

Adaptive acetylcholinesterase splicing patterns attenuate 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinsonism in mice

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Abstract

Balanced dopaminergic cholinergic interactions are crucial for proper basal ganglia function. This is dramatically demonstrated by the worsening of Parkinson's disease symptoms following acetylcholinesterase (AChE) inhibition. Typically, in the brain, the synapse-anchored synaptic AChE (AChE-S) variant is prevalent whereas the soluble readthrough AChE (AChE-R) variant is induced in response to cholinesterase inhibition or stress. Because of the known functional differences between these variants and the fact that AChE-R expression is triggered by various stimuli that themselves are often associated with Parkinson's disease risk, we hypothesized that the splice shift to AChE-R plays a functional role in Parkinsonian progression. After establishing that Paraoxon-induced AChE inhibition indeed aggravates experimental Parkinsonism triggered by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice, we tested the roles of individual AChE variants by exposing transgenic mice overexpressing either the AChE-S or AChE-R variant to MPTP. Differential reductions of tyrosine hydroxylase levels in the striatum and substantia nigra indicated that transgenic AChE-R expression confers resistance as compared with the parent FVB/N strain. In contrast, AChE-S overexpression accelerated the MPTP-induced damage. Survival, behavioral measures and plasma corticosterone levels were also compatible with the extent of the dopaminergic damage. Our findings highlight the functional differences between individual AChE variants and indicate that a naturally occurring stress or AChE inhibitor-induced splicing shift can act to minimize dopaminergic cholinergic imbalances. We propose that inherited or acquired alternative splicing deficits could accelerate Parkinsonism and that, correspondingly, adaptive alternative splicing events may attenuate disease progression.

Introduction

Balanced interactions between the dopaminergic and cholinergic systems are crucial for proper basal ganglia function (Graybiel, 1995; Calabresi *et al.*, 2000; Zhou *et al.*, 2003; Morris *et al.*, 2004). In the context of Parkinson's disease (PD), it has long been known that, whereas muscarinic antagonists alleviate PD symptoms, acetylcholinesterase (AChE) inhibition leads to cholinergic hyperexcitation and exacerbates the symptoms (Duvoisin, 1967). Consistently, epidemiological studies reveal a correlation between rural living associated with chronic exposure to AChE inhibitors and PD (Paolini *et al.*, 2004). Other factors leading to cholinergic hyperexcitation, including psychological stress (Kaufer *et al.*, 1998) and head trauma (Shohami *et al.*, 2000), have also been implicated as PD risk factors (Veldman *et al.*, 1998; Smith *et al.*, 2002). Common to these factors is not only their established or hypothesized involvement in PD progression but also their effect on AChE expression (Kaufer *et al.*, 1998; Meshorer *et al.*, 2002). Specifically, alternative splicing modulations triggered by such challenges modify the relative brain abundance of AChE variants (Darreh-Shori *et al.*, 2004; Nijholt *et al.*, 2004). Normally,

synaptically anchored synaptic AChE (AChE-S) is predominant whereas the soluble readthrough AChE (AChE-R) is scarce. However, following the insults described above, the abundance of AChE-R rises, effectively increasing the ratio between the AChE-R and AChE-S variants (Soreq & Seidman, 2001).

The involvement of AChE in basal ganglia function as well as the correspondence between the PD-promoting and AChE-modulating effects of the abovementioned factors suggest that the splice shift to AChE-R may play a functional role in PD progression. The increase in AChE-R, presumably playing a protective role in diminishing the consequences of increased cholinergic levels, may as an undesired side-effect subsequently contribute to the demise of dopaminergic neurons. Alternatively, the splice shift to AChE-R could play a protective role. Further support for a potential role of AChE splice shifts in PD emerges from recent studies showing that mutations in the regulatory region of AChE that lead to impaired inhibitor-induced up-regulation of AChE-R (Shapira *et al.*, 2000) act synergistically with debilitating mutations in the adjacent PON1 gene to increase PD risk (Benmoyal-Segal *et al.*, 2005). One of the key functions of the PON1 product, Paraoxonase, is degradation of cholinesterase inhibitors (Kardos & Sultatos, 2000). Consequently, acting in concert, these mutations effectively impair restoration of proper cholinergic levels and hence contribute to impairment of the dopaminergic–cholinergic balance.

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To test the role of individual AChE splice variants in PD, we employed the mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model (Przedborski & Vila, 2003; Langston, 1996). First, we established that, as expected from the human studies (Duvoisin, 1967), AChE inhibition indeed exacerbated MPTP-induced dopaminergic intoxication in mice. Second, we tested the MPTP-induced phenotypes of constitutive overexpression of each of the AChE splice variants using transgenic mice. We found not only that AChE-R overexpression was protective but also that AChE-S overexpression was detrimental, highlighting the importance of the splice shift to AChE-R.

Materials and methods

Animals

Naive FVB/N, TgS (carrying a transgene for the human AChE-S variant) and TgR (carrying a transgene for the human AChE-R) adult male mice were used for all behavioral, histochemical, and biochemical analyses. Mice were kept on a 12-h dark/12-h light diurnal schedule. In one experiment (results shown in Figs 1–3 and 5), saline, MPTP, Paraoxon and MPTP + Paraoxon were injected, respectively, into groups of five, six, six and six FVB/N mice (total 23), groups of six, six, six and six TgR mice (total 24) and groups of six, nine, seven and eight TgS mice (total 30). Thus, 77 mice were included in this experiment. In another experiment (Fig. 6A), for each of the three strains we injected three mice with saline and three mice with MPTP, for a total of 18 mice. All experiments were conducted in accordance with the Animal Care and Use Committee of the Hebrew University (approval no. MD-80.03-3).

Injections

The MPTP-HCl (Sigma, Rehovot, Israel) was dissolved in saline and injected in four doses of 20 mg/kg at 2-h intervals for a cumulative dose of 80 mg/kg. Mice used for plasma corticosterone measurements received 60 mg/kg (4×15 mg/kg). The Paraoxon-ethyl (Sigma) cumulative dose was 1 mg/kg administered in two injections of

0.5 mg/kg at a 4-h interval. For combined MPTP + Paraoxon injections, the first and second Paraoxon injections coincided with the first and third MPTP injections. Striatal and midbrain tyrosine hydroxylase (TH) levels were analysed in mice killed 3 or 4 days following injection. These relatively short time periods were selected because they are sufficiently short to minimize death of the sensitive TgS mouse strain but are long enough so that measures of TH loss reflect cell destruction (Jackson-Lewis *et al.*, 1995).

Tissue collection

Following Isoflurane (Rhodia, Bristol, UK) anesthesia, mice were decapitated and blood was collected into EDTA-lined tubes (K2E tubes; BD, Franklin Lakes, NJ, USA) and centrifuged at 420 *g* for 20 min at 4 °C. The serum was collected and stored at –20 °C until assay.

Brain sections

For histology, hemispheres were dissected on ice and immediately transferred to 4% phosphate-buffered saline-paraformaldehyde (pH 7.4) and maintained there for at least 72 h prior to sectioning 10- μ m paraffin-embedded coronal slices.

Cholinesterase activity

Striatal tissue was dissected on ice and immediately transferred to –80 °C. Striata were homogenized in solution D (Tris Triton NaCl EGTA, pH 7.4), kept on ice for 1 h, centrifuged at 20 800 *g* for 15 min at 4 °C and the supernatant was collected. Cholinesterase activity was measured as rates of acetylthiocholine hydrolysis (1 mM; Sigma) according to the method of Ellman *et al.* (1961). The absorbance at 405 nm was measured with a SpectraFluor-Plus instrument (Tecan, Zurich, Switzerland). Following 20 min of incubation with 5×10^{-5} M tetraisopropyl pyrophosphoramidate (Sigma), a specific butyrylcholinesterase inhibitor, kinetic measurements of hydrolysis rates were quantified as nmol substrate hydrolysed/min/ μ l

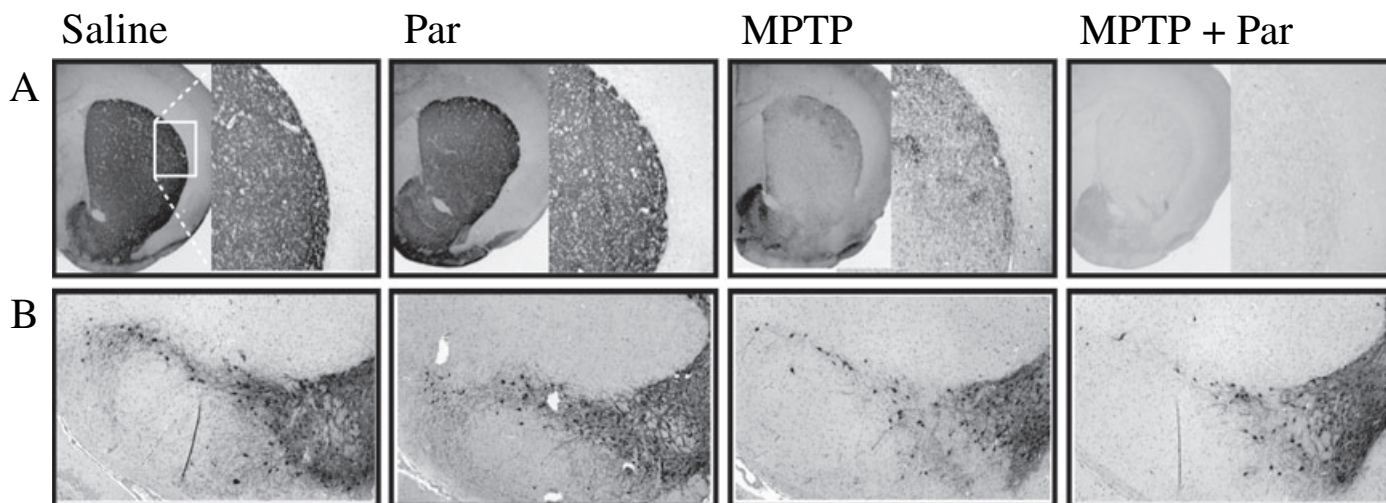


FIG. 1. Examples of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced damage with and without acetylcholinesterase inhibition. (A) Striatal tyrosine hydroxylase (TH) staining for representative mouse brains (coronal sections showing the striatum and surrounding cortex). Magnified view of regions indicated by white rectangles are shown in the right half of each panel. (B) TH staining of midbrain sections for representative brains. Photographs from left to right show brains for saline, Paraoxon (Par), MPTP and a combined MPTP + Paraoxon treatment (MPTP + Par).

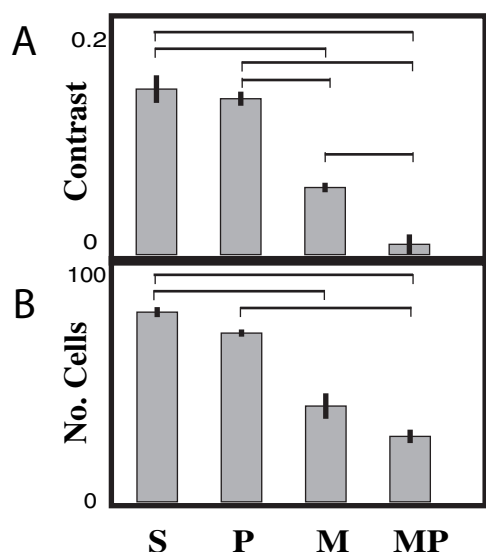


FIG. 2. Quantification of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced damage with and without acetylcholinesterase inhibition. (A) Means and SEMs for tyrosine hydroxylase (TH) staining between dorsal striatum and cortex. Higher contrast values represent more intense staining ($n = 6$ for each group, two brains \times three sections from each brain). (B) Means and SEMs of the number of TH-positive cells in the substantia nigra pars compacta ($n = 4$, two brains \times two sections from similar coordinates, except for the Paraoxon group with $n = 6$, two sections for one brain and four sections from another brain). Horizontal bars represent significantly different groups ($P < 0.05$, non-parametric ANOVA). S, Saline; P, Paraoxon; M, MPTP; MP, MPTP + Paraoxon.

of protein solution. Activity levels were normalized to total protein content as determined with the DC protein assay kit (Bio-Rad, Hercules, CA, USA) to obtain activity values in nmol substrate/min \times μ g protein. An identical procedure was used for plasma cholinesterase activity except that activities were normalized to nmol/min/ μ l plasma. For each of the three strains we used brain or plasma tissues from three mice. Values for each sample were obtained by averaging three replicate reactions.

Corticosterone levels

Serum corticosterone levels were determined using the OCTEIATM corticosterone measurement kit (IDS, Boldon, UK) and quantified with the SpectraFluor-Plus instrument. Three technical replicates of pools, each comprised of brains from five to six mice were used. Comparisons among strains were performed by subtracting the mean

level for saline-injected mice from each of the three replicates for MPTP-treated mice. These values were then compared using non-parametric ANOVA (Kruskal–Wallis test implemented in the statistics toolbox of MATLAB).

Immunohistochemistry

Paraffin-embedded sections were rehydrated using xylene and serial dilutions of ethanol in water. Antigens were retrieved by heating slides immersed in citric buffer in a microwave oven. Sections were blocked (phosphate-buffered saline + 4% serum) and incubated overnight at room temperature (23 °C) with rabbit anti-TH (NB-300-109, Novus Biologicals, Littleton, CO, USA) at a dilution of 1/400 followed by incubation with the secondary biotin-streptavidin-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at a dilution of 1/200. For TH staining, the Vectastain Elite ABC kit (Vector, Ontario, Canada) was used with diaminobenzidine in Tris.

Quantification of striatal tyrosine hydroxylase content and midbrain cell counts

The substantia nigra pars compacta (SNc) was defined according to the conventions described in Zaborszky & Vadasz (2001). When comparing SNc sections, care was taken to compare only sections from analogous coordinates, as determined by hematoxylin (Sigma) counterstaining of cells in the entire section, and by the shape of the TH-stained cells in the ventral tegmental area (Paxinos & Franklin, 2001; Zaborszky & Vadasz, 2001). For quantification of TH depletion in the dorsal striatum [caudate-putamen (CPu)], the contrast of averaged signals between the dorsal striatum (which exhibits clear staining for TH in the intact brain) and the adjacent cortex (which does not contain detectable TH) was calculated as follows: $(CPu - CTX)/(CPu + CTX)$, where CPu and CTX denote the average intensities in the CPu and cortex, respectively. The rationale for contrasting CPu staining with that of the adjacent cortex is to account for any slide- or section-specific effects of background staining. When the CPu is unstained for TH, the contrast will approach 0. When it is strongly stained, values will approach 1. All analyses were performed using the IMAGEPRO PLUS software (version 4). Statistical comparisons between sections (contrasts or number of neurons) were performed using a non-parametric ANOVA.

Behavioral analyses

For open-field tests, mice were placed in the center of a 60 \times 60 \times 50 cm (L \times W \times H) opaque-walled arena. A single inves-

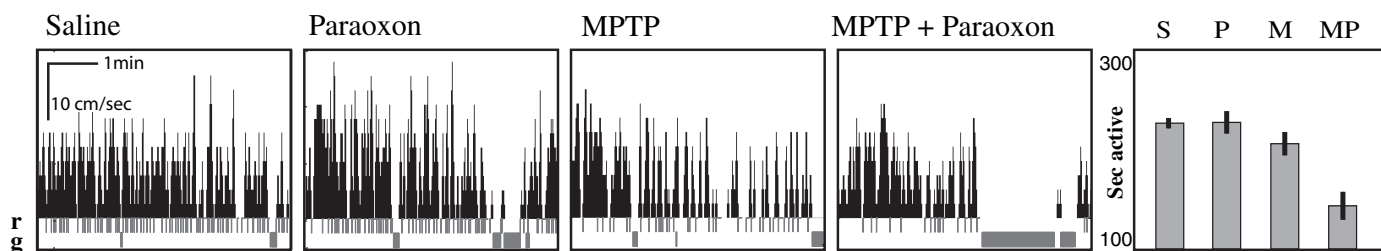


FIG. 3. Behavioral effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and Paraoxon exposure. 5-min open-field sessions from one representative FVB/N mouse from each treatment group. The horizontal axis represents time (300 s). Black bars in the main part of each panel denote instantaneous speed; vertical bars at the bottom of each panel represent episodes of rearing (top, denoted by r) and grooming (bottom, denoted by g). Right: quantification of total seconds of explorative activity comprising time spent moving or rearing. $n = 5, 5, 4$ and 2, respectively, for saline (S), Paraoxon (P), MPTP (M) and combined MPTP + Paraoxon (MP) exposures. Bars denote averages and lines denote SEs.

tigator (Y.B.S.) sat quietly in the same room and counted line crossings (marked on the bottom of the open-field box, at intervals of 10 cm), grooming and rearing episodes using an in-house computer program. Data files were analysed using in-house MATLAB programs to extract movement variables. Care was taken to sequentially test treatment groups to prevent a confounding temporal effect.

Measures of lethality

To quantify and test the statistical significance of differences in survival across groups and treatments following injections, mouse cages were examined at various time points starting immediately after injections and subsequently once every 12 h. These time periods were rank ordered, so that mice that died immediately following the injection were given a score of 1, whereas mice that survived the entire 96 h postinjection (after which mice were killed for other analyses) were given a score of 10. For example, a group of six mice that survived the entire procedure is described by the sample [10, 10, 10, 10, 10] and a group of seven mice of which three were found dead by the second day and four were found dead in the third night are described as [5, 5, 5, 8, 8, 8, 8]. Groups were then compared by a non-parametric ANOVA.

Results

Acetylcholinesterase inhibition accelerates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced deficits

We first tested whether AChE inhibition exacerbates the progression of Parkinsonism in mice following exposure to the neurotoxin MPTP. The pathological hallmark of MPTP intoxication is damage to midbrain dopaminergic neurons and their striatal projections (Dauer & Przedborski, 2003). At 3 days following a cumulative MPTP dose of 80 mg/kg, adult male FVB/N mice revealed a dramatic reduction of striatal TH staining relative to saline-injected controls (saline, 0.15 ± 0.013 , mean \pm SEM, arbitrary units; MPTP, 0.062 ± 0.004 ; $P = 0.02$, non-parametric ANOVA, Figs 1A and 2A). Note that TH staining in the CPu is quantified as the contrast between TH staining in the dorsal striatum and an adjacent cortical region and therefore includes a within-animal control for background staining as well as section-to-section variability. The damage extended to the midbrain cell bodies as shown by the decreased number of TH-positive cells (saline, 79.3 ± 2.1 TH-positive cell counts/section, mean \pm SEM; MPTP, 40 ± 5.3 ; $P = 0.02$, non-parametric ANOVA, Figs 1B and 2B). In contrast, 1 mg/kg of the AChE inhibitor Paraoxon, a blood-brain barrier-permeable metabolite of the commonly used insecticide Parathion (Kardos & Sultatos, 2000), did not significantly affect striatal or midbrain TH staining (CPu contrast, 0.144 ± 0.006 , $P = 1$ vs. saline; SNc cell counts, 70.5 ± 1.5 , $P = 0.06$ vs. saline; Figs 1 and 2). However, when coadministered with MPTP, Paraoxon exacerbated the MPTP toxicity resulting in a further decrease of striatal staining (CPu contrast, 0.0096 ± 0.0092 , $P = 0.020$ vs. saline, $P = 0.020$ vs. MPTP group) and fewer TH-immunoreactive midbrain cells (SNc cell counts, 27.3 ± 2.8 , $P = 0.020$ vs. saline, $P = 0.054$ vs. MPTP). Following a less conservative approach using a two-tailed two-sample *t*-test rather than non-parametric ANOVA, TH-positive SNc cell counts for the MPTP + Paraoxon group were significantly smaller than those in the MPTP group ($P = 0.049$). Thus, Paraoxon acts synergistically with MPTP to damage dopaminergic neurons.

The behavioral outcomes of MPTP treatment are known to vary appreciably among experiments and often reveal opposite responses in different studies (Sedelis *et al.*, 2001). However, within an experiment,

such assays can be informative as to the extent of the damage. In this study, the results of the behavioral analyses correlated with and thus supported the findings at the neurochemical level. We quantified exploratory activity as the total number of seconds spent either running or rearing in a 5-min open field session. At 3 days after the MPTP treatment, mice revealed reduced exploratory open-field activity (saline, 237 ± 6 s; MPTP, 214 ± 13 s, $P > 0.1$, Fig. 3). Consistent with the dopaminergic damage, Paraoxon did not noticeably change the exploratory patterns (237 ± 12 active seconds, $P > 0.01$ vs. saline). However, combined with MPTP it promoted a further marked decrease in activity (146 ± 15 , $P = 0.053$ vs. the saline and Paraoxon groups, $P = 0.064$ vs. MPTP group, Fig. 3), suggesting an interaction between Paraoxon and MPTP. Nevertheless, the limited sample sizes precluded detection of significant differences. Comparison of behavioral measures using a two-tailed *t*-test shows that the MPTP + Paraoxon group differs significantly from the saline ($P = 0.0008$), Paraoxon ($P = 0.009$) and, most importantly, the MPTP ($P = 0.033$) groups. Taken together, these results extend previous reports demonstrating that AChE inhibition mediated by di-isopropylfluorophosphate increases MPTP toxicity as assessed by biochemical measures (Hadjiconstantinou *et al.*, 1994) and provide a framework for testing the roles played by distinct AChE splice variants in this interaction.

Overexpression of the stress-responsive readthrough acetylcholinesterase variant confers protection from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

To investigate whether the splice shift to AChE-R plays a protective role against PD progression, we employed a model of an exaggerated AChE alternative splicing shift, using TgR (Sternfeld *et al.*, 2000) mice expressing the stress-induced human AChE-R (Fig. 4A). As shown in Fig. 4B, these mice exhibit markedly elevated AChE activity in both brain and plasma (CPu activity, 317 ± 10 and 1556 ± 21 nmol molecules hydrolysed/min/mg protein for FVB/N and TgR mice, respectively; plasma activity, 378 ± 42 and 1687 ± 19 nmol molecules hydrolysed/min/ml plasma for FVB/N and TgR mice, respectively).

Compared with their parent strain (FVB/N), TgR mice showed resistance to MPTP as reflected by TH staining 4 days after MPTP injection (CPu TH staining contrast relative to cortex, 0.058 ± 0.005 and 0.081 ± 0.008 for MPTP-injected FVB/N and TgR mice, respectively, $P = 0.037$, non-parametric ANOVA, Fig. 6A). Moreover, although lethality did not significantly differ between FVB/N and TgR mice for the MPTP group ($P = 0.4$, Fig. 5 and Table 1B), there was a significant reduction of lethality for TgR vs. FVB/N mice for combined MPTP + Paraoxon treatment ($P = 0.021$). Qualitative behavioral assessment (data not shown) over the course of 1–4 h after the injections and the following days suggested that TgR mice are behaviorally less affected by the treatment than the strain-matched FVB/N mice. Thus, whereas FVB/N mice displayed marked immobility and frequent episodes of shivering and convulsions a few hours following MPTP injections, TgR mice seemed almost indifferent to the treatment. Additionally, the increase in plasma corticosterone levels 3 days after MPTP exposure was subtler in TgR as compared with FVB/N mice (difference between MPTP-injected and averaged saline-injected samples: FVB/N, 11.9 ± 1.1 ng/mL; TgR, 4.6 ± 0.42 ng/mL; $P = 0.0495$, non-parametric ANOVA, Fig. 6B). This potentially reflects differences in the direct effects of MPTP, consequent neurodegeneration and/or psychological stress due to intoxication symptoms. Overall, transgenic expression of the stress-

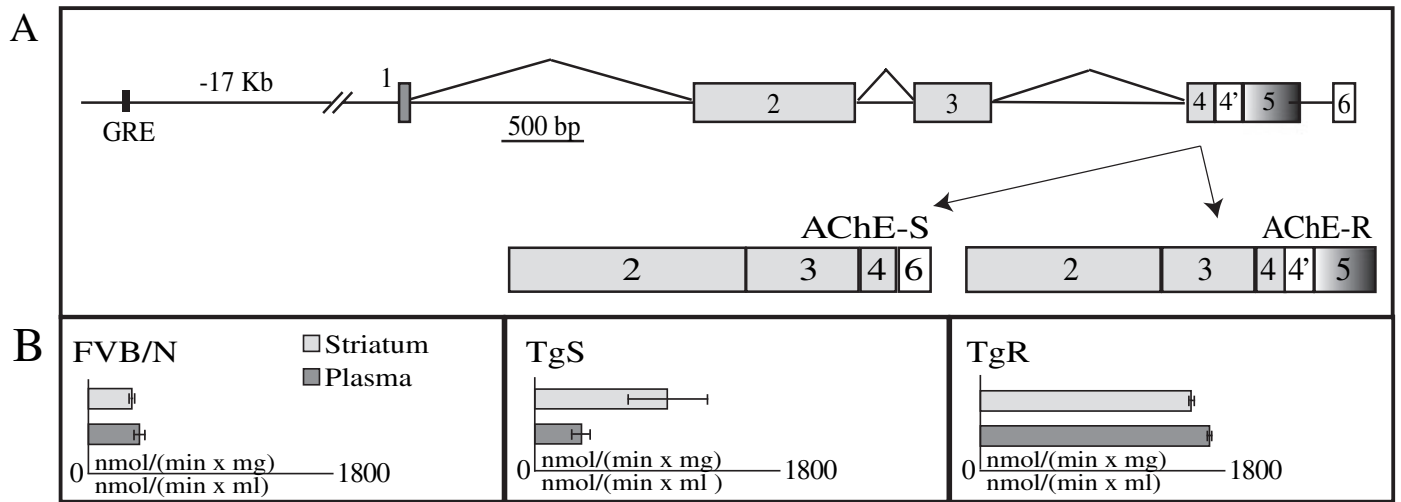


FIG. 4. Transgenic models of acetylcholinesterase (AChE) overexpression. (A) *ACHE* gene structure and alternative splicing scheme of synaptic AChE (AChE-S) and readthrough AChE (AChE-R) variants. (B) Cholinesterase activity in plasma and striatum of FVB/N mice and mice with synaptic acetylcholinesterase transgene (TgS) and readthrough acetylcholinesterase transgene (TgR). GRE, glucocorticoid response element.

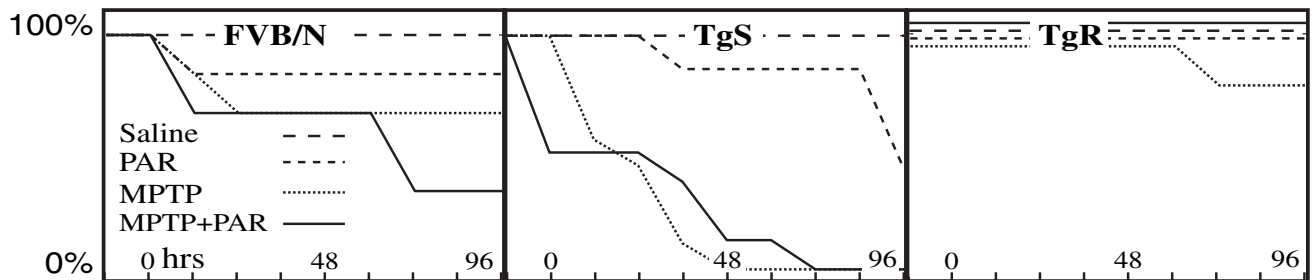


FIG. 5. Survival profiles for different strains and treatments. Horizontal axis denotes time since last 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injection. $n = 6$ mice for each group, except $n = 5$ for saline-injected FVB/N mice and $n = 9, 7$ and 8 for the MPTP, Paraoxon (PAR) and MPTP+Paraoxon (MPTP+PAR) exposed mice with synaptic acetylcholinesterase transgene (TgS), respectively. TgR, mouse with readthrough acetylcholinesterase transgene.

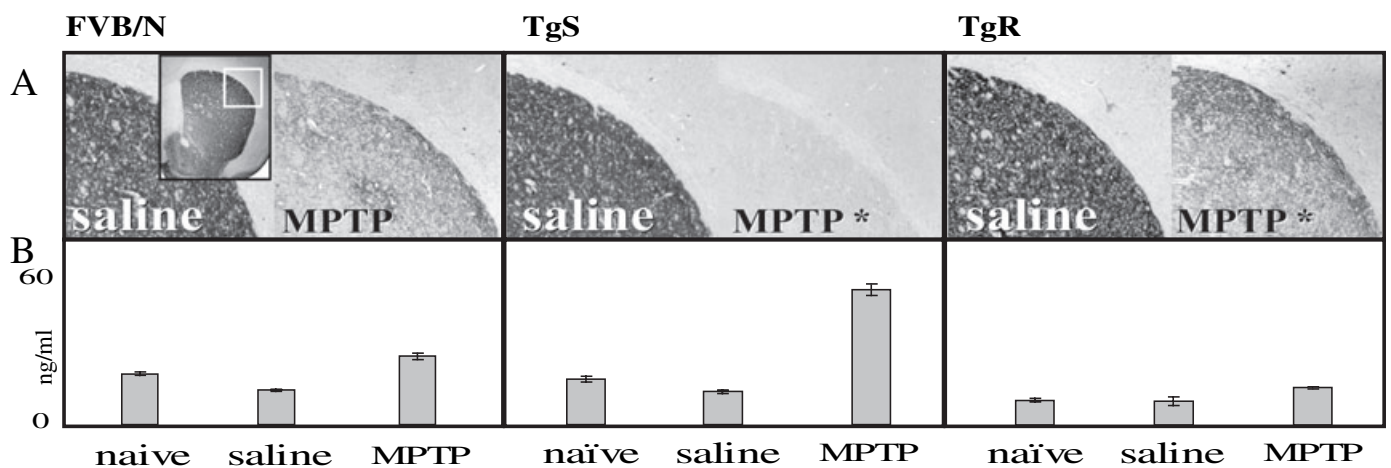


FIG. 6. Striatal tyrosine hydroxylase (TH) staining and corticosterone levels in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. (A) TH immunostaining in striatum of saline- and MPTP-exposed mice from each strain. The inset in the left panel shows the enlarged area shown in the other panels. Asterisks indicate significant differences between TH depletion of FVB/N and mice with synaptic acetylcholinesterase transgene (TgS) (increased depletion relative to FVB/N) and readthrough acetylcholinesterase transgene (TgR) (decreased depletion) ($P < 0.05$ non-parametric ANOVA; $n = 6$ for each strain \times group: three brains \times two sections from each brain). (B) Plasma corticosterone levels of naive, saline- or MPTP-exposed mice 3 days following injection. Each sample comprises pooled plasmas from five to six mice. Error bars indicate SDs of three technical replicates.

TABLE 1A. Comparison of survival scores among different treatments for each of the strains

Comparison	Strain		
	FVB/N	TgR	TgS
S–M	0.176	0.31731	0.00086*
S–P	0.361	1	0.0347*
S–MP	0.033*	1	0.0010*
M–P	0.598	0.31731	0.0017*
M–MP	0.388	0.31731	0.7308
P–MP	0.148	1	0.0038*

Values within each cell indicate the *P*-value for rejection of the null hypothesis of equal survival scores using non-parametric ANOVA. S, saline; M, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP); P, Paraoxon; MP, MPTP + Paraoxon. TgR, mouse with readthrough acetylcholinesterase transgene; TgS, mouse with synaptic acetylcholinesterase transgene. **P* < 0.05.

TABLE 1B. Comparison of survival scores among different strains for each of the treatments

Treatment	Comparison	
	FVB/N vs. TgR	FVB/N vs. TgS
S	1	1
M	0.40	0.059
P	0.317	0.286
MP	0.021*	0.0484*

See footnote to Table 1A.

responsive AChE-R splice variant was clearly protective, revealing an interaction between the AChE-R transgene and the response to MPTP treatment.

Excessive synaptic acetylcholinesterase increases vulnerability to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

That AChE-R confers MPTP resistance raised the question of whether an excess of the normally abundant AChE-S also confers protection. To model increased AChE expression without the concomitant splicing shift to AChE-R, we employed transgenic (TgS) mice selectively expressing human AChE-S in their central nervous system (Beeri *et al.*, 1995). The AChE activity of TgS mice was measured at 980 ± 289 nmol molecules hydrolysed/min/mg protein in striatum and 340 ± 64 nmol molecules hydrolysed/min/ml plasma, both values considerably higher than control FVB/N mice (Fig. 4). Compared with their parent strain (FVB/N) and the TgR mice, TgS mice were remarkably more sensitive to MPTP exposure. Thus, in one set of experiments, all MPTP-injected but not saline-injected TgS mice died within 96 h following injections (TgS saline vs. TgS MPTP, 0.00086; neither FVB/N nor TgR mice showed differences between saline-injected and MPTP-injected mice, *P* > 0.1 for both comparisons, Table 1A). Moreover, although MPTP-injected TgS mouse groups did not differ in lethality from MPTP-injected FVB/N mice (*P* = 0.059, Fig. 5, Table 1B), there was a significant difference between the respective MPTP + Paraoxon groups (*P* = 0.0484). In another set of experiments, mice from all strains survived and were killed 4 days post MPTP exposure. Striatal TH levels presented extreme decreases in TgS mice following MPTP exposure, reflecting accelerated dopaminergic damage as compared with the parent FVB/N strain (CPu TH staining contrast relative to cortex,

0.058 ± 0.005 and 0.034 ± 0.004 for MPTP-injected FVB/N and TgR mice, respectively; *P* = 0.0163, non-parametric ANOVA, Fig. 6A). Thus, over-expressed AChE-S interacted with the MPTP treatment to increase the vulnerability of dopaminergic neurons. Consistent with this, qualitative behavioral assessment over the course of 1–4 h after the injections and the following days revealed that TgS mice are less mobile than the parent strain. Finally, the increase in plasma corticosterone levels 3 days following MPTP exposure was larger in TgS than in FVB/N mice (difference between MPTP-injected and averaged saline-injected samples: FVB/N, 11.9 ± 1.1 ng/mL; TgS, 34.9 ± 1.9 ng/mL; *P* = 0.0495, non-parametric ANOVA, Fig. 6B).

Discussion

In this study we showed that the synaptic (AChE-S) and stress-induced soluble (AChE-R) AChE splice variants confer markedly different phenotypes in the context of the MPTP mouse model of Parkinsonism. This phenotype difference is consistent with an AChE inhibition (or stress)-triggered AChE splicing shift playing a protective role in Parkinsonism. The obvious differences in MPTP sensitivity between the variants is most easily explained by their distinct membrane adherence properties. AChE-R, by virtue of its solubility and its peripheral presence, may scavenge AChE inhibitors and acetylcholine before they reach brain synapses. Thus, with minimal interference with synaptic transmission, AChE-R can protect cholinergic signaling in TgR mice from AChE inhibitors (Fig. 7). At first, it would seem that AChE-S could play a similar protective role but, as we have shown here, this is clearly not the case. We believe that the key to this difference lies in the requirement to maintain cholinergic levels at physiological values, i.e. unlike the case with AChE-R, an excess of synaptically located AChE-S will result in marked deficiencies in cholinergic neurotransmission unless compensated for. Indeed, in TgS mice, the primary AChE overexpression is accompanied by pre- as well as postsynaptic modifications acting to restore normal cholinergic tone (Erb *et al.*, 2001; Svedberg *et al.*, 2003). While essential for viability, this compensation implies that the balance between cholinergic signal release and its termination (i.e. esterase activity) is balanced at excessive gains. This in turn leads to sharp imbalances when one of these components is inhibited (Fig. 7). Important for the experimental MPTP model is that MPTP and its metabolites can also inhibit AChE (Zang & Misra, 1996, 2003), acting in this respect as double-edged swords. Thus, both elements of the dopaminergic/cholinergic balance are disturbed simultaneously and hence act in concert to upset the balance. This can account for the increased sensitivity of the TgS mouse and resistance of the TgR mouse to MPTP even in the absence of the AChE inhibitor Paraoxon. In TgR mice, AChE-R can intercept MPTP before it exerts its effects, whereas in TgS mice the MPTP-induced AChE inhibition would unmask the augmented release elements at the synapse and cholinergic tone would increase.

The primary cholinergic excitation associated with AChE inhibition, stress or trauma elicits a feedback response involving increased AChE expression accompanied by a rapid yet long-lasting shift in AChE mRNA alternative splicing. As a consequence, the fraction of the soluble stress-induced AChE-R variant is increased relative to that of the normally predominant AChE-S variant (Meshorer *et al.*, 2002; Darreh-Shori *et al.*, 2004; Nijholt *et al.*, 2004). TgR and TgS strains represent extreme cases in a continuum of AChE-R : AChE-S ratios whereas FVB/N mice assume values somewhere between these two extremes. We propose that, in humans, mild exposure to stress or AChE inhibition would primarily lead to (an adaptive) increase of

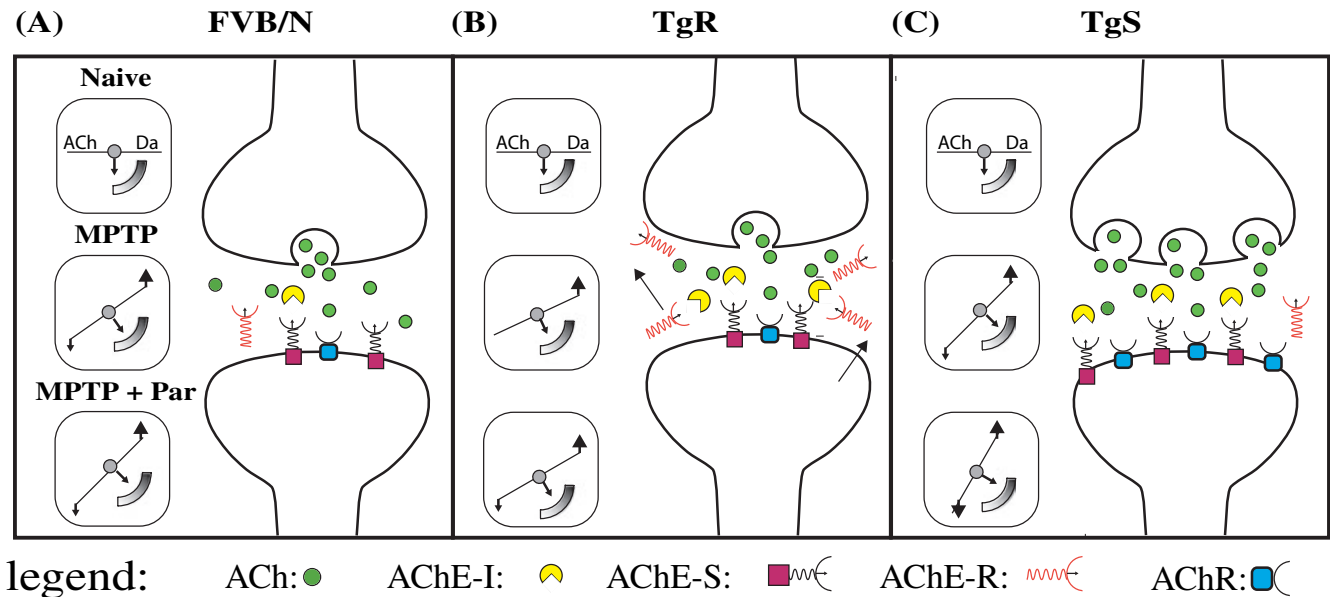


FIG. 7. Model of the complex involvement of splicing in PD. Model of gene–environment interactions linking acetylcholinesterase (AChE) inhibition, stress or trauma to increased 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) risk through AChE overexpression. Each panel shows a synapse and a drawing of the cholinergic–dopaminergic imbalance following MPTP or combined MPTP + Paraoxon (MPTP+Par) exposure. (A) Normal synapse; (B) synapse of TgR mouse with the readthrough acetylcholinesterase transgene (AChE-R). AChE-R protects against circulating toxins and inhibitors. (C) Synapse of TgS mouse with synaptic acetylcholinesterase transgene (AChE-S) with increased acetylcholine release and hydrolysis. Upon inhibition, acetylcholine levels rise sharply and the dopaminergic–cholinergic balance is severely impaired. (C) Mouse with readthrough acetylcholinesterase transgene (TgR) synapse. The readthrough AChE (AChE-R) variant protects against circulating toxins and inhibitors. In all drawings, the extent of the dopaminergic–cholinergic imbalance is depicted by the scales' position. ACh, acetylcholine; Da, dopamine; AChE-I, AChE inhibitor; AChR, ACh receptor.

AChE-R. Although it has been argued that the effects of acute stress do not always lead to an increase in AChE-R, even acute (rather than chronic) exposure to AChE inhibitors leads to overexpression in the striatum (Perrier *et al.*, 2005). It is likely that cumulative chronic effects of stress and AChE inhibitor exposure will lead to more pronounced increases in AChE-R, raising the intriguing prediction that moderate stress or AChE inhibitor exposure can actually serve to protect against PD. This seems plausible in the light of the proposed link between 'adventure-seeking' personalities and decreased PD risk (Kaasinen *et al.*, 2001; Tomer & Aharon-Peretz, 2004). Under natural conditions, there is probably a limit to the extent to which AChE-R overproduction is beneficial or even feasible, i.e. a maintained extreme excess of AChE-R also results in undesired consequences (Brenner *et al.*, 2003; Nijholt *et al.*, 2004), possibly explaining why this splice variant is only induced when a need arises. That blood AChE levels increase with age (Sklan *et al.*, 2004) is consistent with an age-related limitation for further overproduction, compatible with the age-related increased risk of PD (Langston, 1996). Beyond that limit, AChE-S will accumulate, at the price of rendering the system more vulnerable to any further inhibition and/or stressful insult as demonstrated with the TgS mouse.

Parkinson's disease, like other neurodegenerative diseases, involves a complex and only partially understood etiology (Lotharius & Brundin, 2002; Dauer & Przedborski, 2003). Only a small fraction of PD cases can be attributed to discrete genetic elements (Langston, 1996; Vila & Przedborski, 2004). However, a wide variety of environmental factors, including exposure to industrial and agricultural chemicals (Di Monte *et al.*, 2002), psychological stress (Smith *et al.*, 2002) and trauma (Veldman *et al.*, 1998) have also been implicated as risk modifiers. Here we have shown that the splice shift to AChE-R, if properly functioning, can minimize the effects of cholinergic hyperactivation. The other side of this coin is that any

deficits in alternative splicing may be critical risk factors in the progression of PD. Indeed, it may well be that either specific deficits in AChE regulation (Shapira *et al.*, 2000; Benmoyal-Segal *et al.*, 2005) or accumulated damage to the splicing machinery constitute important risk factors for PD onset (Caceres & Kornblihtt, 2002). This suggests that screening for such impairments may enable identification of high-risk individuals, allowing for preventive measures to be exercised. More generally, our results show how a naturally occurring alternative splicing shift can play an adaptive role in the context of parkinsonism and suggest that driving the splicing machinery to follow certain paths may be a promising approach for minimizing the risk of a complex disorder such as PD (Garcia-Blanco *et al.*, 2004).

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Abbreviations

AChE, acetylcholinesterase; AChE-R, readthrough acetylcholinesterase; AChE-S, synaptic acetylcholinesterase; CPU, caudate-putamen; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; SNc, substantia nigra pars compacta; TgR, mouse with readthrough acetylcholinesterase transgene; TgS, mouse with synaptic acetylcholinesterase transgene; TH, tyrosine hydroxylase.

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