Original Article

Galantamine as a Preventive of Diisopropylphosphorofluoridate Toxicity Effects in Rat Brain

(diisopropylfluorophosphate / galantamine / DFP seizures / c-*fos* mRNA / acetylcholinesterase)

M. M. SAGHAFI, M. ŽIVIN, P. PREGELJ

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

Abstract. Diisopropylfluorophosphate exerts its toxic effect by irreversibly inhibiting acetylcholinesterase. This results in over-stimulation of central and peripheral cholinergic activity. The aim of the present study was to evaluate the possible preventive effects of acute treatment with reversible acetylcholinesterase inhibitor galantamine against the signs of cholinergic toxic syndrome provoked by diisopropylfluorophosphate, such as hypothermia, muscular fasciculations, oral dyskinesia and decreased locomotor performance in a rat model of intoxication. The effects of these two anticholinesterases on acetylcholinesterase activity and on the expression of mRNA of the immediate early response gene c-*fos* in the brain were assessed by histochemical acetylcholinesterase staining and by *in situ* hybridization, respectively. Diisopropylfluorophosphate induced rapidly progressing hypothermia, muscular fasciculations, oral dyskinesia and decreased locomotor performance. The increased cholinergic cortical and hippocampal activity due to irreversible acetylcholinerase inhibition were indicated by the increased c-*fos* mRNA autoradiographic signal and by the inhibition of acetylcholinesterase staining, respectively. Galantamine by itself provoked transient and relatively weak inhibition of the acetylcholinesterase staining, while it did not induce increased c-*fos* mRNA expression or significant behavioural signs of cholinergic toxicity. Galantamine significantly reduced the rate of the onset, but not the maximal hypothermia induced by diisopropylfluorophosphate. Importantly, all the above-mentioned behavioural and neurochemical effects of diisopropylfluorophosphate were significantly reduced by galantamine. These results indicate that the acute pre-treatment with galantamine may have prophylactic effects against the intoxication by diisopropylfluorophosphate.

Introduction

Due to the widespread distribution of cholinergic neurons, which mediate numerous physiological functions in different animal species, it is not surprising that the drugs that inhibit acetylcholinesterase (AChE) have received extensive application. Irreversible organophosphorous (OP) inhibitors of AChE were developed as chemical warfare agents (e.g. soman) and agricultural insecticides (e.g. diisopropylfluorophosphate – DFP), while reversible cholinesterases were developed as drugs that, by restoring cholinergic tonus, ameliorate the central and/or peripheral symptoms in patients with deficits of cholinergic transmission (*e.g.* in Alzheimer's disease (AD), myasthenia gravis) (Taylor, 1996). Cholinergic over-activity may cause several pathophysiological changes in the organism with enhanced central and peripheral muscarinic and nicotinic receptor stimulation. The cholinergic pathways in the central nervous system (CNS) play a critical role in the control of body temperature and the stimulation of CNS muscarinic pathways appears to be a primary cause of the acute hypothermic response elicited by cholinesterase inhibitors (Gordon et al., 2006).

This leads to cholinergic toxic syndrome, which is characterized by numerous signs and symptoms of central and peripheral origin and perturbations of other neuroactive chemicals (Taylor, 1996; Solberg and Belkin, 1997). Specific and effective treatment of peripheral and central symptoms includes muscarinic antagonists and AChE reactivators that, when taken immediately after intoxication, may help to regenerate the activity of the enzyme well before the slow recovery of AChE activity,

Received March 9, 2012. Accepted November 23, 2012.

This work was supported by Slovenian Research Agency, grants M3-0142 and P-0171.

Correspondning author: Peter Pregelj, University of Ljubljana, Medical Faculty, Institute of Pathophysiology, Brain Research Laboratory, Zaloska 4, 1000 Ljubljana, Slovenia. Phone: (+386) 41 691 512 ; Fax: (+386) 1 543 7021; e-mail: peter.pregelj@psih-klinika.si

Abbreviations: AChE – acetylcholinesterase, AD – Alzheimer's disease, Cl – clearance, CNS – central nervous system, DFP – diisopropylfluorophosphate, GAL – galantamine, IEG – immediate early response gene, OP – organophosphorous, ROD – relative optical density, SAL – saline.

by *de novo* AChE synthesis, eventually takes place. Muscarinic antagonist, such as atropine, in sufficient dosage to get appreciable concentrations within CNS and AChE reactivator pralidoxime may be used for this purpose (Taylor, 1996; Watson et al., 2009). Increasing amounts of data from animal studies have suggested that more centrally acting anticholinergic agents (i.e. scopolamine, trihexyphenidyl, and benztropine) are more effective than atropine in preventing effects of the irreversible AChE inhibitors (Lallement et al., 2001; Janowsky, 2002).

Another possible approach to prevention of irreversible AChE inhibitor toxicity has been to pre-treat the animals with relatively low, non-lethal doses of reversible AChE inhibitors. These agents, by reversible binding to the AChE molecule, prevent the subsequent irreversible binding of organophosphorus AChE inhibitors. Some data suggested that peripherally acting reversible AChE inhibitor pyridostigmine could have some protective effects against organophosphate nerve agents (Xia et al., 1981; Walday et al., 1993). On this presumption, many allied soldiers in the Persian Gulf War were given this peripherally acting antidote (Keeler et al., 1991). There is even some evidence that the central effects of OP agents may actually be potentiated by pre-treatment with pyridostigmine (Amourette et al., 2009).

It has therefore been proposed that newly developed centrally acting reversible anticholinesterases that are used for the treatment of AD may be a better prophylactic choice against OP intoxication than pyridostigmine. However, more recent research indicates that the ability of pyridostigmine to antagonize AChE toxicity in the central nervous system is limited (Leadbeater et al., 1985; Miller et al., 1993) and even that the central effects of irreversible AChE inhibitors may actually be potentiated by the pre-treatment with peripherally acting AChE inhibitors, supposedly by reducing the binding of the toxin to peripheral cholinesterases and thus increasing the availability of irreversible AChE inhibitor in the central nervous system (Lallement et al., 2001). Acute pre-treatment with oral donepezil, with and without scopolamine, decreased the hypothermic, hypokinetic, and diarrhoea-inducing effects of DFP, while after subchronic treatment some protection was observed even when the last treatment of the chronic donepezil protocol was given 24 h before the DFP injection (Janowsky et al., 2004, 2005). The objective of the current research was to further evaluate the prophylactic potential of the acute pre-treatment with reversible anticholinesterase inhibitor galantamine (GAL) against the effects of DFP. GAL is a novel agent that is clinically used for the treatment of AD (Maelicke, 2000; Albuquerque et al., 2001; Farlow, 2003).

The aim of the present study was to evaluate the possible protective effects of acute treatment with GAL on the signs of cholinergic toxic syndrome, such as hypothermia, muscular fasciculations, oral dyskinesia and decreased locomotor performance induced by DFP. These behavioural signs were selected on the basis of the reports in the literature indicating that central cholinergic and, in part, dopaminergic mechanisms are involved in anticholinesterase-induced hypothermia, decreased locomotor performance and oral dyskinesia, while fasciculation is a valid index of peripheral cholinergic activation (Ogura et al., 2001). In addition, the central effects of these two anticholinesterases separately and in combination were assessed by histochemical acetylcholinesterase staining, and by *in situ* hybridization of the immediate early gene c-*fos* mRNA in the brain.

Material and Methods

Animals

Adult male Wistar rats (220–300 g, The Wistar Institute, Philadelphia, PA) were used in our experiments. They were housed in groups of four in polycarbonate cages under standard housing conditions (22–24 °C, 50% humidity) and a 12-h light/dark cycle (light on from 07.00 AM to 19.00 PM) with free access to food pellets and tap water. The rats were handled according to the European Community Council Directive of 24th November 1986 (86/609/EEC) and National Veterinary Institute Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering. At the end of the experiment, the rats were sacrificed by decapitation under CO_2 anaesthesia.

Drugs

Diisopropylfluorophosphate (DFP, Sigma, St. Louis, MO) was dissolved in 0.9% saline (SAL) and injected subcutaneously (*s.c.*) in the dorsal neck region in a volume of 1 ml/kg. Galantamine (GAL, E. Merck, Darmstadt, Germany) was also dissolved in 0.9% isotonic SAL and administered intraperitoneally (*i.p.*) in a volume of 1 ml/kg. The doses and timing of GAL and DFP injections was inferred from the literature (Saghafi et al., 2010). It is known that the time to reach the peak plasma concentration (t_{max}) after single oral administration of GAL is estimated to be 0.33 h (Mannens et al., 2002), while plasma levels after single intravenous administration of GAL (1.25–2.5 mg/kg) declined bi- or triphasically, with an elimination half-life of 3.5 h in male, and 5.1 h in female rats (van Beijsterveldt et al., 2004). The plasma clearance (Cl) averaged 1.9 l/kg/h (male rats) and 0.9 l/kg/h (female rats), and the volume of distribution was about 5 l/kg for rats (van Beijsterveldt et al., 2004). Following oral administration (2.5–10 mg/kg), GAL was rapidly absorbed, with the absolute oral bioavailability of 77 % (Monbaliu et al., 2003; van Beijsterveldt et al., 2004). Distribution studies after oral administration of 3H-galantamine showed an almost immediate equilibrium between plasma and tissues (van Beijsterveldt et al., 2004).

Experimental protocol

Two experiments were performed to further explore the prophylactic potential of GAL against the effects of

DFP in the brain. The aim of the first experiment was to evaluate the effect of GAL on the symptoms of central and peripheral origin, including: hypothermia, oral dyskinesia, muscular fasciculation, and locomotor performance (tested on the activity wheel and high bar), and signs of cholinergic intoxication with DFP using recognized behavioural observation methods. The aim of the second experiment was to assess the effects of these two anti-cholinesterases on brain AChE activity, by semi-quantitative densitometric analysis of brain sections stained histochemically for AChE activity and of the intensity of *in situ* hybridization autoradiographic signal of immediate early response gene (IEG) c-*fos* mRNA, used here as a marker of enhanced neuronal activity (Gupta et al., 2000). Eight groups consisting of six adult male Wistar rats were used. They were treated in two separate experiments to explore the prophylactic potential of GAL against the central and peripheral cholinergic effects of DFP in the brain as follows: in the first experiment we evaluated whether the pre-treatment with GAL (2 mg/kg *i.p.*) could affect the onset of hypothermia, muscular fasciculations, oral dyskinesia, locomotor performance at the activity wheel and balance at the high bar tests, induced by DFP (1 mg/kg *s.c.*).

In this experiment three groups of animals (N = 6 each) were treated as follows: the control group was treated with 0.9% SAL followed after 1 h by second injection of SAL, while the experimental groups were pre-treated with vehicle (SAL+DFP) or GAL (GAL+DFP) 1 h prior to being treated with DFP. In the second experiment we evaluated whether the pre-treatment with GAL could affect the induction of c-*fos* and AChE inhibition induced by DFP. In this experiment five groups of animals (N = 6 each) were treated as follows: the first group (SAL+SAL) of animals was treated with SAL and after 1 h with SAL, and sacrificed 1 h after the second injection. The second group (SAL+DFP) was treated with SAL and after 1 h with DFP (1 mg/kg *s.c.*), and sacrificed 1 h after the second injection. The third group (GAL) was treated with GAL (2 mg/kg *i.p.*) only and sacrificed 1 h after the injection. The fourth group (GAL+SAL) was treated with GAL and after 1 h with SAL, and sacrificed 1 h after the second injection. The fifth group (GAL+DFP) was treated with GAL (2 mg/kg *i.p.*) and after 1 h with DFP (1 mg/kg *s.c.*), and sacrificed 1 h after the second injection.

Implantation of a thermistor probe and temperature measurements

The electrical cord between the small atraumatic thermistor probe and the digital thermometer (Physiotemp, Clifton, NJ) was cut in the vicinity of the probe and both sides were fitted with a connector. The probes were then implanted subcutaneously in the interscapular region so that the connector was sticking out of the skin. Before the experiment, the setup was verified for accuracy of temperature measurements. At the beginning of the experiment, the probes were again connected with the digital thermometer. Baseline temperature was manually recorded approximately 1 min before the pre-treatment injection. The effects of the treatments on body temperature were then manually recorded in 10 min intervals for 2 h in all groups except for the group of animals treated only with GAL.

Behavioural observation

During the experiment, we evaluated the presence/absence of peripheral (muscle fasciculations) and central signs of cholinergic hyperactivity (abnormal perioral movements) characterized by the appearance of: chewing movements, jaw opening and closing, tongue protrusions and jaw tremors. The animals were placed individually in small plastic observation cages (20 × 20 × 15 cm) and observed during the experiment. Intensity of muscle fasciculations and abnormal perioral movements was graded in arbitrary units by a trained observer who was unaware of the treatment protocols. The intensity of abnormal perioral movements was graded as follows: 0 – absent or borderline, 1 – one to three events per hour, 2 – four to six events per hour, 3 – more than six events per hour. The intensity of fasciculation was classified into three grades as follows: 0 – none or borderline, 1 – mild and intermittent fasciculation on some of the digits, 2 – moderate fasciculation on limbs and digits, 3 – severe fasciculation observed all over the body.

Just before the end of the experiment (approximately 1 min after the last recording of the temperature), the rats were placed on a turning wheel (diameter 3.5 cm, 17 turns per min) for 1 min. We recorded the time before the rat fell from the wheel. Each animal was placed on the wheel for three times and the average duration of the time before the rat fell from the wheel was calculated. Approximately 1 min after the wheel experiment, animals were placed on a wooden elevated bar (2.5 cm in diameter placed horizontally 15 cm above the rubber floor) for 1 min. The time before the rat fell to the ground was recorded. Each rat was placed on the elevated bar three times and the average duration of the time before the rat lost its balance was calculated.

Preparation of brain cryo-sections

The animals in all experimental groups were sacrificed by decapitation under CO_2 anaesthesia. Brains were rapidly removed and quickly frozen on dry-ice powder, wrapped in Parafilm to prevent desiccation and stored at –80 °C. Before cutting, the brains were allowed to equilibrate with the temperature of the cryostat chamber that had been adjusted to –20 °C. Coronal sections (10 μm) were cut at three evenly spaced rostro-caudal levels through the striatum (approx. between +1.7 mm to –0.3 mm from bregma) and the rostral part of hippocampus (approx. between –2.6 mm to –3.6 mm from bregma). Each section was thaw-mounted onto a RNAse-free glass slide coated with 0.01% solution of (poly)L-lysine in dimethylpyrocarbonate. The sections were vacuum-packed together with a small amount of silica gel and stored at -80 °C until further processing.

Oligonucleotide probe

We designed an oligodeoxyribonucleotide 'antisense' probe (45 bases long) complementary to the reported sequences of 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).

In situ hybridization histochemistry

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

Acetylcholinesterase staining

The AChE activity staining procedure was done according to a histochemical reaction described by Koelle and Friedenwald (1949). Briefly, the sections described above were brought to room temperature, 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. Following incubation, sections were again rinsed (4 × 3 min) in distilled water and then reacted (1 min, constant agitation) in a freshly prepared solution of 1% sodium sulphide (pH = 7.5). The staining reaction was terminated by rinses in distilled water (4 × 3 min). Finally, the sections were dehydrated through a graded ethanol series (70, 90, 100%; 4 min in each ethanol solution) followed by xylene (4 min) and cover-slipped with Canada balsam.

Visualization of c-fos mRNA level and of AChE activity

The autoradiograms of the *c-fos* mRNA level and of the cover-slipped sections stained for AChE activity were trans-illuminated in the visual field of a black and white digital camera (DAGE MTI, CCD72) connected to the MCID, M5 image analyser (Imaging Research, Inc. (Canada), St. Catherines, 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. The mean ROD values were first calculated for individual animals, by averaging ROD values pertaining to regions of interest of both hemispheres. The mean ROD values measured for the regions of interest of different treatment groups were then calculated for statistical analysis.

Statistical analysis

The effects of GAL on DFP-induced hypothermia, muscular fasciculations, oral dyskinesia, locomotor performance and balance on the high bar tests were statistically assessed by calculation for each experimental group. Average and standard deviations and differences between the groups were compared using the Student's *t*-test. The effect of pre-treatment with GAL on AChE activity and on the expression of IEGs c-*fos* mRNA 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

Effects of GAL on the changes in temperature and locomotor activity induced by DFP

The effects of pre-treatment with GAL on temperature changes induced by DFP are shown in Fig. 1. Decreased temperature was observed in the group of animals pre-treated with GAL, even as early as 50 min after GAL injection. At the 70 min time point, GAL it-

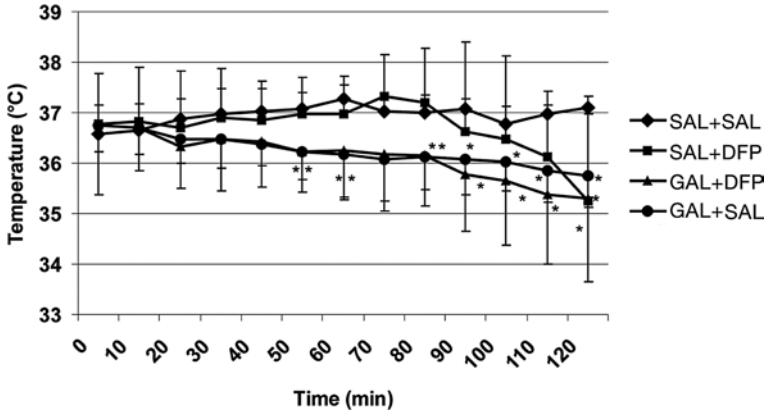

Fig. 1. Changes in temperature induced by DFP following pre-treatment with GAL. Rats were pre-treated with vehicle or GAL 60 min prior to being treated with vehicle or DFP. Temperatures were then recorded every 10 min for 2 h. The values represent the mean ± standard deviation (SD), N = 6 for all groups, temperature (°C). *Significantly different ($P < 0.01$) from the control group according to *t*-test.

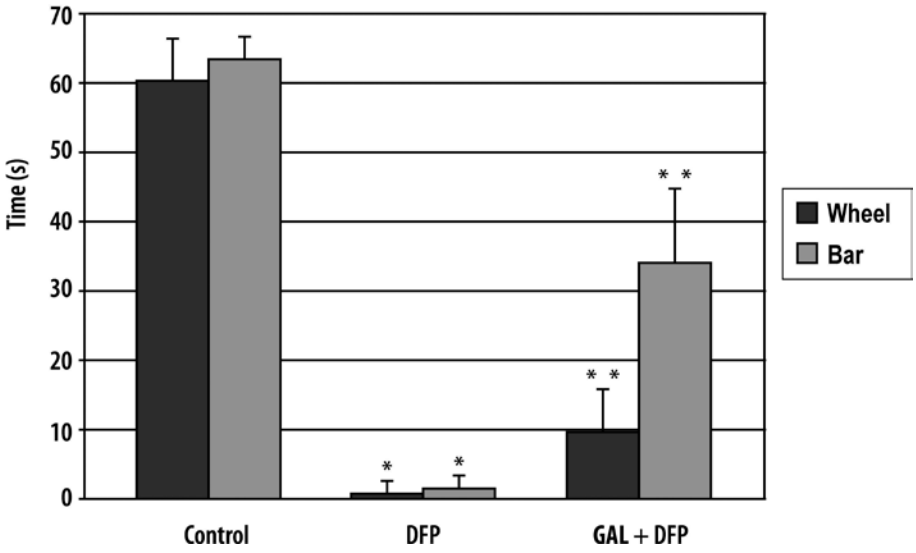

Fig. 2. Changes in locomotor activity induced by DFP following pre-treatment with GAL. Rats were pre-treated with vehicle or GAL 60 min prior to being treated with vehicle or DFP. Approximately 1 h post-DFP or vehicle injection, the rats were placed on the turning wheel (black columns) and thereafter on the balance bar (gray columns). Every animal was placed on the wheel three times and the average time for the animal was calculated. The values represent the mean ± standard deviation (SD). Time (s), N = 6 for all groups. Group codes: Control = vehicle–vehicle; DFP = vehicle–DFP; GAL+DFP = GAL–DFP. *Significantly different ($P < 0.05$) from the control group according to *t*-test. **Significantly different from the DFP group according to *t*-test.

self induced a decrease in temperature that was greater than that induced by DFP. When DFP was added, a greater hypothermic effect was observed. At the 2 h time point, a different pattern emerged: the temperature of the animals treated with DFP alone dropped to the level observed in the animals pre-treated with GAL. There were significant differences in temperature between animals receiving DFP and control animals receiving vehicle 1 h after the injections. Thus, GAL itself exhibits early hypothermia but protects against the late-developing hypothermia induced by DFP. There were no changes in the animals treated with vehicle alone during the observational time. In the animals receiving DFP after injection of vehicle, the temperature decreased after 80 min. However, in the animals that underwent pre-treatment with GAL, the temperature decreased even earlier.

After treatment with DFP, the locomotor activity was substantially decreased, as indicated by the wheel and balance bar test (Fig. 2). In the groups of animals pre-treated with GAL, the locomotor activity when running on the wheel was decreased in comparison to the control, but still statistically significantly higher than in the DFP group ($P < 0.05$). In this group of animals, the locomotor activity on the balance bar was not significantly different from the control group ($P < 0.05$). Decreased locomotor activity was observed in the subgroup of animals pre-treated with GAL 60 min before DFP exposure. Even further decline in locomotor activity 60 min after DFP exposure was observed in the subgroup of animals exposed to DFP alone. Pre-treatment with GAL enabled animals, even after exposure to DFP, to perform on the balance bar almost as long as animals treated with vehicle only and significantly longer than animals exposed to the DFP toxicity alone. However, the difference in the time animals spent on the rotating wheel between the subgroup exposed to DFP alone and the subgroup pre-treated with GAL and exposed to DFP was small but significant (Fig. 2).

Effects of GAL on muscle fasciculation and oral dyskinesia induced by DFP

Muscle fasciculation and oral dyskinesia was observed in the group of animals treated with DFP. The severity of fasciculations 60 min after DFP exposure was two times higher in the subgroup of animals exposed to DFP only compared to that of the animals pre-treated with GAL and then exposed to DFP. The appearance of oral dyskinesia was also about two times higher in the subgroup of animals exposed to DFP only than in animals pre-treated with GAL 60 min prior to DFP exposure (Fig. 3). These results suggest that the GAL pre-treatments counteracted all of the effects of DFP that were measured.

The effects of GAL on DFP induction of c-fos mRNA

The results of semi-quantitative analysis of c-*fos* mRNA levels are presented in Fig. 4. In rats receiving saline pre-treatment followed by DFP, a significant up-regulation of cortical c-*fos* mRNA signal in the cerebral cortex was found 1 h after the injection of DFP, as compared to the saline-only treated control group (Fig. 4). GAL by itself did not significantly up-regulate cortical c-*fos* mRNA signal 1 or 2 h after the injection of the

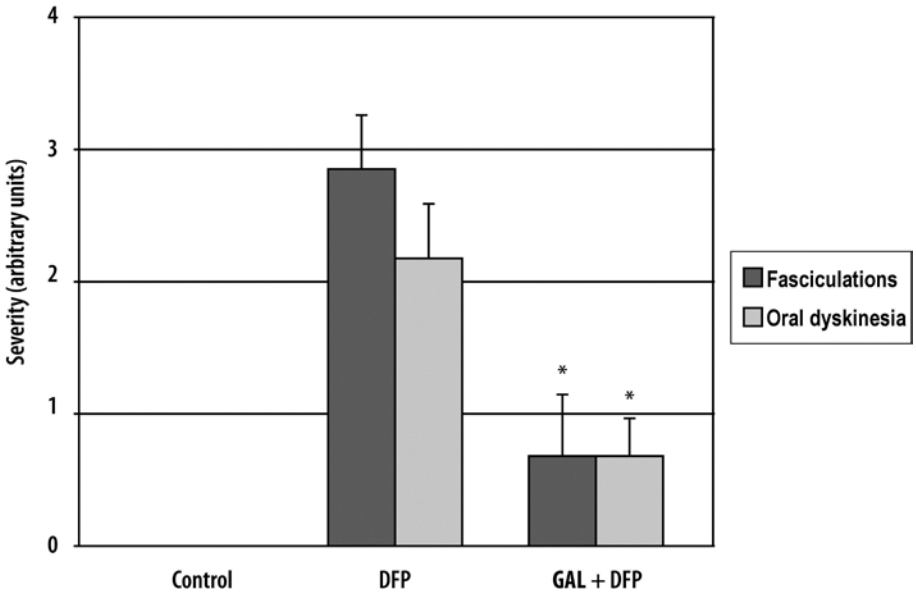

Fig. 3. Intensity of muscle fasciculation and oral dyskinesia induced by DFP following pre-treatment with GAL. Rats were pre-treated with vehicle or GAL (2 mg/kg) 60 min prior to being treated with vehicle or DFP. At 1 h post DFP or vehicle injection, the rats were observed for appearance of muscle fasciculations (black columns) and oral dyskinesia (gray columns). The values represent the mean ± standard deviation (SD). Time (s), N = 6 for all groups. Group codes: Control = vehicle–vehicle; DFP = vehicle–DFP; GAL+DFP = GAL–DFP. *Significantly different ($P < 0.05$) from the DFP group according to *t*-test.

drug. On the contrary, GAL significantly prevented up-regulation of the cortical c-*fos* mRNA signal induced by DFP (Fig. 4). GAL also attenuated DFP-induced hippocampal up-regulation of c-*fos* mRNA (Fig. 4).

Acetylcholinesterase staining

The results of semi-quantitative analysis of AChE histochemical staining are presented in Fig. 4. In this experiment the AChE activity visualized by AChE staining showed almost complete inhibition of AChE staining in the coronal hippocampal (quantified changes are shown in *gyrus dentatus*, CA regions of hippocampus) and striatal brain sections of the animals receiving vehicle and DFP treatment (SAL+DFP group), as compared to vehicle-only treated controls (SAL+SAL group) (Fig. 4). The inhibition of AChE by GAL was reverible, since GAL by itself significantly reduced hippocampal and striatal AChE staining (group GAL) 1 h after GAL treatment, while after 2 h (GAL+SAL group) AChE reverted to the intensity observed in the control (SAL+SAL group). GAL significantly prevented the subtotal inhibition of striatal and hippocampal AChE staining induced by DFP (GAL+DFP group) (Fig. 4).

Discussion

Recent studies indicate that reversible anticholinesterases could be able to block the effects of subsequently administered toxic doses of irreversible AChE inhibitors. In our experiment, DFP induced rapidly progressing hypothermia. The dose of GAL was selected according to reports in the literature that showed a modest hypothermic, hypoactivity and diarrhoea effect and protection against the more severe hypothermic effect of DFP (Janowsky et al., 2004; Albuquerque et al., 2006). Concerning the effect of GAL on DFP-induced hypothermia, our results somewhat differ from the results published in the literature, since in our hands GAL did not decrease the level of hypothermia induced by DFP (Janowsky et al., 2004). However, we probably did not observe the maximal hypothermic response for the employed dose of DFP, since the maximal hypothermia after DFP was described to occur at four hours after DFP injection (Janowsky et al., 2005), while our experiment ended one hour after the injection of DFP.

On the other hand, we found that GAL significantly reduced the relative rate of decrease of body temperature during this period. It thus appears that the methodological limitation of the present study may have obscured the protective effect of GAL against the maximal hypothermic effect of DFP described in previous research (Janowsky et al., 2005). Namely, our intention was to use c-*fos* mRNA as the marker of cholinergic hyperactivation of the brain, while in our experience, four hours after the treatment with DFP the transiently increased levels of c-*fos* mRNA may well return to the baseline. The second reason for this discrepancy may be attributed to the difference in the location of temperature measurements, *i.e*. subcutaneous interscapular *vs*. rectal. In our experiment DFP induced intensive perioral behaviour, as described above. The induction of behavioural hypoactivity with anticholinesterases is also considered to be of central origin. In accordance with previous observations (Janowsky et al., 2004), we also observed almost complete reduction of locomotor activity in the subgroup of animals treated with DFP. In our

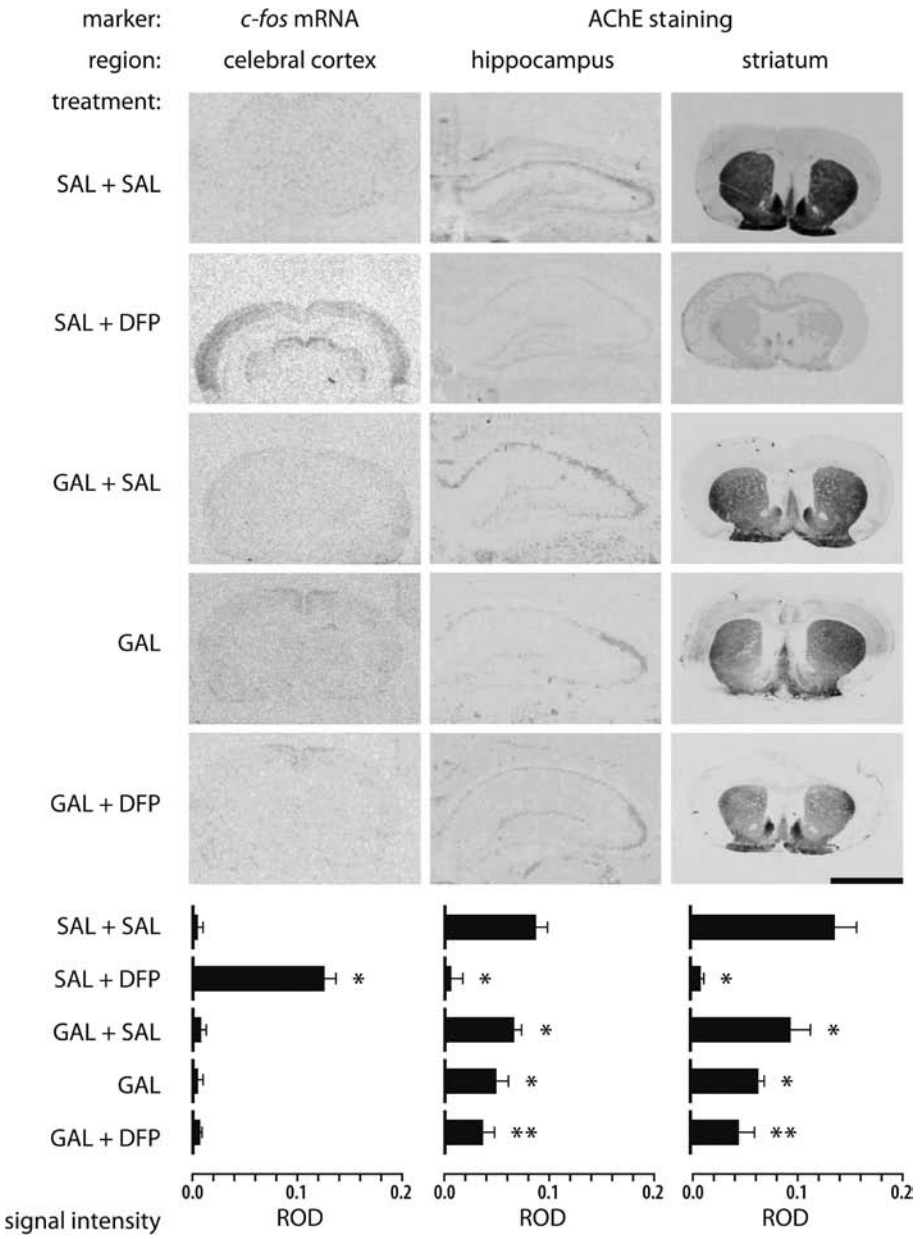

Fig. 4. The effect of GAL on DFP-induced c-*fos* mRNA expression and AChE activity in the brain. The panel represents relative optical density images of the c-*fos* mRNA and AChE activity of coronal brain sections. The effects of DFP on the c-*fos* mRNA induction and inhibition of AChE activity was reduced following pre-treatment with GAL. Note that in the SAL+DFP group, DFP significantly reduced striatal/hippocampal AChE staining and increased the levels of c-*fos* mRNA in cerebral cortex. In the GAL+DFP group, GAL completely prevented the DFP-induced up-regulation of c-*fos* mRNA in the cerebral cortex. GAL also significantly attenuated the inhibition of striatal/hippocampal AChE induced by DFP. GAL by itself significantly reduced striatal/hippocampal AChE staining (group GAL, 1 h after GAL treatment), while after 2 h (group GAL+SAL) the AChE staining intensity reverted toward higher levels, although it was still significantly lower than the intensity observed in the control SAL+SAL group. GAL by itself did not induce any changes in the level of c-*fos* mRNA. Statistics: ANOVA followed by Tukey's HSD Multiple-Comparison test, N = 6 for all groups, $P < 0.05$. Calibration bars represent 5 mm. ROD – relative optical density; mean ± standard deviation (SD). *Significantly different from the SAL+SAL group. **Significantly different from the SAL+DFP group.

experiment we recorded the time before the rat fell from the turning wheel and its capability to maintain balance on the elevated bar. The DFP-treated animals were seriously incapacitated in both tests.

After treatment with DFP, the locomotor activity was substantially decreased, as indicated by the wheel and balance bar test (Fig. 2). In the groups of animals pre-treated with GAL, locomotor activity when running on the wheel was decreased in comparison with the control, but still statistically significantly higher than in the DFP group ($P < 0.05$). In this group of animals, locomotor activity on the balance bar was not significantly different from the control group ($P < 0.05$). However, decreased locomotor activity was also observed in the sub-

group of animals pre-treated with GAL even before DFP exposure, indicating that AChE inhibition could be involved in the observed locomotor impairment. Indeed, even further decline in locomotor activity after DFP exposure was observed in the subgroup of animals exposed to DFP after GAL exposure or after DFP exposure alone. However, the difference in the time animals spent on the rotating wheel between the subgroup exposed to DFP alone and the subgroup pre-treated with GAL and exposed to DFP was small but significant.

It is not clear whether the observed locomotor impairment is associated with mechanisms related exclusively to the central nervous system or also to the mechanisms involved in the activation of neuromuscular junction. In the present study GAL by itself induced a mild degree of acute peripheral/central cholinergic hyperactivity, as revealed by occasional muscle fasciculations, abnormal perioral movements, slowly progressing hypothermia, and behavioural inhibition. Anticholinesterases and other cholinomimetic drugs are known to induce changes in perioral behaviour in rodents that are of central origin (Ogura et al., 2001). Cholinergic stimulation of ventrolateral striatum namely produces dose-dependent induction of mouth movements that are not directed toward any stimulus and are characterized by chewing movements, jaw opening and closing, tongue protrusions and jaw tremors (Kelley et al., 1989).

In the present experiment, treatment with GAL resulted in a modest and reversible decrease of AChE histochemical staining by the Koelle method. Reversible inhibitors, such as GAL, are usually washed out during this staining procedure, which results in underestimation of the degree of *in situ* inhibition. Even so, we found a significant reduction of AChE staining at 60 min, but at 120 min AChE reverted to the intensity observed in the control. It could be speculated that the observed reduction of AChE staining may correlate with a peak concentration of GAL in the rat brain that occurs approximately 60 min after acute injection of 2 mg/kg GAL (Liang and Tang, 2004). In rats, DFP toxicity is also associated with the induction of c-*fos* mRNAs in the cerebral cortex (Saghafi et al., 2010). We therefore speculated that the changes of c-*fos* mRNA levels in the brain may also serve as a good marker for evaluation of the proposed prophylactic mechanisms of GAL against DFP toxicity in the brain. The absence of behavioural seizure activity in our experiments is in agreement with the data from the literature indicating that DFP by itself rarely induces overt motor convulsions and status epilepticus (McDonough and Shih, 1997).

It is generally assumed that increased cholinergic activity in the brain could induce the first phase of seizures, whereas sustained seizures leading to status epilepticus are linked to increased glutamatergic activity (McDonough and Shih, 1993; Carpentier et al, 2001). In any case, in our experimental paradigm *c-fos* mRNA up-regulation by DFP represented a valuable marker for the evaluation of DFP toxicity that corroborated the prophylactic effects of GAL in the brain.

GAL significantly reduced the DFP-induced inhibition of AChE and completely prevented the up-regulation of *c-fos* mRNA in the brain. Since the massive induction of *c-fos* mRNA is indicative of pathologic hyperactivation of the affected brain regions, which is often also associated with the onset of excitotoxic neurodegenerative changes, we assume that prophylaxis with GAL could also prevent some of the neurodegenerative changes induced by DFP. Although it may seem unusual that two centrally acting anticholinesterases do not have additive pathophysiologic effects, it should be remembered that DFP by itself induced almost total inhibition of AChE (i.e. maximal effect). Due to the irreversibility of AChE inactivation, the only physiological mechanism for restoring AChE activity depends on the new synthesis of AChE.

By comparison, the data from the literature suggest that the dose of GAL used in our experiment induced only partial non-competitive and reversible inhibition of AChE that protected the enzyme against the irreversible inhibition by DFP. The comparatively low increase of the intensity of central cholinergic activity by this dose of GAL was indicated by the absence of *c-fos* mRNA up-regulation. This is in agreement with clinical experience with GAL for the treatment of cognitive deficits in AD that showed a low propensity of the drug for the induction of central side effects. The prophylactic effects of GAL on AChE inhibition and c-*fos* mRNA induction revealed by the present study are similar to the effects of donepezil, another anticholinesterase drug currently used for the treatment of AD (Saghafi et al., 2010). GAL is a novel agent that is clinically used for the treatment of AD with a dual mode of action, since it also modulates the activity of nicotinic acetylcholine receptors (Maelicke 2000; Albuquerque et al., 2001; Farlow 2003). At present, there are still insufficient experimental data demonstrating how such pharmacological profile might eventually be favourable in relation to prophylactic effects of other reversible anticholinesterases, such as donepezil. Our data only confirm that at least in the animal model, the pre-treatment with GAL prevents the maximal AChE inhibition induced by DFP, indicating its potential usefulness for the prophylaxis against peripheral and central signs of toxicity induced by irreversible OP AChE inhibitors.

Acknowledgement

We thank Mrs. Helena Kupšek for technical assistance.

References

Albuquerque, E. X., Santos, M. D., Alkondon, M., Pereira, E. F, Maelicke, A. (2001) Modulation of nicotinic receptor activity in the central nervous system: a novel approach to the treatment of Alzheimer disease. *Alzheimer Dis. Assoc. Disord*. **15** (**Suppl 1)**, S19-S25.

Albuquerque, E. X., Pereira, E. F., Aracava, Y., Fawcett, W. P., Oliveira, M., Randall, W. R., Hamilton, T. A., Kan, R. K.,

Romano, J. A. Jr., Adler, M. (2006) Effective countermeasure against poisoning by organophosphorus insecticides and nerve agents. *Proc. Natl. Acad. Sci. USA* **103**, 13263-13264.

Amourette, C., Lamproglou, I., Barbier, L., Fauquette, W., Zoppe, A., Viret, R., Diserbo, M. (2009) Gulf War illness: Effects of repeated stress and pyridostigmine treatment on blood-brain barrier permeability and cholinesterase activity in rat brain. *Behav. Brain Res*. **203,** 207-214.

Carpentier, P., Foquin, A., Kamenka, J. M., Rondouin, G., Lerner-Natoli, M., de Groot, D. M., Lallement, G. (2001) Effects of thienylphencyclidine (TCP) on seizure activity and brain damage produced by soman in guinea-pigs: ECoG correlates of neurotoxicity. *Neurotoxicology* **22**, 13-28.

Farlow, M. (2003) Clinical pharmacokinetics of galantamine. *Clin Pharmacokinet*. **42**, 1383-1392.

Gordon, C. J., Herr, D. W., Gennings, C., Graff, J. E., McMurray, M., Stork, L., Coffey, T., Hamm, A., Mack, C. M. (2006) Thermoregulatory response to an organophosphate and carbamate insecticide mixture: testing the assumption of dose-additivity. *Toxicology* **5**, 1-13.

Gupta, R. P., Damodaran, T. V., Abou-Donia, M. B. (2000) C-fos mRNA induction in the central and peripheral nervous systems of diisopropyl phosphorofluoridate (DFP)-treated hens. *Neurochem. Res*. **25**, 27-34.

Janowsky, D. S. (2002) Central anticholinergics to treat nerve-agent poisoning. *Lancet* **19**, 265-266.

Janowsky, D. S., Davis, J. M., Overstreet, D. H. (2004) Antagonism of anticholinesterase (DFP) toxicity by donepezil plus scopolamine: a preliminary study. *Pharmacol. Biochem*. *Behav*. **77**, 337-343.

Janowsky, D. S., Davis, J. M., Overstreet, D. H. (2005) Anticholinesterase (DFP) toxicity antagonism by chronic donepezil: A potential nerve agent treatment. *Pharmacol. Biochem. Behav.* **8**, 917-922.

Keeler, J. R., Hurst, G. G., Dunn, M. A. (1991) Pyridostigmine used as a nerve agent pre-treatment under wartime conditions. *JAMA* **266**, 693-695.

Kelley, A. E., Bakshi, V. P., Delfs, J. M., Lang, C. G. (1989) Cholinergic stimulation of the ventrolateral striatum elicits mouth movements in rats: pharmacological and regional specificity. *Psychopharmacology (Berl.)* **99**, 542-549.

Koelle G. G., Friendenwald, J. S. (1949) A histochemical method for localizing acetylcholinesterase activity. *Exp. Biol. Med*. **70**, 617-622.

Lallement, G., Foquin, A., Dorandeu, F., Baubichon, D., Carpentier, P. (2001) Subchronic administration of various pre-treatments of nerve gas poisoning: II. Compared efficacy against soman toxicity. *Drug Chem. Toxicol*. **24**, 165-180.

Leadbeater, L., Inns, R. H., Rylands, J. M. (1985) Treatment of poisoning by soman. *Fundam*. *Appl*. *Toxicol*. **5**, 225-231.

Liang,Y. Q., Tang, X. C. (2004) Comparative effects of huperzine A, donepezil and rivastigmine on cortical acetylcholine level and acetylcholinesterase activity in rats. *Neurosci. Lett*. **6**, 56-59.

Mannens, G. S., Snel, C. A., Hendrickx, J., Verhaeghe, T., Le Jeune, L., Bode, W., van Beijsterveldt, L., Lavrijsen, K., Leempoels, J., Van Osselaer, N., Van Peer, A., Meuldermans, W. (2002) The metabolism and excretion of galantamine in rats, dogs, and humans. *Drug Metab Dispos*. **30**, 553-563.

Maelicke, A. (2000) Allosteric modulation of nicotinic receptors as a treatment strategy for Alzheimer's disease. *Dement. Geriatr. Cogn. Disord.* **11** (**Suppl 1**), 11-18.

McDonough, J. H. Jr., Shih, T. M. (1993) Pharmacological modulation of soman-induced seizures. *Neurosci. Biobehav. Rev*. **17**, 203-215.

McDonough, J. H. Jr., Shih, T. M. (1997) Neuropharmacological mechanisms of nerve agent-induced seizure and neuropathology. *Neurosci. Biobehav. Rev*. **21**, 559-579.

Miller, S. A., Blick, D. W., Kerenyi, S. Z., Murphy, M. R. (1993) Efficacy of physostigmine as a pre-treatment for organophosphate poisoning. *Pharmacol. Biochem. Behav*. 442, 343-347.

Monbaliu, J., Verhaeghe, T., Willems, B., Bode, W., Lavrijsen, K., Meuldermans, W. (2003) Pharmacokinetics of galantamine, a cholinesterase inhibitor, in several animal species. *Arzneimittelforschung* **53,** 486-495.

Ogura. H., Kosasa, T., Kuriya, Y., Yamanishi, Y. (2001) Central and peripheral activity of cholinesterase inhibitors as revealed by yawning and fasciculation in rats. *Eur. J. Pharmacol.* **415**, 157-164.

Saghafi, M. M., Pregelj, P., Zivin, M. (2010) Donepezil inhibits diisopropylfluorophosphate-induced seizures and up-regulation of synaptotagmin 4 mRNA. *Folia Biol. (Praha)* **56**, 256-262.

Solberg. Y., Belkin, M. (1997) The role of excitotoxicity in organophosphorous nerve agents central poisoning. *Trends Pharmacol. Sci*. **18**, 183-185.

Taylor, P. (1996) Anticholinesterase agents. In: *The Pharmacological Basis of Therapeutics,* eds. Hardman, J. G., Limbird, L. E., Molinoff, P. B., Luddon, R. W., Gilman, A. G., pp. 131-149, McGraw-Hill, New York.

van Beijsterveldt, L., Geerts, R., Verhaeghe, T., Willems, B., Bode, W., Lavrijsen, K., Meuldermans, W. (2004) Pharmacokinetics and tissue distribution of galantamine and galantamine-related radioactivity after single intravenous and oral administration in the rat. *Arzneimittelforschung* **54,** 85-94.

Walday, P., Aas, P., Haider, T., Fonnum, F. (1993) Effect of pyridostigmine pre-treatment, HI-6 and Toxogonin treatment on rat tracheal smooth muscle response to cholinergic stimulation after organophosphorus inhalation exposure. *Arch. Toxicol.* **67**, 212-219.

Watson, A., Opresko, D., Young, R., Hauschild, V., King, J., Bakshi, K. (2009) Organophosphate nerve agents. In: *Handbook of Toxicology of Chemical Warfare Agents*, ed. Gupta, R. C., pp. 43-67, Academic Press/Elsevier, London.

Xia, D. Y., Wang, L. X., Pei, S. Q. (1981) The inhibition and protection of cholinesterase by physostigmine and pyridostigmine against Soman poisoning in vivo. *Fundam. Appl. Toxicol*. **1**, 217-221.

Zivin, M., Milatovic, D., Dettbarn, W. D. (1999) Nitrone spin trapping compound N-tert-butyl-alpha-phenylnitrone prevents seizures induced by anticholinesterases. *Brain Res*. **850**, 63-72.