular mechanism of NR2B in learning and memory is still less understood.

Clinical significance: Understanding the molecular basis of NR2B regulation on age-related memory storage will provide new potential therapeutic targets for age-related disorders, such as memory impairments and neurodegenerative diseases.

Academic terminology: KEGG, the Kyoto Encyclopedia of Genes and Genomes, was initiated by the Japanese human genome programme in 1995. It is a collection of online databases dealing with genomes and enzymatic pathways.

Peer review: Using microarray technology, this study found 457 genes with altered expression levels in the neocortex of aged NR2B transgenic mice. Further preliminarily observations identified relationships between specific altered genes and pathways. Although the hypothesis is not novel, and there are no important technological innovations, our study does provide new insight into the molecular mechanisms of NR2B regulated age-related memory storage, normal organismal aging and age-related disease.

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