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| Article CopyRight | Springer-Verlag<br>(This will be the copyright line in the final PDF) | |
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|---|---|---|
| Schedule | Received | 24 March 2009 |
| | Revised | |
| | Accepted | 27 July 2009 |




Abstract

It is generally believed that the cholinergic system plays an important role in normal cognitive functioning. Botulinum toxin is the most potent toxin of the peripheral cholinergic system and today it is used in the treatment of a variety of neurological disorders. However, it is surprising that its effect on cognitive processes has been investigated in only two publications. Short-term effects of the central application of botulinum toxin (BTX) type B have been associated with cognitive impairment in animals, while results with type A are ambiguous. In the present study, we have investigated the duration of memory impairment after an intracerebroventricular administration of BTX-A in rats. Two experiments were performed, lasting 12 and

5 months, respectively. In both experiments, the same dose of BTX-A was applied (2 U/kg) and the Morris water maze test was used in the assessment of memory performance. Results show that a single icv injection of a small dose of BTX-A significantly impairs the water maze performance. In both experiments, impairment was apparently of a slow onset and long lasting (up to 12 months). The length and pattern of attenuation suggest development of dementia-like deficits. In addition to providing a potentially new experimental model of memory impairment, these results question the idea of an intracranial application of BTX in the treatment of CNS disorders.

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|---|---|
| Keywords (separated by '-') | Botulinum toxin - Botulinum toxin type A, cognitive impairment - Intracerebroventricular application - Morris water maze, rat, dementia |
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J Neural Transm
DOI 10.1007/s00702-009-0285-y

MOVEMENT DISORDER - SHORT COMMUNICATION

# Single intracerebroventricular injection of botulinum toxin type A produces slow onset and long-term memory impairment in rats

**Zdravko Lacković · Veseljka Rebić · Peter F. Riederer**



Received: 24 March 2009 / Accepted: 27 July 2009


**Abstract** It is generally believed that the cholinergic system plays an important role in normal cognitive functioning. Botulinum toxin is the most potent toxin of the peripheral cholinergic system and today it is used in the treatment of a variety of neurological disorders. However, it is surprising that its effect on cognitive processes has been investigated in only two publications. Short-term effects of the central application of botulinum toxin (BTX) type B have been associated with cognitive impairment in animals, while results with type A are ambiguous. In the present study, we have investigated the duration of memory impairment after an intracerebroventricular administration of BTX-A in rats. Two experiments were performed, lasting 12 and 5 months, respectively. In both experiments, the same dose of BTX-A was applied (2 U/kg) and the Morris water maze test was used in the assessment of memory performance. Results show that a single icv injection of a small dose of BTX-A significantly impairs the water maze performance. In both experiments, impairment was apparently of a slow onset and long lasting (up to 12 months). The length and pattern of attenuation suggest development of dementia-like deficits. In addition to providing a potentially new experimental model of memory impairment, these results question the idea of an intracranial application of BTX in the treatment of CNS disorders.

**Keywords** Botulinum toxin · Botulinum toxin type A, cognitive impairment · Intracerebroventricular application · Morris water maze, rat, dementia

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## Introduction

The classic mechanism of the botulinum toxin (BTX)-type A action is cleavage of the synaptosomal associated protein of 25 kD (SNAP-25) which is required for vesicle docking and fusion with the plasma membrane. Accordingly, in the peripheral nervous system, BTX-A prevents acetylcholine release into the synaptic cleft (Kao et al. 1976; Bach-Rojecky et al. 2007).

Cholinergic activity is considered to play an important role in animal and in human memory function. A suppressed cholinergic function impairs, and an enhanced one improves learning and memory (for review see Gold 2003). Moreover, a selective loss of cholinergic neurons in the brain of Alzheimer's patients was already well documented decades ago (Davies and Maloney 1976). In line with that, cholinomimetics (cholinesterase inhibitors) are an accepted therapy in Alzheimer's disease (Birks 2006). Accordingly, if the outcome of a centrally administered BTX is similar to the effects on the peripheral cholinergic nerves, cognitive impairment could be expected. Contrary to such expectations, some authors recently suggested that a central administration of BTX A could be useful in the therapy of seizures and several other CNS disorders (Bozzi et al. 2006; Verderio et al. 2007). Therefore, further investigation of the centrally applied BTX-A seems increasingly important.

Here, we report that an icv application of small doses of BTX A impairs hippocampal-dependent memory in rats, tested with the standard version of the Morris water maze (MWM) task. This effect was long lasting, possibly permanent and developed slowly after the BTX A application.

## Methods

### Animals

A total of 32 male Wistar rats (Zagreb University School of Medicine, Zagreb, Croatia), 3-month-old and weighing 250–300 g at the beginning of treatment, were used in two experiments. Additional 23 rats were used for preliminary experiments in which dose dependency of BTX-A effects was examined.

Rats were housed in standard transparent plastic cages, in groups of four per cage, under standard animal room conditions (free access to food and water, 12 light:12 dark cycle, room temperature of 23°C). The experiments were carried out between 09.00 and 18.00 hours. The experiments were carried out according to the Croatian Act on Animal Welfare (Narodne novine 19/1999). The Principles of Laboratory Animal Care (NIH Publication No. 86-23, 1985) were followed. The experiments were approved by the Ethical Committee of the Zagreb University School of Medicine (permit No. 07-76/2005-43).

### Drugs

Botulinum toxin type A (BOTOX®, Allergan, Inc., Irvine, USA); containing per vial 100 U (~4.8 ng) of purified *Clostridium botulinum* toxin type A. BTX-A was reconstituted in a 0.9% saline solution. In preliminary experiments, BTX-A was used in doses of 0.5, 2 and 4 U/kg (Table 1). All three doses had similar effect on the decline of results in memory test, and only the dose of 2 U/kg was employed in all further experiments. Chloral hydrate (Sigma, St. Louis, MO, USA) was used for anaesthesia.

### Drug administration

Rats were randomly divided into groups (5–6 per group in Experiment 1, 10–11 per group in Experiment 2) and given general anaesthesia (chloral hydrate 300 mg/kg, i.p), followed by an icv injection of either saline as vehicle or selected BTX–saline solutions. An icv-injection of botulinum toxin type A was applied bilaterally into the left and the right lateral ventricle, according to the procedure described by Noble et al. (1967). Drug concentration and solution volume were adjusted according to the animal body weight, and a volume of 4 µL per 300 g body weight was administered (2 µL/ventricle). The same procedure was applied for dose-dependent pre-screening.

**Table 1** Time course of memory impairment for saline and BTX-A icv treated rats

| | Time after injection | | |
|---|---|---|---|
| | 15 days | 1 month | 3 months |
| Saline injected | | | |
| Mean | 60,75 | 91,875 | 71,375 |
| SE | 4,283 | 6,634 | 7,969 |
| *N* | 8 | 8 | 8 |
| BTX-A 0.5U | | | |
| Mean | 70,50 | 80,25 | 54,00 |
| SE | 4,787 | 6,909 | 9,958 |
| *N* | 4 | 4 | 4 |
| BTX-A 2U | | | |
| Mean | 56,00 | 76,75 | 48,5 |
| SE | 7,012 | 11,736 | 7,577 |
| *N* | 4 | 4 | 4 |
| BTX-A 4U | | | |
| Mean | 65,14 | 62,714 | 43,86 |
| SE | 7,731 | 8,909 | 5,152 |
| *N* | 7 | 7 | 7 |
| Kruskall-Wallis test | | | |
| Chi-square | 3,154 | 4,653 | **7,507*** |
| df | 3 | 3 | 3 |

BTX-A was injected in doses of 0.5, 2 and 4 U/kg. Memory impairment was assessed with the Morris water maze task. Results present time spend in the goal quadrant (max 120 s) during a probe trial for different periods of assessment (0.5, 1 and 3 months). The mean difference was tested using Kruskal–Wallis test (*$p < 0.05$)

### Acquisition of motor skills

Motor learning was assessed using the Rotarod test. The apparatus consisted of a horizontal rod (7 cm in diameter, 10 cm long), situated 30 cm above the landing platform. The animals were placed on the rod with their head directed against the direction of the rotation so they had to progress forward to maintain equilibrium. Duration of their holding without falling down was measured. The test was run up to 180 s.

The rotarod test used acclimation sessions and training sessions. The acclimation sessions were performed over two consecutive days (one session per day). The training





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sessions started on the day following the last acclimation session. Each (daily) training session consisted of four trials with an inter-trial interval of 10 min. The training sessions were performed over four consecutive days. This assessment was part of Experiment 1 and was applied 6 months after the drug administration.

### Spatial learning and memory tests

Spatial memory was evaluated in the hidden platform version of the MWM task. Rats were required to learn spatial location of a hidden platform in a square pool filled with clear water (25°C). A circular transparent platform (10 cm in diameter) was placed 1.5 cm below the water surface in the middle of the northeast quadrant. There were large high-contrast visual cues throughout the room and on the pool walls.

The following MWM procedure was the same for all experiments:

A rat was placed in the pool with its head facing the pool wall. A different starting point was used for each trail in pseudo-random order. If the rat did not find the platform within 120 s, it was gently guided by hand to the platform. When finding the platform, rats were allowed to remain there for 30 s. For each point of testing, rats were trained in this task for four consecutive days, performing block of three trials per day. An inter-trial interval of 5–10 min was given. On the fifth day, rats were given a retention probe test in which the platform was removed from the quadrant.

For dose-dependent pre-screening, following procedure matched one described for Experiment 1, except that testing was done within 3 months and the cut-off time in probe test was set at 120 s.

#### *Experiment 1*

No training in the task prior to drug administration was conducted. The MWM performance was evaluated at day 15 after the surgery, 1 month after the surgery and from that point once per month for 12 months in total. During a probe trail (cut-off time 90 s), the total time each rat swam in the former platform quadrant was recorded.

#### *Experiment 2*

Prior to drug administration all animals were trained in the MWM task for 10 sessions (10 consecutive days), each session consisting of three trials, following the general procedure described above. At the end of the training phase, all animals were able to find the platform within 10 s. After the training phase, animals were randomly assigned to the control (saline-infusion) group ($N = 11$) or the BTX-A-infusion group ($N = 10$), and an icv drug administration was conducted. Time period between the end of the training phase and the surgical procedure was 2 days. The MWM performance was evaluated 3, 10, 20, 30, 40, 60, 120, and 150 days after the BTX-A application. During a probe trail (cut-off time 60 s), the total time each rat swam in the former platform quadrant was recorded. A larger pool was used than in Experiment 1 (1.75 vs. 1.2 m), so the absolute magnitudes of time spent in the goal quadrant were not comparable between the experiments.

### Statistics

All values are expressed as the mean ± SE. In all three data sets (Rotarod test; MWM 1 and MWM 2), the Friedman test was used to analyse data in each group within the time course. Depending on data set characteristics, either the Kruskal–Wallis or the Mann–Whitney *U* test was used for comparison of the data among groups at the same time point. A $p$ value less than 0.05 was considered statistically significant.

## Results

### Dose-dependent pre-screening experiment

To investigate weather BTX-A affects memory and possible dose-dependency of such effects; a MWM task was assessed in four groups: (1) saline-treated; (2) BTX-A 0.5 U/kg; (3) BTX-A 2 U/kg and (4) BTX-A 4 U/kg. During the probe test, the time rats spent swimming in the goal quadrant was recorded at multiple points: 0.5, 1 and 3 months after the treatment. The Kruskal–Wallis test showed marginally significant difference in group performance for probe tests conducted 3 months post-treatment ($p = 0.51$), while differences on the probe tests conducted prior to that point were not significant (Table 1).

### Experiment 1

#### *Motor learning*

To examine whether the central administration of the BTX-A impairs acquisition of skilled behaviour, performance on the Rotarod test of the BTX-A- and the saline-treated rats was assessed. Results of four trials per training session were averaged and treated as one trial. The changes in performance over four training sessions, analysed with the Friedman test, were statistically significant both in the saline-injected group ($\chi^2 = 8.846$, df = 3, $p < 0.05$) and the BTX-A injected group ($\chi^2 = 14.455$, df = 3, $p < 0.01$). Differences in the groups' performance were not statistically significant for any of the training sessions, as confirmed with the Kruskal–Wallis



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test ($\chi^2_{day1} = 0.409$, df = 1, $p > 0.05$; $\chi^2_{day2} = 0.186$, df = 1, $p > 0.05$; $\chi^2_{day\ 3} = 0.001$, df = 1, $p > 0.05$; $\chi^2_{day4} = 0.012$, df = 1, $p > 0.05$).

Results show that both the BTX-A-injected and the control rats were equally able to improve their Rotarod performance, displaying continuous increase of time spent walking on the rotating rod during the 4 days of training sessions, as shown in Fig. 1.

*MWM—no previous training*

To investigate onset and duration of the BTX-A-induced memory impairment, a MWM task was assessed and during a probe test, the time rats spent swimming in the goal quadrant was recorded at multiple points: day 15 after the treatment and from that point once per month for 12 months in total. A clear trend of impaired performance in the BTX-A group is notable in all probe trials (Fig. 2). The Friedman test showed that changes in performance within each group over the time course were not statistically significant, neither for the BTX-A ($\chi^2 = 13.555$, df = 12, $p > 0.05$) nor the saline-injected group ($\chi^2 = 13.872$, df = 12, $p > 0.05$;), i.e. results for each group were stabile over time.

The groups' performance on each of the multiple probe trials was compared using the non-parametrical Mann–Whitney *U* test. The between-group difference was confirmed statistically significant on the 2nd to 5th month of testing, then on 8th and 10th to 12th month of testing

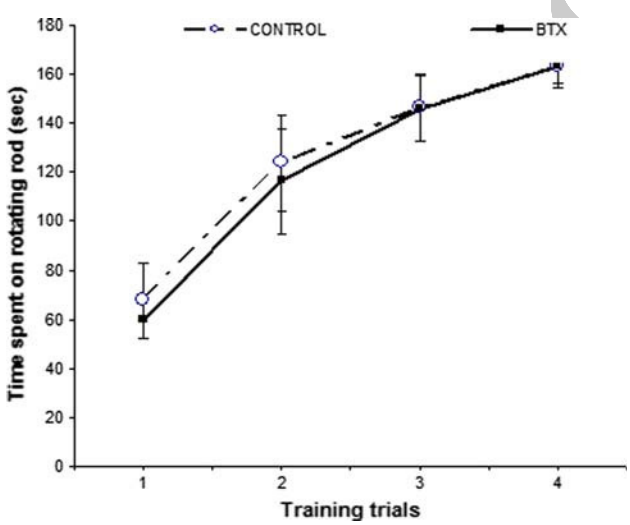


**Fig. 1** Results of the Rotarod test for the BTX-A-treated and the control rats. Results present time spent on the rotating rod (max 180 s) during four daily trials. Results are presented as the mean ± SEM, $n = 5$–6. Statistical analyses shows a significant improvement of within groups' performance over the training trials (Friedman test), and no significant difference in between group performance at any of the trials (Kruskal–Wallis test)

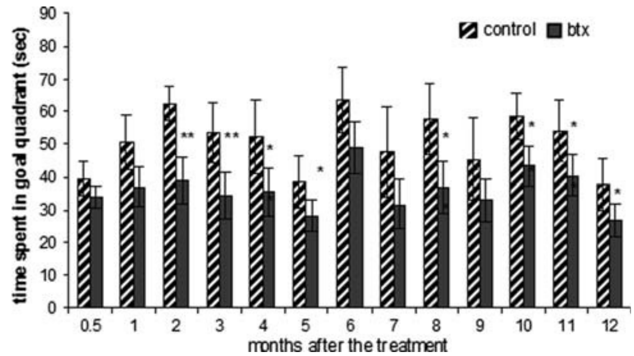


**Fig. 2** Time course of the memory impairment after an icv-injection of BTX-A (2 U/kg); assessed with the Morris water task, with no training in task prior to the drug administration. Results present time spent in the goal quadrant (max 90 s) during a probe trail for different periods of assessment (15 days, 1–12 months). Results are presented as the mean ± SEM, $n = 5$–6. The mean difference was tested using the Mann–Whitney *U* test (**$p < 0.01$; *$p < 0.05$)

($p < 0.05$). A marginally significant difference ($p = 0.052$) was found on the 1st month of testing, and performance at other time points (15th day, 6th, 7th, 9th month) was non-significant ($p > 0.05$). Non-significant difference in some of the points of testing could be a result of random variations, due to small number of animals per group and relatively high variability of tested behaviour. As random variations are inconsistently related to the true results of measurement, they should cancel themselves when results are aggregated over multiple data-points, resulting with significant difference in all aggregated time points.

To test this hypothesis, four quartiles were calculated from the original data set, i.e. individual results for each 3 months were averaged and treated as one. No changes in the amount of time that rats spend in the goal quadrant were found in any of the groups (Friedman test; $\chi^2_{saline} = 2.28$, df = 3, $p > 0.05$;; $\chi^2_{btx\text{-}A} = 2.80$, df = 3, $p > 0.05$) and group differences at all four quartile points were found significantly different (Kruskal–Wallis test; $\chi^2_{quartile1} = 7.50$, df = 1, $p < 0.01$; $\chi^2_{quartile2} = 4.03$, df = 1, $p < 0.05$; $\chi^2_{quartile3} = 7.50$, df = 1, $p < 0.01$; $\chi_{quartile4}{}^2 = 5.66$, df = 1, $p < 0.05$), showing impaired performance in the BTX-treated group.

## Experiment 2

*MWM—training in task prior to the treatment*

To examine whether an icv injection of BTX-A could affect retrieval of the previously learned spatial information, rats were trained in the MWM task prior to treatment. The MWM test was performed after the drug administration. During a probe test, the time that rats spent swimming in the goal quadrant was recorded 3, 10, 20, 30, 60, 120 and 150 days after the treatment (Fig 3). For the saline group,



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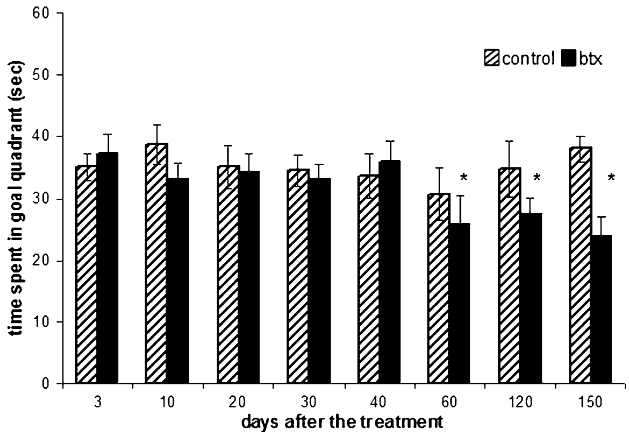


**Fig. 3** Time course of the memory impairment after an icv-injection of BTX-A (2 U/kg); assessed with the Morris water task, with the training in task prior to the drug administration. Results present time spent in the goal quadrant (max 60 s) during a probe trail for different periods of assessment. Results are presented as the mean ± SEM, $n = 10$–$11$. The mean difference was tested using the Kruskal–Wallis test (**$p < 0.01$; *$p < 0.05$)

the Friedman test showed no significant changes in performance over time ($\chi^2 = 11.634$, df = 1, $p > 0.05$); but for the BTX-A-injected group a decrease in performance after 2nd month of testing was found ($\chi^2 = 15.165$, df = 7, $p < 0.05$). The Kruskal–Wallis test showed significant difference in group performance for probe tests conducted on 2nd ($\chi^2 = 4.492$, df = 1, $p < 0.05$), 3rd ($\chi^2 = 4.576$, df = 1, $p < 0.05$) and 5th ($\chi^2 = 11.148$, df = 1, $p < 0.01$) month after the treatment, while group performance on the probe test conducted prior to 2nd month after the treatment were not significant ($\chi^2_{\text{day3}} = 0.549$, df = 1, $p > 0.05$; $\chi^2_{\text{day10}} = 1.541$, df = 1, $p > 0.05$; $\chi^2_{\text{day20}} = 0.013$, df = 1, $p > 0.05$; $\chi^2_{\text{day30}} = 0.150$, df = 1, $p > 0.05$; $\chi^2_{\text{day40}} = 0.245$, df = 1, $p > 0.05$).

## Discussion

It is generally accepted that modulation of the central cholinergic system influences cognitive function, primarily attention processes and capacities (Everitt and Robbins 1997; Sarter and Bruno 1997), and consequently learning and memory (Torres et al. 1994; Sarter et al. 2003).

One of the most frequently used laboratory tools for investigating spatial memory in rats is the MWM task (for review, see D'Hooge and De Deyn 2001). The standard version of the MWM task requires an animal to learn to escape from water onto a hidden platform, using distal extra-maze cues to map the platform location. This spatial version of the task is considered to be largely dependent on the neuronal integrity of the hippocampus (Morris et al. 1986).

Effects of central cholinergic system manipulations are extensively investigated using the MWM (for review, see Myhrer 2003). In general, impaired performance in the MWM task was related to a systemic administration of cholinergic antagonists (Miyamoto et al. 1989; Cozzolino et al. 1994; Fishkin et al. 1993; Puumala et al. 1996; Jackson and Soliman 1996; von Linstow Roloff et al. 2007; Herrera-Morales et al. 2007), or icv infusions of toxic agents, such as cholinergic immunotoxin 192IgG-saporin (Nilsson et al. 1992; Leanza et al. 1995; Garcia-Alloza et al. 2006) and cholinergic neurotoxin AF64A (Opello et al. 1993). Surprisingly, however, botulinum toxin, usually assumed the most potent cholinergic neurotoxin, was investigated only sporadically. So far, only two studies on experimental animals addressed the effect of BTX on cognitive performance. The only experimental finding that indicates a long-term cognitive impairment after a central application of BTX was done with clinically less important BTX-B. Ando et al. (2002) found that entorhinal injections of BTX-B lead to an impaired performance on several memory tasks and a long-lasting reduction of LTP formation in aged rats. In a short-lasting experiment (9 days), Luvisetto et al. (2004) found that an intracerebroventricular (icv) injection of BTX-B and BTX-A (7.5 pg/animal) in mice is associated with impaired performance on the novel recognition test, but had no effect on the avoidance acquisition. Both studies indicate that the BTX-related cognitive impairment develops relatively shortly after the drug administration (1–2 weeks). Although different species, doses, types of BTX, mode of application as well as nature of cognitive test used do not allow direct comparison between those two studies, it is evident that the central administration of botulinum toxin in animals contributes to some sort of cognitive decline.

In spite of these preclinical observations of potentially deleterious effects of the central administration of BTX, some authors recently suggested that centrally applied BTX A could be useful in therapy of seizures and some other CNS disorders (Bozzi et al. 2006; Verderio et al. 2007).

In this paper, we investigated duration and possible variations in the magnitude of cognitive deficit in rats after an icv application of the clinically most important BTX type A. In the preliminary experiment, the rats' performance on the MWM test demonstrated that significant memory impairment after BTX-A icv application becomes statistically significant after 3 months (Table 1). Based on that observation, we decided for a long-term follow up. In that preliminary experiment, there was no difference among doses of 2 and 4 U/kg. Small number of animals might account for that. We did not investigate dose-dependent relations in more detail because a slow onset of





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the effect and its potentially long duration appeared more intriguing.

In the follow-up experiment (Experiment 1), rats' performance on the MWM test was monitored for 12 months after the drug administration. Results showed that a single icv injection of 2 U/kg of BTX-A leads to impaired performance. The effect lasted up to 12 months, with no sign of recovery; and became significant between the 1st and 2nd month after the toxin administration. However, due to the high intra-group variability and the fact that the Friedman test did not show significant changes in performance over time for neither group, we cannot exclude the possibility that significant differences in performance between the BTX-A- and the saline-injected rats could exist prior to the observed ones (Fig. 2). Onset of memory impairment between 1st and 2nd month seems especially important because other BTX_A effects like muscle weakness or antinociceptive effect become evident within few days and after 2 months they are not visible any more. (Aoki 2002; Bach-Rojecky et al. 2005).

Additionally, using the Rotarod test, we investigated the effect of BTX-A on motor skill acquisition. Motor learning is a model of procedural learning, which is known to largely depend on the basal ganglia (Salmon and Butters 1995). In spite of the important role of cholinergic nerves in the central motor control performance, the Rotarod performance was not affected by the employed dose of BTX A (Fig. 1). Some research suggests that selective ablation of cholinergic neurons in the striatum impairs procedural learning only in reward-related tasks, but not in simple motor tasks (Kitabatake et al. 2003) which could account for our findings.

It is known that the pre-training in MWM can restore impaired spatial performance in some cases (Gage 1985; Handelmann and Olton 1981; Jarrad 1978). Accordingly, in Experiment 2, we examined whether the pattern of BTX-A-induced cognitive impairment would be the same if rats were pre-operatively trained for the MWM task. Results were very similar to those obtained in Experiment 1; a decreased MWM performance in the BTX-A-treated group was detected 2 months after the treatment, showing that pre-training does not influence the pattern of the dementia-like deficit in the BTX-A-treated rats as found in Experiment 1.

Results of both experiments indicate a slow onset of BTX-A-induced memory deficit, regardless of the amount of pre-training to the task. A slow onset may be the reason why the effects of the centrally induced BTX-A on cognition were not reported more frequently.

This is the first report of long-term memory deficits induced by the central administration of BTX-A. In the neuromuscular junctions, BTX damages the function of cholinergic nerve endings by cleavage of SNAP-25, which prevents release of acetylcholine (Jankovic 2004). For the reasons which are not completely understood, near non-functioning neuromuscular junctions, the sprouting of cholinergic nerve endings takes place (Meunier et al. 2002). Accordingly, in interpreting our results, the first assumption could be that in the CNS, like at neuromuscular junctions, the function of cholinergic nerves is prevented, but if this were the case, we should expect cognitive deficit to be detectable much earlier. On the other hand, the lack of expected effects on motor performance cannot be explained by the difference in the distance of the basal ganglia and hippocampus from the cerebral ventricles into which the toxin was injected, but it could be a consequence of different vulnerability of cholinergic neurons depending on their length and myelinisation (Braak et al. 2006). However, slow onset of cognitive decline could hardly be explained with direct and acute loss of cholinergic function. Highly speculative possibilities may be related to a misguided slow sprouting after damage of the axonal function. However, there is evidence that BTX does not only affect the release of acetylcholine but, at least in vitro, also some other neurotransmitters like glutamate, GABA, dopamine, serotonin, etc. (Ashton and Dolly 1988; Bergquist et al. 2002; Verderio et al. 2007; Najib et al. 1999).

Our results indicate that additional experiments are needed before central application of botulinum toxin type A could be recommended as therapy for different CNS diseases, as suggested by some authors (Bozzi et al. 2006; Verderio et al. 2007). Additionally, icv BTX-A application could be used as a new model of memory impairment. At present, there are several animal models of dementia, but none of them can completely reflect the complexity of the human disorder (McDonald and Overmier 1998). Although, at present, we cannot offer a complete explanation of the effects described here. Like some other models of animal dementia (Murray and Fibiger 1986; Itoh et al. 1997), the BTX-A icv model could also potentially be used in assessing the validity of therapeutic interventions with cholinergic drugs.

## Conclusion

Previous research confirmed that BTX type A and type B affect cognitive processes in rats and mice. Prolonged duration of the effects was determined for BTX-B only. This is the first study confirming that an icv administration of BTX-A produces long-term damage of memory retrieval. The effect is long-lasting, clearly detectable 2 months after the treatment, with no sign of recovery over a longer time period (1 year). These results, combined with recent evidence indicating an axonal transport of BTX-A after an unilateral hippocampal infusion (Antonucci et al. 2008),





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seriously compromise the idea of using botulinum toxin in the treatment of CNS disorders, as it was recently suggested (Verderio et al. 2007; Donovan 2001, 2006; Donovan and Francis, 2008). On the other hand, the BTX-A-induced cognitive deficit might be a new animal model for research on memory impairment.

**Acknowledgments** Supported by the Croatian Ministry of Science, Education and Sport and the Deutscher Akademischer Austausch Dienst (DAAD). We thank Bozica Hrzan for her excellent technical assistance.



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MS Code : JNT-D-09-00069 | CP | DISK