

Nicotine Relieves Anxiogenic-Like Behavior in Mice that Overexpress the Read-Through Variant of Acetylcholinesterase but Not in Wild-Type Mice^[S]

R. Salas, A. Main, D. A. Gangitano,¹ G. Zimmerman, S. Ben-Ari, H. Soreq, and M. De Biasi

Baylor College of Medicine, Department of Neuroscience, Houston, Texas (R.S., A.M., D.A.G., M.D.B.); Interdisciplinary Center of Neural Computation, the Hebrew University of Jerusalem, Jerusalem, Israel (G.Z., H.S.); and Department of Biological Chemistry, the Institute of Life Sciences, the Hebrew University of Jerusalem, Jerusalem, Israel (S.B.A., H.S.)

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ABSTRACT

Stress increases vulnerability and causes relapse to drugs of abuse. The usually rare read-through variant of acetylcholinesterase (AChE-R) is causally involved in stress-related behaviors, and transgenic mice constitutively overexpressing AChE-R (TgR) show behaviors characteristic of chronic stress. We measured anxiety-like behavior on TgR and control mice under normal conditions and under long-term nicotine treatment. In addition, we measured epibatidine binding in the brain and transcription status in the striatum, using microarrays, in wild-type and TgR mice. TgR mice behaved as more anxious than controls, an effect normalized by long-term nicotine intake. In control mice, long-term nicotine augmented epibatidine binding in several areas of the brain, including the hippocampus and striatum. In TgR transgenics, long-term nicotine increased epibatidine binding in some areas but not

in the hippocampus or the striatum. Because the striatum is involved in the mechanisms of drug addiction, we studied how the transgene affected striatal gene expression. Whole-genome DNA microarray showed that 23 transcripts were differentially expressed in TgR mouse striata, including 15 known genes, 7 of which are anxiety-related. Subsequent reverse-transcriptase polymerase chain reaction validated changes in 7 of those 15 genes, confirmed the increase trend in 5 more transcripts, and further revealed changes in 5 genes involved in cholinergic signaling. In summary, we found that nicotine acts as an anxiolytic in TgR mice but not in control mice and that continuously overexpressed AChE-R regulates striatal gene expression, modulating cholinergic signaling and stress-related pathways.

Tobacco smoke is a leading cause of death (Peto et al., 2000). Despite the widely publicized negative effects of smoking, most smokers return to the habit within a year of quitting (Dani and De Biasi, 2001). Nicotine, which acts at nicotinic acetylcholine receptors (nAChRs) in the central and peripheral nervous systems, is the major addictive compo-

nent of tobacco. A complex relationship between stress and nicotine exists (Sinha, 2001). In humans, exposure to long-term stress activates the hypothalamus-pituitary-adrenal (HPA) axis and the autonomic nervous system, produces alterations in anxiety levels, and reduces cognitive and affective abilities (Gold and Chrousos, 2002). Relief from the symptoms produced by stress is a frequently cited reason for smoking, and smoking rates are higher in people suffering from anxiety-related disorders (Morissette et al., 2007). In addition, the desire of smoking is increased by stress, a phenomenon observed not only after traumatic experiences but also after modestly stressful conditions (Todd, 2004). Finally, stressors and the state of stress exacerbate nicotine withdrawal symptoms and increase vulnerability to relapse (Morissette et al., 2007). Based on these observations, a better understanding of the interaction between nicotine and

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¹ Current affiliation: Department of Forensic Sciences, College of Criminal Justice, Sam Houston State University, Huntsville, Texas.

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; TgR, acetylcholinesterase-R overexpressing transgenic mice; AChE, acetylcholinesterase; PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase polymerase chain reaction; AChE-R, read-through variant of acetylcholinesterase; OFA, test of locomotor activity in the open field; EPM, elevated-plus maze; TST, tail suspension test; LDB, light/dark box exploration; GO, Gene-Ontology; BDNF, brain-derived neurotrophic factor; VIP, vasoactive intestinal polypeptide; PTPRS, protein tyrosine phosphatase receptor type S; HPA, hypothalamus-pituitary-adrenal.

stress may provide insight into therapeutic targets for smoking cessation.

Laboratory animals can be used to study the behavioral effects of stress and the interaction between stress and nicotine. Physical or social stressors are usually used to elicit stress-related symptoms in rodents and study the effects of nicotine. For example, short-term foot-shock stress has been shown to reinstate nicotine but not sucrose seeking (Buczek et al., 1999). Long-term stress has also been used to study the effects of nicotine on the HPA axis (Lutty et al., 2006).

Among the cellular responses to stress, there is an increase in expression levels of the R splice variant of the enzyme acetylcholinesterase (AChE) (Kaufer et al., 1998), which hydrolyzes acetylcholine at brain cholinergic synapses. Alternative splicing generates three AChE isoforms called AChE-S, -E, and -R (Meshorer and Soreq, 2006). AChE-S or "synaptic" AChE is the most abundant isoform in the nervous system. During stress, the expression of the -R form is greatly increased. The levels of the AChE-R protein in TgR (a transgenic mouse harboring a constitutively expressed human AChE-R gene) mice are similar to those observed for wild-type FVB/N mice subjected to forced-swim stress (Sternfeld et al., 2000). In addition, the TgR mice display histopathological features characteristic of stress responses (Kaufer et al., 1998).

To determine whether AChE-R expression represents a link between stress, anxiety, and addiction, we tested nicotine's behavioral effects in the TgR mice. Specifically, we performed a battery of anxiety-related behavioral tests after long-term nicotine treatment and determined the levels of nicotine-induced nAChR up-regulation in several areas of the brain. Finally, to find candidate genes for the phenotypes in TgR mice, we performed microarray and RT-PCR experiments on wild-type and control mice.

Materials and Methods

Animals

We analyzed wild-type FVB/N mice and FVB/N transgenic mice expressing the AChE-R transgene under the control of the CMV promoter (Sternfeld et al., 1998, 2000). We used 2- to 6-month-old female and male mice. After weaning at 21 days after birth, same-sex litter mates were housed in cages containing a maximum of five animals. The animals had ad libitum access to water and food pellets (Labdiet 5001; PMI, Brentwood, MO) and were maintained on a 12-h light/dark cycle. Mice were tested between 9 AM and 4 PM. Behavioral experiments were conducted on two or three different batches of approximately 10 mice per genotype. All of the procedures complied with the directives of Baylor's Institutional Animal Care and Use Committee and Center for Comparative Medicine.

Long-Term Nicotine Treatment

Control mice had unlimited access to a solution of tap water containing 2% saccharine, whereas nicotine-treated mice had 2% saccharine and 200 μ g/ml nicotine in the water (Sparks and Pauly, 1999; Brunzell et al., 2003). According to Brunzell and colleagues (2003), this treatment yields plasma cotinine levels of 215.8 ± 12.4 ng/ml. Mice were treated for 5 weeks before and during behavioral testing.

Behavioral Tests

Mice were used in all four behavioral tests, with at least 1 day between tests.

Locomotor Activity in the Open Field. Mice were placed in a clear Plexiglas arena ($40 \times 40 \times 40$ cm), and locomotor activity was measured for 30 min using a computer-operated tracking system (Any-maze; Stoelting, Woods Dale, IL; used in all behavioral tests). The total distance moved and the distance moved in a center square (20×20 cm) were recorded. The ratio of the distance moved in the center to the total distance moved was used as a measure of anxiety-related behavior.

Elevated-Plus Maze. The maze consisted of four arms (25×7 cm), two with 15-cm high black walls and two without walls, elevated 50 cm from the floor. Mice were placed in the intersection between the arms, and the percentage of open to total arm entries and the time spent into the open arms were recorded for 5 min. These two parameters were taken as measures of anxiety-related behavior.

Light/Dark Box Exploration. The light/dark box exploration (LDB) test for anxiety-related behavior was performed by placing the mouse in a maze ($44 \times 21 \times 21$ cm) that has two chambers, one bigger (28 cm long) and bright, and the other smaller (16 cm long) and dark. Transitions between sides and the time spent in each division were recorded for 10 min.

Tail Suspension Test. The tail suspension test (TST) is a model of depression-related behavior (Trullas et al., 1989). Animals were suspended by the tail for 6 min, and immobility behavior was analyzed using the Any-maze system with a setting of 80 in the relative mobility scale provided in the program. This procedure was validated by manually scoring some mice and comparing the results obtained by both methods.

Toxin Binding

125 I-Epipatidine binding was performed as published previously (Salas et al., 2003a). In brief, slide-mounted brain sections were incubated at 22°C for 1 h in buffer (50 mM Tris-base, pH 7.4, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , and 1 mM MgCl_2) containing 500 pM 125 I-epibatidine (specific activity, 2200 Ci/mmol; PerkinElmer Life and Analytical Sciences, Waltham, MA). Brain sections were washed twice for 3 min each in buffer, dipped in water, air-dried, and apposed to BioMax film (Carestream Health, Rochester, NY) for 3 to 12 h.

DNA Microarrays

Total RNA was extracted from the striatum of adult (2–3 months old) male mice ($n = 12$) using the Rneasy Mini Kit for lipid tissues (Qiagen, Hilden, Germany) and four different pools (two pools for each genotype, three mice in each pool) were prepared. Pooled RNA was used to prepare 5'-biotinylated target RNA for the microarray tests. In brief, approximately 5 μ g of total RNA was used to generate first-strand cDNA by using a T7-linked oligo(dT) primer. After second-strand synthesis, in vitro transcription was performed with biotinylated nucleotide triphosphates, resulting in approximately 100-fold amplification of RNA, which was then processed according to the manufacturer's recommendation using an Affymetrix GeneChip Instrument System (Affymetrix, Santa Clara, CA). For hybridization, spike controls were added to 15 μ g of fragmented cRNA for overnight incubation. Arrays were then washed and stained with streptavidin-phycoerythrin before being scanned on an Affymetrix GeneChip scanner.

Quality and amount of starting RNA were confirmed by agarose gel electrophoresis. After scanning, array images were assessed by eye to confirm scanner alignment and the absence of significant bubbles or scratches. 3'/5' ratios for glyceraldehyde-3'-phosphate dehydrogenase and β -actin were confirmed to be within acceptable limits range from Affymetrix quality control parameters. When scaled to a target intensity of 150 (using Affymetrix GCOS array analysis software), scaling factors for all arrays were within acceptable limits, as were background, Q values, and mean intensities. All experiments were performed using Affymetrix Mouse 430A 2.0 oli-

gonucleotide arrays (as described at <http://www.affymetrix.com/products/arrays/specific/mgu74.affx>).

Microarray Analysis

Because probes with low signal intensity usually present higher levels of variance and rates of false-positive detection, we decided to relate only 60% of the probe set, which shows the highest signals levels (in general, this distinction between the “low signal” and the “high signal” probes is compatible with the “absent” and “present” calls determined by the Affymetrix software). Probes with an inter-group variance/average ratio greater than 10 were considered highly variable and were further excluded to reduce false results. Choosing this specific criterion for variability was based on our former experience with similar data (G. Zimmerman, S. Ben-Ari, H. Soreq, unpublished results). Examining the remaining probe set, probes with a \log_2 fold-change above or below ± 1 were considered “changed.” To characterize sets of functionally related genes we used Onto-Express (Khatri et al., 2002) and classified changed genes according to Gene-Ontology (GO) categories. To reduce false-negative results, we included only categories in which the expression of at least three genes was altered. For further explanations on the method, refer to Khatri et al. (2002). To estimate the significance of our results, we reshuffled the subject labels and performed all of the analyses on these permuted groups.

RT-PCR

Total RNA was extracted from five TgR and five strain- and sex-matched controls using the RNeasy Lipid Tissue Mini Kit and then reverse-transcribed (200 ng) using random primers and ImProm-II reverse transcriptase (Promega, Madison, WI) according to manufacturer's indications. Real-time PCR was performed using ABsolute QPCR SYBR Green ROX (ABgene, Epsom, Surrey, UK) in a 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). Quantification was assessed at the logarithmic phase of the PCR reaction. The PCR annealing temperature was 60°C for all primer pairs (Sigma, Rehovot, Israel) presented in Supplementary Data Table 1.

Data Analysis and Statistics

Behavioral and binding data were examined by analysis of variance followed by Duncan's post hoc comparisons or by Student's *t* test when appropriate. Behavioral data were first analyzed using gender as a variable, and because no differences were observed, data were collapsed and analyzed without regard to gender. X-ray film analysis and quantification were performed using computer-assisted densitometry (NIH Image: <http://rsbweb.nih.gov/nih-image/>). Relative optical densities from discrete brain regions were measured and presented as a percentage of control sections in the same film.

Results

Anxiety-Like Behavior in TgR Transgenic Mice. Control mice and transgenic mice carrying the human AChE-R isoform were subjected to the elevated-plus maze (EPM), locomotor activity in the open field (OFA), LDB, and TST behavioral tests, in that order. In the EPM, TgR mice showed an anxiogenic-like phenotype, as evidenced by less time spent in the open arms (Fig. 1A) and a smaller percentage of open entries (Fig. 1B). In the OFA, no significant difference was found on total distance traveled (Fig. 1C), but TgR mice showed significantly less center exploration (Fig. 1D). Therefore, TgR transgenic mice behave as more anxious than controls in two of the experiments we performed. In the light/dark box, no statistically significant difference was observed (Fig. 1E). In addition, TgR transgenic and control mice had

comparable behavior in the TST, a rodent model of depression-like behavior (Trullas et al., 1989) (Fig. 1F).

Long-Term Nicotine Relieves the Anxiogenic-Like Phenotype in TgR Transgenic Mice. We performed three anxiety-like behavior-related experiments in control and TgR transgenic mice continuously exposed to nicotine in the drinking water. In the EPM, nicotine treatment produced a significant anxiolytic-like effect in TgR transgenic [$F(3,109) = 7.55$, $P < 0.05$; Fig. 2, A and B] but not in control mice. The effect of nicotine was test-specific, and as in the OFA, no statistically significant effect of chronic nicotine was found: TgR transgenic mice behaved as more anxious than controls when treated with nicotine or control solution [$F(3,124) = 1.51$; Fig. 2, C and D]. In the LDB test, no statistically significant differences were found [$F(3,81) = 0.64$; Fig. 2E]. Likewise, no effect of genotype or nicotine treatment was found on the TST [$F(2,113) = 1.9$; Fig. 2F]. It is noteworthy that these data also replicate the effects seen in Fig. 1 in a different cohort of mice.

Epibatidine Binding in the Brains of TgR Mice after Long-Term Nicotine Treatment. Long-term exposure to nicotine results in the up-regulation of $\beta 2$ -containing nAChRs (Flores et al., 1992; Sparks and Pauly, 1999). To confirm that our nicotine treatment was effective and to study the possibility of an interaction between nicotine and stress at the level of $\beta 2$ -containing nAChR expression levels, we performed binding studies with 125 I-epibatidine, a ligand that binds to different heteromeric nAChR sub-

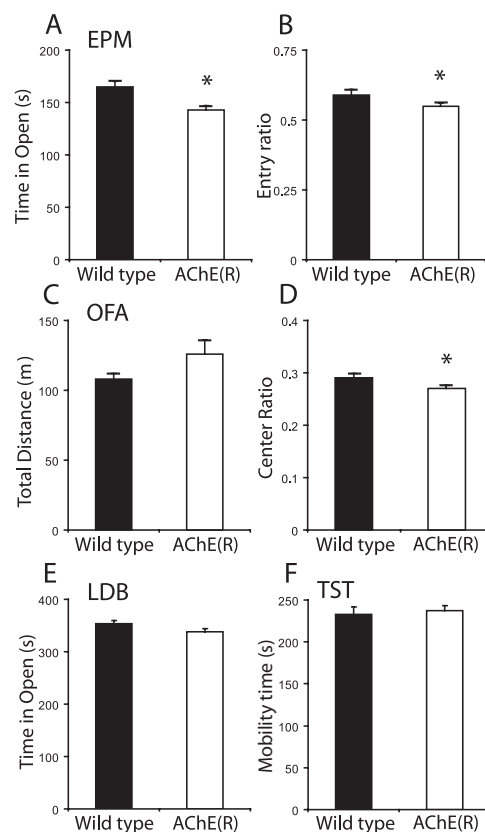


Fig. 1. Anxiety-like behavior in TgR mice. A, elevated-plus maze, time in open arms; B, elevated-plus maze, open/total entries ratio; C, open-field activity test, total distance moved; D, open-field activity test, distance traveled in center/total distance ratio; E, light/dark box test, time in open area; F, tail suspension test, mobility time. Averages \pm S.E.M. are shown. $n_{\text{wild-type}} = 35$, $n_{\text{TgR}} = 57$ except for TST, $n = 37$ and 65 , respectively; *, $P < 0.05$.

types, including those containing $\beta 2$. As shown in Fig. 3, we found the expected increase in ^{125}I -epibatidine binding in several areas of the brain, including striatum, cingulate cortex, somatosensory cortex, and hippocampus. It is interesting that TgR animals treated with nicotine showed decreased nicotine-induced up-regulation of epibatidine binding in the striatum and the hippocampus but normal up-regulation of binding in the cingulate and somatosensory cortices (Fig. 3E).

Expression Changes in the Striatum of TgR Mice.

From the 22,693 probes analyzed in the microarray, 16,489 (~72%) were considered present according to the intensity of their signals, compatible with the known complexity of brain-expressed genes (Lein et al., 2007). To eliminate probes with high variance between replicates, we filtered out probes with

an intergroup variance/average ratio greater than 10, which further reduced the number of subsequently analyzed probes to 14,199. The \log_2 fold-change of the remaining transcripts presented a normal distribution ($P \leq 0.0001$, Kolmogorov-Smirnov test) with a median value of 0.009, showing that the transgene did not globally alter the striatal pattern of transcript expression (Fig. 4A).

From the 14,199 filtered probes, 23 showed a \log_2 fold-change above/below ± 1 (Table 1). This number highly exceeds the expected number of 3.5 changed genes, as calculated by permuting replicates from the different groups (Fig. 4B). The 23 changed probes included 15 well characterized mouse genes. The remaining eight transcripts belong to yet-unidentified genes. Further RT-PCR validated the changes observed in seven of these genes (Fig. 4C and Table 1).

It is noteworthy that most of the differentially expressed genes were up-regulated and included several enzymes (sulphatase, peptidase, phosphatases, and a kinase) in addition to two receptor subunit genes (muscarinic acetylcholine receptor 4, and γ amino butyric acid receptor α -2). Four additional transcripts presented a similar trend for up-regulation but with nonsignificant change; only one of these encodes an

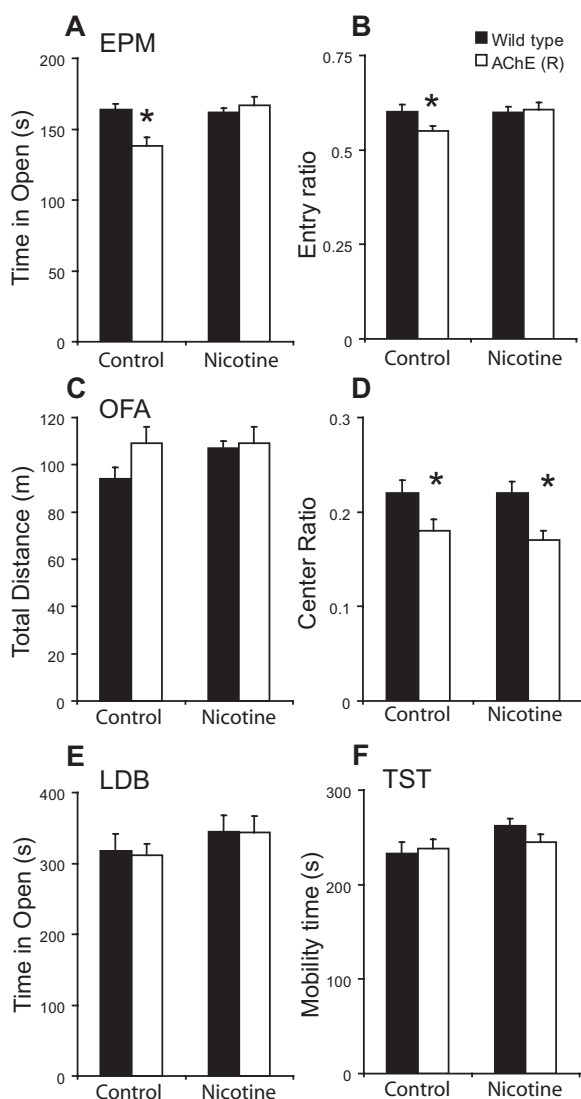


Fig. 2. Anxiety-like behavior in transgenic TgR mice after long-term nicotine treatment. A and B, elevated-plus maze ($n_{\text{control/PBS}} = 33$, $n_{\text{AChE-R/PBS}} = 27$, $n_{\text{control/nicotine}} = 25$, $n_{\text{AChE-R/nicotine}} = 28$). A, time in open arms; B, open/total entries ratio; C and D, open-field activity test ($n_{\text{control/PBS}} = 37$, $n_{\text{TgR/PBS}} = 32$, $n_{\text{control/nicotine}} = 29$, $n_{\text{TgR/nicotine}} = 30$); C, total distance moved; D, distance traveled in center/total distance ratio; E, light/dark box test ($n_{\text{control/PBS}} = 25$, $n_{\text{TgR/PBS}} = 20$, $n_{\text{control/nicotine}} = 28$, $n_{\text{TgR/nicotine}} = 22$), time in open area; F, tail suspension test ($n_{\text{control/PBS}} = 32$, $n_{\text{TgR/PBS}} = 31$, $n_{\text{control/nicotine}} = 27$, $n_{\text{TgR/nicotine}} = 27$), mobility time. Averages \pm S.E.M. are shown. *, $P < 0.05$.

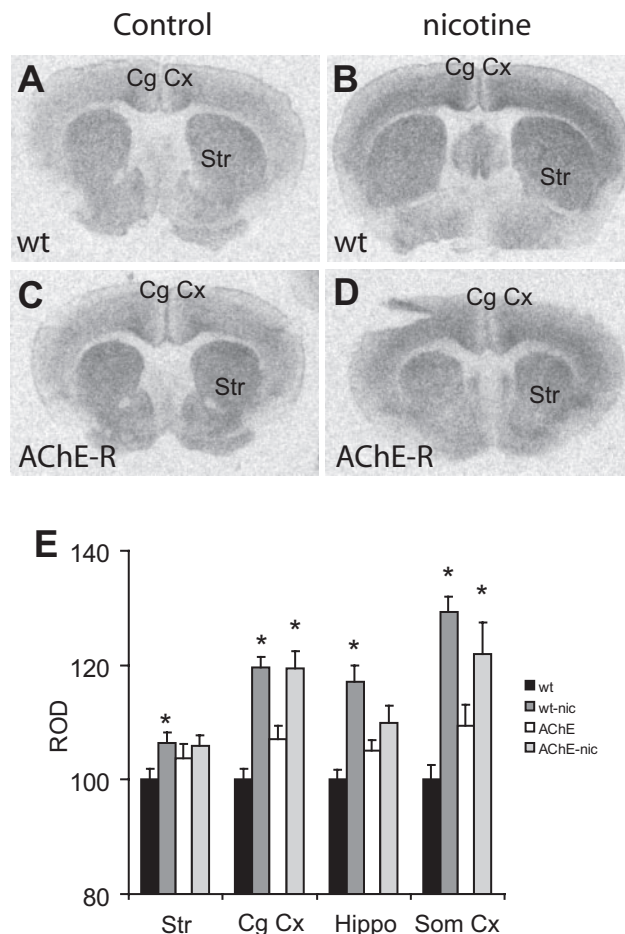


Fig. 3. Epibatidine binding in wild-type and transgenic TgR mouse brains. ^{125}I -epibatidine binding at the level of the striatum in control mice treated with saline (A), control mice treated with nicotine (B); TgR mice treated with saline (C), TgR mice treated with nicotine (D), and quantitation of epibatidine binding in the striatum (Str), cingulate cortex (Cg Cx), hippocampus (Hippo), and somatosensory cortex (Som Cx) (E). Averages \pm S.E.M. are shown; $n = 8, 8, 8$, and 10 , respectively. ROD, relative optical density. *, $P < 0.05$.

enzyme subunit, ATPase (*ATP6V0A1*). Two other transcripts presented an increase change by RT-PCR but decreased in the microarray. Finally, vasoactive intestinal polypeptide (VIP) and protein tyrosine phosphatase receptor type S (*PT-PRS*) transcripts were down-regulated in both microarrays and RT-PCR tests.

To further characterize the sets of functionally related genes, we searched among the changed genes in the TgR striatum for corresponding GO categories. We discarded categories that contained fewer than three changed genes. A *P* value cutoff of 0.01 yielded 11 changed categories (Table 2), with the expected number of changed categories when permuting replicates from different groups being only three (Fig. 4B). It is interesting that all changed categories found with permuted replicates belonged to the “cellular component” ontology, suggesting that it might be more error-prone.

To determine whether AChE-R overexpression affects additional genes involved in cholinergic signaling, we further assayed by RT-PCR the expression levels of 7 key cholinergic genes in striata of TgR mice. Five of those genes exhibited significant changes (up to 326% greater

than controls) relative to strain-matched control animals (Table 3). These included nicotinic and muscarinic receptor subunits and the vesicular acetylcholine transferase transporter, probably reflecting global change in cholinergic signaling.

Discussion

Stress has been shown to induce anxiogenic-like behavior in mice. For example, predator odor (Belzung et al., 2001) and unpredictable mild stress (Ducottet and Belzung, 2005), were shown to decrease exploration in the EPM. The “readthrough” AChE-R transcript is not abundant in the adult brain, but exposure to stressors shifts the expression of splice variants and increases the levels of AChE-R in various brain areas, including the striatum (Pick et al., 2004; Perrier et al., 2005). We showed that continuous overexpression of AChE-R in mice leads to an anxiogenic-like phenotype, much like long-term stress does. This provides a molecular link between long-term stress (ongoing elevated AChE-R expression) and anxiogenic-like behavior.

We also report that long-term exposure to nicotine mitigates the anxiety-like behaviors observed in TgR mice but has no effect on control mice. Stress may be the reason why certain people begin to smoke or increase their tobacco consumption (Boos and Croft, 2004), and smokers often report an anxiolytic effect of short-term nicotine exposure. It has been suggested that the “anxiolytic” effect of nicotine mainly reflects the relief of the withdrawal symptoms that arise between cigarettes (Parrott, 2003). These observations imply that nicotine might be anxiolytic only during stress.

In TgR transgenic mice, nicotine relieved the anxiogenic-like phenotype in the elevated-plus maze but not in the center exploration in the open field. It has been reported that these two experimental paradigms measure different dimensions of anxiety-like behaviors (Belzung and Le Pape, 1994; Ramos et al., 1997), and our group showed that mutant mice lacking the $\beta 4$ subunit of nAChRs display an anxiolytic-like genotype in the EPM but not in the OFA or the LDB (Salas et al., 2003b).

One of the hallmarks of long-term nicotine treatment is the increase in epibatidine binding due to a greater number of plasma membrane $\beta 2$ subunit-containing nAChRs (Flores et al., 1992), an effect hypothesized to be important for the development of nicotine addiction and withdrawal (Dani and Heinemann, 1996). Although we have shown previously that nicotine withdrawal symptoms in mice are mediated by the $\beta 4$ and not the $\beta 2$ subunit (Salas et al., 2004), the up-regulation of nAChRs after long-term nicotine exposure is probably involved in the development of nicotine dependence (Dani and Heinemann, 1996). As expected, we found significant nicotine-induced up-regulation of epibatidine binding in several areas of control brains (Flores et al., 1992). Brains from TgR mice had similar nicotine-dependent increases in epibatidine binding, except for the hippocampal and striatal areas. Given the importance of the striatum and hippocampus in the mechanisms of anxiety, stress responses, and drug addiction (Carlezon et al., 2005), the anxiolytic-like effect of nicotine we observed on TgR mice might be related to the lack of $\beta 2$ -containing nAChR up-regulation in those areas.

Next, we explored the genomic pathways by which AChE-R overexpression may exert its stress-related effects using

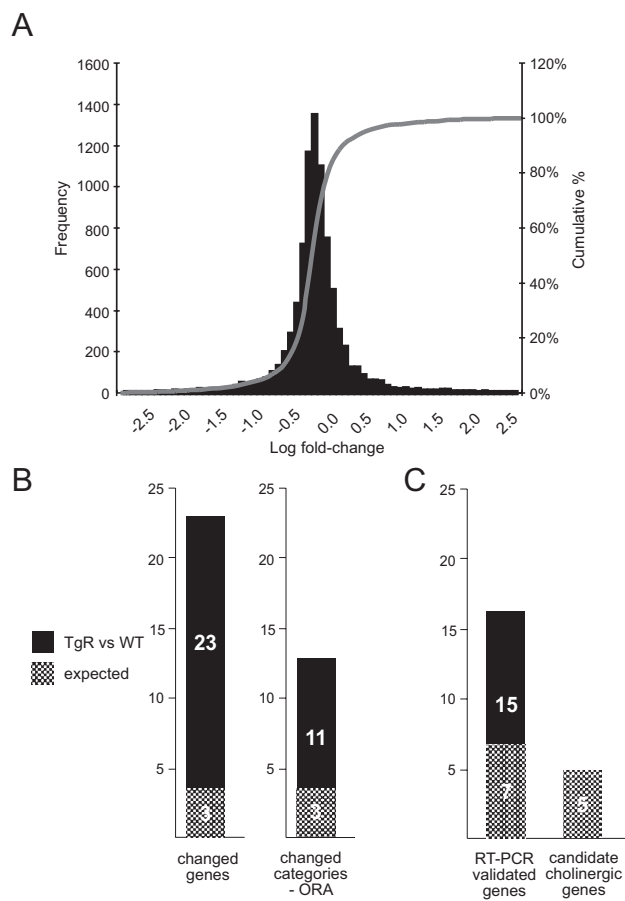


Fig. 4. Overview of changed genes and over-represented GO categories in the TgR striatum. A, the ratio TgR/wild type of all probes (\log_2) distributes normally with a median value of 0.009; B, 23 transcripts were changed by more than 1-fold in the TgR striatum compared with only 3 genes that would be expected to display such change by chance. Eleven GO categories were found to be over-represented among the changed genes, whereas only three were expected to be found by chance. C, DNA microarray analysis revealed 15 known genes to be differentially expressed in TgR mice striatum, 7 of those were positively validated by RT-PCR. Five genes associated with cholinergic signaling were found to be differentially expressed by RT-PCR.

TABLE 1

Differentially expressed genes in the TgR striatum

Genes differentially regulated by more than 1-fold according to DNA microarray analysis. Note the high proportion of changed genes related to anxiety behavior. RT-PCR-validated changes ($P < 0.05$) are indicated in column 3. Characterized genes are in boldface type.

Rank	\log_2 TgR/WT (DNA microarray)	\log_2 Change over Control (RT-PCR)	Symbol	Gene Title	Description	Reference
1	-1.00	0.27 ($P < 0.45$)	CSPG2	Chondroitin sulfate proteoglycan 2	Connects cells with the extracellular matrix, regulating cell motility, growth and differentiation. It is increased after CNS injury.	Wight 2002; Morgenstern et al., 2002
2	1.01		<i>FRGQ</i>	Frequenin homolog		
3	1.02		<i>SIN3b</i>	Transcriptional regulator, SIN3B (yeast)		
4	1.02	0.77 ($P < 0.17$)	ATP6V0A1	ATPase, H+ transporting, lysosomal V0 subunit A1	Involved in synaptic vesicle exocytosis.	Hiesinger et al., 2005
5	-1.03	-0.10 ($P < 0.27$)	SLC34A2	Solute carrier family 34 (sodium phosphate), member 2	Involved in phosphate transport into cells.	Feild et al. 1999
6	1.06		<i>NR2C2</i>	Nuclear receptor subfamily 2, group C, member 2		
7	1.07		<i>TRIM44</i>	Tripartite motif-containing 44		
8	1.07	1.00 ($P < 0.01$)	IDS	Iduronate 2-sulfatase	Required for the lysosomal degradation of heparan sulfate and dermatan sulfate. Catalyzes proteolytic activation of BDNF, which is in turn related to anxiety.	Wilson et al., 1990
9	1.09	1.50 ($P < 0.01$)	MBTPS1	Membrane-bound transcription factor peptidase, site 1		Seidah et al., 1999; Govindarajan et al., 2006
10	1.10	-0.42 ($P < 0.34$)	NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	BDNF receptor. BDNF mutations are related to anxiety. BDNF transgenic mice show increased anxiety-like behavior.	Chen et al., 2006; Govindarajan et al., 2006
11	1.11		<i>CTDSPL2</i>	CTD small phosphatase-like 2		
12	-1.12	-0.46 ($P < 0.07$)	VIP	Vasoactive intestinal polypeptide	Different stress stimuli differentially increase VIP. Intravenous VIP infusion increases (10-fold) plasma adrenocorticotropin levels.	Nakamura et al., 1994; Ceccatelli et al., 1991
13	-1.14		<i>CALM4</i>	Calmodulin-like 4		
14	1.15	1.31 ($P < 0.01$)	CHRM4	Cholinergic receptor, muscarinic 4	CHRM4 knockout mice exhibit anxiolytic response in shock-probe burying model.	Degroot et al., 2006
15	1.16		<i>TOM1l2</i>	Target of myb1-like 2 (chicken)		
16	1.17	0.87 ($P < 0.05$)	GABRA2	GABA_A receptor, subunit $\alpha 2$	Benzodiazepine target. Inhibits HPA axis and is down-regulated in anxiety patients.	Tiihonen et al., 1997, and others
17	1.19	0.75 ($P < 0.05$)	PTPN11	Protein-tyrosine phosphatase σ	Controls energy balance and metabolism. It is required for complete MAPK activation by BDNF	Zhang et al., 2004; Easton et al., 2006
18	1.21	-0.76 ($P < 0.05$)	PTPRS	Protein tyrosine phosphatase, receptor type, S	PTPRS(-/-) mice exhibit more than 50% less cholinergic neurons. PTPRS(-/-) mice show aberrant pituitary gland morphology, with an increase of corticotrope cells.	Wallace et al. 1999; Elchebly et al., 1999
19	-1.23		<i>HKDC1</i>	Hexokinase domain containing 1		
20	1.23		<i>KLF9</i>	Kruppel-like factor 9		
21	1.33	0.38 ($P < 0.20$)	PEDF	Serine (or cysteine) peptidase inhibitor, clade F, member 1	Neurotrophic and neuroprotective protein	Tombran-Tink et al., 2003
22	1.68	0.18 ($P < 0.27$)	PAIP1	Polyadenylate binding protein-interacting protein 1	Coactivator in the regulation of translation initiation of poly(A)-containing mRNAs.	Craig et al., 1998
23	2.27	0.85 ($P < 0.01$)	PIP5K1C	Phosphatidylinositol-4-phosphate 5-kinase, type 1 γ	Plays a role in focal adhesion dynamics and clathrin-mediated endocytosis	De Pereda et al., 2005

DNA microarrays and RT-PCR in striata of TgR and strain-matched control mice. We chose the striatum because it is one of the two areas in which AChE-R overexpression blunted the molecular response to nicotine. In addition, the striatum is a common area for the effects of drugs of abuse. DNA microarrays identified 15 well characterized genes differentially expressed in TgR mice, and further RT-PCR analysis validated 7 of those genes (Table 1). In addition, RT-PCR analysis revealed five differentially expressed genes directly related to acetylcholine metabolism (Table 3). We hypothesize that the marked up-regulation of vesicular acetylcholine transferase, *CHRM1*, *CHRM4* (muscarinic receptors), and *CHRNA7* (nicotinic receptor $\alpha 7$) serves to counteract the inhibitory effect on cholinergic signaling, which is predictably produced by AChE-R overexpression. The *CHRNA4* (nAChR $\alpha 4$ subunit), however, was down-regulated in TgR mice. This down-regulation of the $\alpha 4$ subunit in the striatum by the presence of the transgene might explain the lack of nicotine-induced up-regulation of nAChRs. Upon nicotine treatment, $\beta 2$ -containing receptors are up-regulated in the plasma membrane. New receptors are probably assembled mainly from subunits that were already expressed and localized to the *trans*-Golgi network (Keller et al., 2001). A decrease in $\alpha 4$ mRNA might result in a decrease in Golgi $\alpha 4$ subunits, which might impair the ability of a cell to up-regulate receptors upon nicotine treatment.

Beyond cholinergic genes, a notable part of the changed genes in TgR mice is related to anxiety-like behavior. For example, the expression of three genes related to brain-derived neurotrophic factor (BDNF) metabolism was up-regulated: BDNF's proteolytic activator *MBTP1*; BDNF's receptor *NTRK2* (with a similar trend, although nonsignificant in RT-PCR); and BDNF's coactivator of mitogen-activated protein kinase; *PTPN11*. Up-regulation of these three proteins intensifies BDNF signaling, which in turn has been causally linked to anxiety-like behaviors (Chen et al., 2006). Several

interactions between BDNF and addiction, including nicotine addiction, have been reported before. For example, proteomic analysis of two-dimensional electrophoresis in rat striatum revealed that BDNF is one of the nicotine-addiction associated proteins, because long-term treatment with nicotine tends to decrease BDNF levels in rats (Yeom et al., 2005). In the CA1 area of the hippocampus, stress has been shown to decrease BDNF levels, and that effect is normalized by long-term nicotine injections (Aleisa et al., 2006). Finally, a genetic interaction between *NTRK2* and the nicotinic receptor subunit $\alpha 4$ has been reported in a population of human smokers (Lou et al., 2007). Other modified genes related to anxiety are the protein kinase A activator VIP, which is coregulated with cholinergic proteins (Gozes et al., 1997) and was reported to increase after stressful stimuli (Nakamura et al., 1994); the GABA_A receptor, broadly implicated in the pathophysiology of anxiety (Lydiard, 2003); and the PTPRS, which has been linked to pituitary gland development (Elchebly et al., 1999).

It is noteworthy that none of the ACh-related candidate transcripts that were identified as changed in the RT-PCR tests showed up as changed in the microarray analysis. This outlines the need for caution when using microarray tests as a single approach. Taken together, our findings present evidence that constitutive overexpression of AChE-R induces changes in diverse stress-related gene pathways that may explain its association with enhanced anxiety-like behaviors.

In conclusion, we show that in a mouse overexpressing AChE-R, but not in control mice, long-term nicotine exposure acts as an anxiolytic. Toxin-binding data indicate the striatum as a potential target for the interaction between AChE-R and nicotine. Microarray and RT-PCR data reinforced the view that AChE-R overexpression affects the levels of several anxiety-related and cholinergic genes. Overall, our data contribute to the understanding of the relationship between tobacco addiction and stress and might help explain why

TABLE 2
GO categories over-represented among changed genes in TgR mice striatum

Category	GO ID	Function Name	Changed Genes	Total Genes in Category	P Value
MF	GO:0004721	Phosphoprotein phosphatase activity	3	99	0.00
MF	GO:0004872	Receptor activity	5	1032	0.00
MF	GO:0016787	Hydrolase activity	5	988	0.00
MF	GO:0016301	Kinase activity	3	610	0.01
MF	GO:0016740	Transferase activity	3	1028	0.05
MF	GO:0005515	Protein binding	3	2094	0.34
BP	GO:0006811	Ion transport	3	347	0.00
BP	GO:0006810	Transport	3	1130	0.08
CC	GO:0016021	Integral to membrane	8	2678	0.01
CC	GO:0005615	Extracellular space	5	1773	0.03
CC	GO:0016020	Membrane	7	2848	0.03

MF, molecular function; BP, biological process; CC, cellular component.

TABLE 3
Key cholinergic genes differentially expressed in the striatum of TgR mice

Shown genes were differentially regulated in the striatum of TgR mice as revealed by RT-PCR. Significantly changed genes ($P < 0.05$) are in boldface type.

Symbol	Gene Title	log ₂ Change over Control (RT-PCR)	P Value (U Test)
<i>VACHT</i>	Vesicular acetylcholine transferase	1.18	0.01
<i>CHRM1</i>	Muscarinic acetylcholine receptor 1	1.71	0.01
<i>CHRM2</i>	Muscarinic acetylcholine receptor 2	-0.16	0.149
<i>CHRM4</i>	Muscarinic acetylcholine receptor 4	1.31	0.01
<i>CHRNA7</i>	Nicotinic acetylcholine receptor $\alpha 7$	0.69	0.05
<i>CHRNA4</i>	Nicotinic acetylcholine receptor $\alpha 4$	-0.98	0.03
<i>CHRNA2</i>	Nicotinic acetylcholine receptor $\beta 2$	0.64	0.17

stress is one of the major contributors to nicotine addiction and relapse.

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Address correspondence to: Dr. Mariella De Biasi, Department of Neuroscience, Baylor College of Medicine, Houston, TX 77030. E-mail: debiasi@bcm.tmc.edu