Original Article

Donepezil Inhibits Diisopropylfluorophosphate-Induced Seizures and Up-regulation of Synaptotagmin 4 mRNA

(diisopropylfluorophosphate / LiCl / donepezil / epileptic seizures / c-*fos* mRNA / *Syt4* mRNA)

M. M. SAGHAFI, P. PREGELJ, M. ŽIVIN

University of Ljubljana, Medical Faculty, Institute of Pathophysiology, Brain Research Laboratory, Ljubljana, Slovenia

Abstract. Reversible acetylcholinesterase inhibitor donepezil displays prophylactic effects against intoxication with irreversible organophosphorous acetylcholinesterase inhibitors. We used behavioural observation of yawning and epileptic seizures, histochemical acetylcholinesterase staining, and *in situ* hybridization of the immediate early genes, c-*fos* and synaptotagmin 4 (*Syt4*) mRNAs in the brain, to evaluate whether donepezil could protect the brain against the effects of the organophosphate anticholinesterase, diisopropylfluorophosphate, in a rat model of intoxication. Diisopropylfluorophosphate-treated animals exhibited frequent yawning, significant inhibition of acetylcholinesterase staining and upregulation of c-*fos* mRNA, but not the epileptic seizures or significant change of *Syt4* mRNA levels. In order to reduce the threshold for the induction of cholinergic seizures, additional groups of rats were pre-treated with LiCl 24 h before the treatment with diisopropylfluorophosphate. These rats exhibited the seizures, a significant inhibition of acetylcholinesterase staining and significant upregulation of c-*fos* and *Syt4* mRNA levels. All the above-mentioned effects of diisopropylfluorophosphate were inhibited by donepezil pre-treatment. Donepezil pre-treatment by itself induced only a comparatively weaker inhibition of acetylcholinesterase staining and infrequent yawning. We conclude that donepezil protects the brain against diisopropylfluorophosphate-induced effects and that *Syt4* mRNA upregulation may serve as a novel marker for organophosphate-induced seizures.

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Corresponding author: Marko Živin, University of Ljubljana, Medical Faculty, Institute of Pathophysiology, Brain Research Laboratory, 1000 Ljubljana, Slovenia. Phone: (+386) 1 543 7058; Fax: (+386) 1 543 7021; e-mail: zivin@mf.uni-lj.si

Abbreviations: ACh – acetylcholine, AChE – acetylcholinesterase, AD – Alzheimer's disease, BBB – blood-brain-barrier, CNS – central nervous system, DFP – diisopropylfluorophosphate, DON – donepezil, IEG – immediate early gene, NMDA – *N*-methyl-D-aspartate, OP – organophosphorous, PB – pyridostigmine bromide, ROD – relative optical density, SD – standard deviation, *Syt4* – synaptotagmin 4.

Introduction

Irreversible organophosphorous (OP) inhibitors of acetylcholinesterase (AChE) were developed as chemical warfare agents (*e.g.,* soman) and agricultural insecticides (*e.g.,* diisopropyl fluorophosphate – DFP). These compounds exert their toxic effects by (pseudo)irreversible inhibition of AChE, resulting in prolonged acetylcholine (ACh) activity, with subsequent excessive stimulation of both muscarinic and nicotinic ACh receptors (Taylor, 1996). Due to the localization of cholinergic synapses in the central nervous system (CNS), autonomic ganglia, and neuromuscular junction, this leads to a cholinergic toxic syndrome, characterized by numerous symptoms of central and peripheral origin. Since OP anticholinesterases are highly soluble in lipid membranes, they easily cross the blood-brain-barrier (BBB). The spectrum of acute CNS effects includes confusion, ataxia, slurred speech, loss of reflexes, Cheyene-Stokes respiration, coma, and central respiratory paralysis (Watson et al., 2009). Cholinergic mechanisms can also trigger the onset of seizures (Turski et al., 1983), although the propagation and maintenance of *status epilepticus* occurs primarily *via* activation of excessive glutamatergic transmission (Smolders et al., 1997) and subsequent excitotoxic brain damage (Solberg and Belkin, 1997). Conventional treatment of organophosphate poisoning includes combined administration of a cholinesterase reactivator (an oxime), a muscarinic cholinergic receptor antagonist (atropine) and a benzodiazepine anticonvulsant (diazepam) (Watson et al., 2009).

One of the strategies for prevention of irreversible inhibition of AChE by OP is the use of reversible inhibitors, such as carbamates (Lallement et al., 2001). AChE inhibited by carbamates is resistant against irreversible inhibition by OP nerve agents and the reversibly inhib-

ited enzyme recovers spontaneously, without the use of AChE reactivators. The peripherally acting carbamate AChE inhibitor, pyridostigmine bromide (PB), has been advocated as a prophylaxis against potential threat with OP warfare agents (Keeler et al., 1991). However, PB does not cross the BBB and thus may not counteract CNS symptoms. Indeed, the CNS effects of OP agents may actually be potentiated by pre-treatment with PB (Amourette et al., 2009). In this regard, centrally acting reversible anticholinesterases that have been used for the treatment of Alzheimer's disease (AD) may offer a better prophylactic choice against OP intoxication than PB.

Donepezil (DON) is a reversible, primarily non-competitive, selective inhibitor of AChE used for the treatment of AD. Acute pre-treatment with oral DON, with and without scopolamine, decreased the hypothermic, hypokinetic, and diarrhoea-inducing effects of DFP (Janowsky et al., 2004, 2005). A combined pre-treatment with DON and procyclidine, a muscarinic receptor antagonist, protected against soman-induced seizures (Haug et al., 2007).

Here, we further explored the prophylactic potential of DON against the central effects of DFP in the brain, such as yawning (Ogura et al., 2001) and seizures (Zivin et al., 1999). In addition, we performed AChE histochemical staining and *in situ* hybridization of immediate early genes (IEGs) c-*fos* and synaptotagmin 4 (*Syt4*) mRNA for this purpose. c-*fos* induction in the brain is a well-established marker of neuronal hyperactivity induced by cholinergic seizures (Zivin et al., 1999), while there is rising evidence for the pathophysiological and adaptive changes of membrane trafficking in the brain induced by seizures, thus implicating the potential role of the plasticity of *Syt4* expression in these processes (Glavan et al., 2009).

Material and Methods

Animals

Adult male Wistar rats (220–300 g) were housed in groups of four in polycarbonate cages under standard housing conditions (50% humidity) and were maintained in a 12-h light-dark cycle (light on: 07.00 h – 19.00 h) in a temperature-controlled colony room at 22–24 °C with free access to food pellets and tap water. At the end of the experiment, we sacrificed the rats by decapitation under CO_2 anaesthesia. We made all efforts to minimize the number of animals and their suffering. The handling of rats followed the European Communities Council Directive of 24th of November 1986 (86/609/EEC) and National Veterinary Institute Guide for the Care and Use of Laboratory Animals.

Drug

Lithium chloride (LiCl, Sigma, St. Louis, MO) and donepezil (DON, obtained by donation from Krka Pharmaceutical Company, Novo Mesto, Slovenia) were dissolved in 0.9% saline (SAL) and were administered *i.p.* in a volume of 1 ml/kg, DFP (Sigma) was dissolved in 0.9% SAL and injected *s.c.* in the dorsal neck region in a volume of 1 ml/kg.

Experimental protocol

We performed two experiments to explore the prophylactic potential of DON against the central effects of DFP in the brain.

In the first experiment we evaluated whether the pre-treatment with DON could act against the onset of intoxication induced by DFP. We used five groups of animals treated with DFP (1 mg/kg *s.c.*). Experimental groups (N = 5 each) were treated as follows: groups A (SAL+SAL) and B (SAL+DFP) – with 0.9% saline (SAL), followed after 1 h with saline or DFP (1 mg/kg), respectively; groups C (DON+DFP) and D (DON+SAL) – with 2 mg/kg of DON, followed after 1 h by treatment with 1 mg/kg of DFP or saline, respectively. The rats from groups A–D were sacrificed 1 h after the second injection. Group E (DON) was treated with DON (2 mg/kg) only and was sacrificed 1 h after the treatment with DON.

In the second experiment we evaluated whether the pre-treatment with DON could protect against the onset of epileptic seizures induced by DFP. Experimental groups were treated as follows: groups F (LiCl+SAL+SAL), G (LiCl+SAL+DFP), and H (LiCl+DON+DFP) (N = 6 rats each) received three injections of drugs and/or 0.9% saline in the following manner. All animals first received LiCl (3 mEq/kg). After 24 h, the rats from groups F and G received saline, while the rats from group H received DON (2 mg/kg). One hour after the second injection, group F received saline, while groups G and H received DFP (1.3 mg/kg). The rats from these three groups were sacrificed 4 h after the last injection.

Behavioural observations

Behaviour was recorded by a trained investigator, who was unaware of the treatments. During the first experiment, we evaluated signs of central cholinergic hyperactivity in the form of yawning. Yawning was recorded for 1 h after the second injection (groups A–D) or for 1 h after DON (group E). Intensity of yawning was graded as follows: absent (-), occasional, one to six events per hour (+), or frequent, more than six events per hour (++).

During the second experiment, we performed the behavioural ranking of seizures for 1 h after the injection of DFP. In seizures induced with OP anticholinesterases, the behaviour that precedes the onset of overt seizures and epileptic status is often obscured by other signs of cholinergic toxic syndrome. We therefore ranked seizures by using a modified Racine scale (Racine, 1972): absent (-), weak, short bursts of convulsive activity (+) (forelimb clonus with rearing, *i.e.*, Racine grade 4), or pronounced and prolonged convulsive activity (++) (loss of balance and falling, *status epilepticus*, *i.e.*, Racine grade 5).

Preparation of brain cryo-sections

Animals in all experimental groups were sacrificed by decapitation under CO_2 anaesthesia. Brains were rapidly removed and quickly frozen on dry-ice powder. Coronal cryo-sections (10 µm) were cut at three evenly spaced rostro-caudal levels through the striatum (approx. between +1.7 to -0.3 mm from bregma) and the rostral part of hippocampus (approx. between -2.6 to -3.6 mm from bregma).

Oligonucleotide probes

We used oligodeoxyribonucleotide 'antisense' probes (45 bases long) complementary to the rat c-*fos* mRNA (bases encoding 135–179, sequence 5'-CTC CTT TAC ACA GGA TGT CCA TAT TAG GAC ATC TGC GTC AGG TTT-3, GenBank, accession number UO2631) and rat *Syt4* mRNA (bases encoding 1082–1126, sequence 5'-CAG AGG GAG ACC AGA AGT TCA CCC CGT CCA GAA GAC TTC TTA GCA-3', GenBank, accession number L38247).

In situ hybridization histochemistry

The standard autoradiographic procedure used by Zivin et al. (1999) was performed. The autoradiograms were exposed at room temperature for 2–3 weeks and developed using standard darkroom techniques.

Histochemical staining of AChE activity

AChE activity staining was done according to a histochemical reaction described by Koelle and Friedenwald (1949). For striatal staining, the sections were fixed for 5 min in 4% paraformaldehyde, rinsed (4 × 3 min) in distilled water and incubated in 'Koelle' medium (3.1 mM copper (II) sulphate, 10 mM glycine, 50 mM sodium acetate with 4 mM acetylthiocholine iodide as the substrate for AChE and 0.14 mM ethopropazine as the inhibitor of non-specific esterase activity; pH = 5) for 2 h at 37 °C. Additional staining was performed where incubation at 37 °C was prolonged for 24 h to achieve higher intensity of staining of hippocampal and cortical AChE.

Image analysis

The autoradiograms of c-*fos* and *Syt4* mRNA levels and of the cover-slipped sections that were histochemically stained for AChE activity were trans-illuminated in the visual field of a 12-bit digital Photometrics camera CoolSNAP cf (Spectra Services Inc, Ontario, NY) connected to an MCID, M5 Elite image analyzer (Imaging Research Inc., St. Catherines, Ontario, Canada) and visualized as relative optical density (ROD) images. The ROD measurements in the regions of interest and the subtraction of the background signals were performed according to the principles of computerized densitometric image analysis.

Statistical analysis

Effects of DON on DFP-induced yawning and seizures were subjected to Kruskal-Wallis one-way ANOVA on Ranks Hypotheses, followed by Kruskal-Wallis Multiple-Comparison Z-Value test. The effect of pre-treatment with DON on AChE activity and on the expression of IEGs in DFP-treated animals was subjected to one-way ANOVA followed by Tukey's HSD Multiple-Comparison test. Statistical analysis was performed by SOLO60 statistical analysis software. Statistical significance for both tests was set at $P < 0.05$. All data in the bar charts are expressed as means ± SEM.

Results

Behavioural observations

In both experiments DFP induced signs of cholinergic hyperactivation of presumably peripheral (severe muscle fasciculation), vegetative (increased salivation, urination and defecation), and central (frequent orofacial automatisms, tremors, yawning) origin.

In the first experiment we aimed to evaluate the effect of DON on yawning, a sign of central cholinergic intoxication. In our preliminary experiments the selected dose of DFP (1 mg/kg) induced frequent yawning but never induced behavioural seizures that could interfere with behavioural observation of yawning. This behavioural sign was selected on the basis of the reports in the literature indicating that central cholinergic and, in part, dopaminergic mechanisms are involved in anticholinesterase-induced yawning (Ogura et al., 2001). We found that DON by itself induced a milder form of cholinergic intoxication, including the yawning, did not induce the seizures, but significantly attenuated DFP-induced yawning (Table 1A).

In the second experiment we evaluated whether the pre-treatment with DON could protect against the onset of epileptic seizures induced by DFP. We have used a protocol that in our hands reliably provokes *status epilepticus* (Zivin et al., 1999), but without significant mortality, *i.e*. DFP (1.3 mg/kg) in combination with LiCl (3 mEq/kg) pre-treatment, when given somewhere between 24–8 h before treatment with DFP. LiCl is thought to facilitate the coupling of cholinergic excitation and phosphoinositol-1-phosphate transmembrane signalling, thus effectively reducing the concentration of cholinergic agents needed to induce seizures (Ogura et al., 2001). In this experiment, in addition to yawning, DFP induced short-lasting bursts of severe convulsive activity that progressed to epileptic status. Pre-treatment with DON prevented induction of the seizures (Table 1B).

Acetylcholinesterase staining

The results of semi-quantitative analysis of AChE histochemical staining are presented in Figs. 1 and 2.

In the first experiment there was almost complete inhibition of AChE staining 1 h after the injection of DFP

Table 1. Effects of donepezil on central signs of DFP intoxication

A. *In groups A–E (N = 5), DFP induced intensive yawning but no seizures (group B). DON pre-treatment significantly reduced the frequency of yawning that was induced by DFP (group C). DON by itself induced infrequent yawning, but only during the 1-h pre-treatment period (group E), while during the second hour of observation, yawning was no longer present (group D). Yawning was recorded for 1 h after the second injection (groups A–D) or for 1 h after DON (group E). Intensity of yawning was graded as follows: absent (-), occasional, one to six events per hour (+), or frequent, more than six events per hour (++). Statistics: Kruskal-Wallis one-way ANOVA on Ranks Hypotheses, followed by Kruskal,Wallis Multiple-Comparison Z-Value test. *significantly different from the indicated groups*

B. *In groups F–H (N = 6) that were pre-treated with LiCl, DFP induced epileptic status (group G) that was completely prevented by DON (group H). Seizures: absent (-), weak, short bursts of convulsive activity (+), pronounced and prolonged convulsive activity (++). Statistics: Kruskal-Wallis one-way ANOVA on Ranks Hypotheses, followed by Kruskal-Wallis Multiple-Comparison Z-Value test. *significantly different from the indicated groups*

1A

group	**treatment**	**effect:** yawning
A	SAL+SAL	–
B	SAL+DFP	++ * **(A,C,D,E)**
C	DON+DFP	+ * **(A,B,D)**
D	DON+SAL	–
E	DON	+ * **(A,B,D)**

1B

group	**treatment**	**effect:** seizures
F	LiCl+SAL+SAL	–
G	LiCl+SAL+DFP	++ * **(F,H)**
H	LiCl+DON+DFP	–

(Fig. 1, group B), as compared to saline-only treated controls (Fig. 1, group A). DON by itself also significantly inhibited striatal AChE staining (Fig. 1, group E). The inhibition of AChE by DON was reversible, since it was significant only at 1 h, but not at 2 h after the injection of the drug (Fig. 1, DON, group D). DON significantly attenuated the inhibition of striatal AChE staining induced by DFP (Fig. 1, group C). In the second experiment, there was almost complete inhibition of striatal, cortical, and hippocampal AChE staining 4 h after the injection of DFP, as compared to saline-only treated controls (Fig. 2, groups G and F, respectively). DON significantly attenuated the inhibition of AChE staining induced by DFP (Fig. 2, group H).

c-fos and Syt4 mRNA levels

The results of semi-quantitative analysis of AChE c-*fos* and *Syt4* mRNA levels in rats without seizures are presented in Fig. 1. In rats receiving saline pre-treatment followed by DFP, a significant up-regulation of cortical c-*fos* but not *Syt4* mRNA signal was found 1 h after the injection of DFP, as compared to saline-only treated controls (Fig. 1, B and A, respectively). DON by itself did not significantly up-regulate cortical c-*fos* or *Syt4* mRNA signal at 1 or 2 h after the injection of the drug (Fig. 1, D and E, respectively). On the other hand, DON significantly prevented up-regulation of the cortical c-*fos* mRNA signal induced by DFP (Fig. 1, C). DON also attenuated DFP-induced hippocampal upregulation of c-*fos* mRNA (data not shown).

In the second experiment (Fig. 2), the animals were sacrificed 4 h after the induction of seizures with DFP. This timing was selected according to our preliminary experiments that showed that the maximal *Syt4* mRNA upregulation occurs about 4 h after the onset of seizures (unpublished observation). In the brains of animals with DFP-induced seizures we found massive and significant up-regulation of c-*fos* and *Syt4* mRNA in several brain regions (quantified changes are shown in gyrus dentatus, CA regions of hippocampus, cerebral cortex and striatum (Fig. 2, G). Again, all these changes were completely prevented by DON (Fig. 2, H).

Discussion

The main findings of our experiments confirmed that DON, which is the dominant drug in the treatment of Alzheimer's disease, could also display prophylactic activity against central signs of intoxication with OP nerve agents.

AChE staining showed that DFP almost fully inhibited striatal, cortical and hippocampal AChE. Although to a lesser extent, DON also inhibited AChE in these regions. However, it should be remembered that DFP induces irreversible inhibition of AChE, while the inhibition of AChE by DON is mostly reversible. The only physiological mechanism for restoring DFP-inhibited AChE activity thus depends on the relatively slow process of *de novo* synthesis of AChE. It may be speculated that the pre-treatment with DON has protected AChE against the irreversible inhibition by DFP, and that the relatively high AChE activity observed in our experiments 1–4 h after the treatment with DFP reflects spontaneously recovered activity of AChE.

In rats without seizures, we did not find cortical and hippocampal c-*fos* mRNA up-regulation after DON, as compared to the clear up-regulation in DFP-treated animals. DON in fact completely prevented the DFP-induced up-regulation of c-*fos* mRNA levels. IEGs such as c-*fos* respond to various stimuli and their protein products transactivate other genes, resulting in long-term changes in the nervous system. Various types of noxious stimulation (*e.g.,* thermal, mechanical, and chemical), including DFP, have been shown to induce

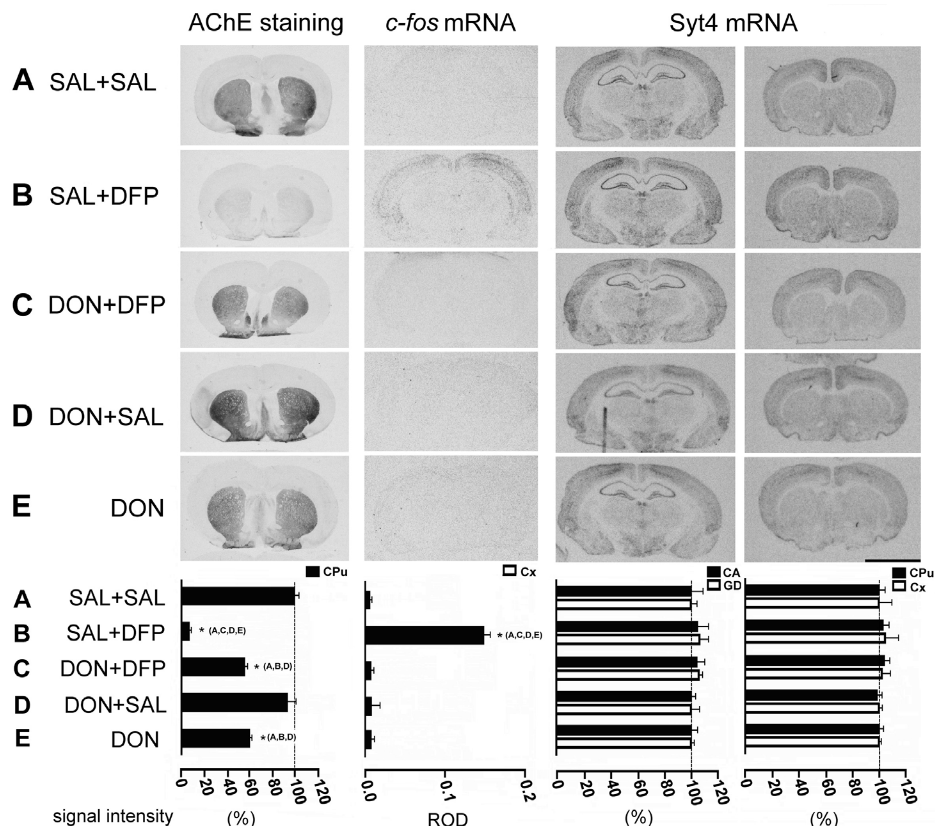

Fig. 1. Effect of DON on DFP-induced changes in AChE staining and on the levels of c-*fos* and *Syt4* mRNAs in animals without seizures. Panels show adjacent coronal striatal/hippocampal sections from the same animal that were stained for AChE activity by Koele method (AChE staining) or processed for autoradiographic procedure of *in situ* hybridization (c-*fos* and *Syt4* mRNA). Calibration bars represent 5 mm. **Cx** – cerebral cortex; **CPu** – dorsal striatum, **CA** – cornu Ammonis, **GD** – gyrus dentatus of hippocampus. Graphs below each column of images show the results of densitometric analysis of AChE staining/autoradiographic signals. Bars on the graphs represent average signal intensity (expressed as % of signal intensity *vs*. control group) ± standard deviation (SD). Since the basal levels of c-*fos* on autoradiograms were extremely low, we expressed the intensity of c-*fos* mRNA signal in ROD instead of % *vs.* control.
*statistical significance: one-way ANOVA followed by Tukey's HSD Multiple-Comparison test, for all groups N = 5, $P < 0.05$. Note that in group B, DFP significantly reduced striatal AChE staining and increased the levels of c-*fos* mRNA in cerebral cortex, but did not affect the levels of *Syt4* mRNA in the examined brain regions. DON completely prevented the up-regulation of c-*fos* mRNA in the hippocampus and cerebral cortex (group C). DON by itself significantly reduced striatal AChE staining (group E, 1 h after DON treatment), while after 2 h (group D) AChE reverted to the intensity observed in the control group A. DON did not induce any changes in the level of c-*fos* or *Syt4* mRNAs. DON significantly attenuated the inhibition of striatal AChE staining induced by DFP and completely prevented the DFP-induced up-regulation of c-*fos* mRNA (group C).

c-*fos* in the brain and spinal cord of various species (Zivin et al., 1999; Gupta et al., 2000). Since the massive induction of c-*fos* mRNA is indicative of pathologic processes, such as excitotoxic and neurodegenerative changes (Gupta et al., 2000), the prevention of DFP-induced upregulation of c-*fos* mRNA indicates that prophylaxis with DON may prevent neurodegenerative changes induced by DFP. *Syt4* is also considered to be an IEG, since its induction does not depend on previous protein synthesis (Vician et al., 1995). However, *Syt4* does not seem to be involved in the regulation of delayed gene response. *Syt4* has rather been recognized to be implicated in phenotypic changes involving membrane trafficking (Glavan et al., 2009). *Syt4* mRNA levels were not significantly affected by either DON or DFP. This suggests that in rats without seizures, the membrane trafficking may not be significantly affected by the cholinergic stimulation and that the increase of c-*fos* mRNA levels is a more rapid and sensitive marker for increased brain cholinergic activity.

So far, no data exist in the literature in regard to the prophylactic effects of DON against DFP-induced seizures. *Syt4* mRNA is known to be strongly up-regulated in the brains of animals with seizures induced with glutamate analogue kainic acid (Glisovic et al., 2007). There is rising evidence for the pathophysiological and adaptive changes of membrane trafficking in neurodegeneration in neuronal plasticity and in glial activation induced by seizures, thus implicating the potential role of the plasticity of *Syt4* expression in these processes.

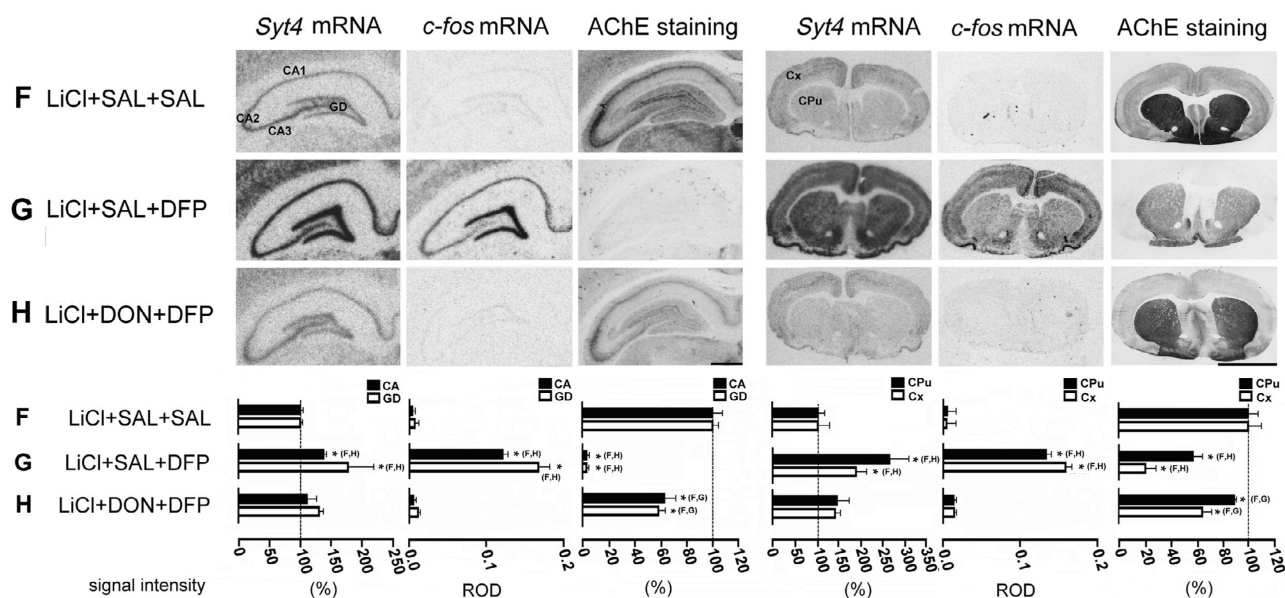

Fig. 2. Effect of DON on changes of AChE staining and on the levels of c-*fos* and *Syt4* mRNAs in animals with DFP-induced seizures. Panels show coronal striatal/hippocampal sections from the same animal that were stained for AChE activity by Koele method (AChE staining) or processed for autoradiographic procedure of *in situ* hybridization of c-*fos* and *Syt4* mRNAs. Calibration bars represent 1 mm. **Cx** – cerebral cortex; **CPu** – dorsal striatum, **CA** – cornu Ammonis, **GD** – gyrus dentatus of hippocampus. Graphs below each column of images show the results of densitometric analysis of AChE staining/autoradiographic signals. Bars on the graphs represent average signal intensity (% of signal intensity *vs.* control group) ± standard deviation (SD). Since the basal levels of c-*fos* on autoradiograms were extremely low, we expressed the intensity of c-*fos* mRNA signal in ROD instead of % *vs.* control.
*statistical significance: one-way ANOVA followed by Tukey's HSD Multiple-Comparison test, for all groups N = 6, P < 0.05. Note that in group G, DFP significantly reduced striatal, cortical and hippocampal AChE staining and increased the levels of c-*fos* and *Syt4* mRNAs in cerebral cortex, striatum and hippocampus. DON significantly attenuated the inhibition of striatal AChE staining induced by DFP and completely prevented the DFP-induced up-regulation of c-*fos* and *Syt4* mRNA (group H).

So far, *Syt4* mRNA levels (Tocco et al., 1996) and protein (Glisovic et al., 2007) were found to be up-regulated in the brain regions associated with the propagation of temporal lobe epilepsy only following kainic acid-induced seizures, and in hemi-seizures in rats after unilateral striatal injection of glutamate analogue and excitotoxin quinolinic acid (Glavan et al., 2009). Numerous studies have demonstrated that excitatory amino acid glutamate also plays a prominent role in the maintenance of organophosphate-induced seizures and in the subsequent neuropathology especially through over-activation of the *N*-methyl-D-aspartate (NMDA) receptor subtype (Lallement et al., 1999). We have shown previously that prevention of hemi-seizures in rats after unilateral striatal injection of glutamatergic NMDA agonist quinolinic acid by pre-treatment with NMDA antagonist dizolcipine (MK-801) prevented up-regulation of c-*fos* and *Syt4* mRNAs (Glavan et al., 2009). In the present experiment, we also found that the prerequisite for the up-regulation of *Syt4* mRNA was the glutamatergic hyperactivity due to the induction of seizures, and not just cholinergic hyperactivity by itself. The increase of cholinergic activity in DON-treated rats without seizures or in rats in which LiCl/DFP-induced seizures were prevented by DON namely did not result in increased *Syt4* mRNA levels. It is noteworthy in this regard that DON has been shown to protect cortical neurons against glutamate-induced neurotoxicity and apoptotic death *via* nicotinic ACh receptors (Takada et al., 2003). It remains to be determined whether this may be an additional pharmacological mechanism by which DON could prevent the up-regulation of *Syt4* mRNA in animals with seizures.

We conclude that the pre-treatment with DON protects the brain against the effects of central cholinergic and glutamatergic over-activity induced by DFP. This study has also revealed that up-regulation of *Syt4* mRNA may be considered as a novel marker for increased membrane trafficking in animals with OP-induced seizures.

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