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

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

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8 **Abstract** It is generally believed that the cholinergic  
9 system plays an important role in normal cognitive func-  
10 tioning. Botulinum toxin is the most potent toxin of the  
11 peripheral cholinergic system and today it is used in the  
12 treatment of a variety of neurological disorders. However,  
13 it is surprising that its effect on cognitive processes has  
14 been investigated in only two publications. Short-term  
15 effects of the central application of botulinum toxin (BTX)  
16 type B have been associated with cognitive impairment in  
17 animals, while results with type A are ambiguous. In the  
18 present study, we have investigated the duration of memory  
19 impairment after an intracerebroventricular administration  
20 of BTX-A in rats. Two experiments were performed,  
21 lasting 12 and 5 months, respectively. In both experiments,  
22 the same dose of BTX-A was applied (2 U/kg) and the  
23 Morris water maze test was used in the assessment of  
24 memory performance. Results show that a single icv  
25 injection of a small dose of BTX-A significantly impairs  
26 the water maze performance. In both experiments,

impairment was apparently of a slow onset and long lasting 27  
(up to 12 months). The length and pattern of attenuation 28  
suggest development of dementia-like deficits. In addition 29  
to providing a potentially new experimental model of 30  
memory impairment, these results question the idea of an 31  
intracranial application of BTX in the treatment of CNS 32  
disorders. 33

**Keywords** Botulinum toxin · 35  
Botulinum toxin type A, cognitive impairment · 36  
Intracerebroventricular application · 37  
Morris water maze, rat, dementia 38

**Introduction** 39

The classic mechanism of the botulinum toxin (BTX)-type 40  
A action is cleavage of the synaptosomal associated protein 41  
of 25 kD (SNAP-25) which is required for vesicle docking 42  
and fusion with the plasma membrane. Accordingly, in the 43  
peripheral nervous system, BTX-A prevents acetylcholine 44  
release into the synaptic cleft (Kao et al. 1976; Bach- 45  
Rojecky et al. 2007). 46

Cholinergic activity is considered to play an important 47  
role in animal and in human memory function. A sup- 48  
pressed cholinergic function impairs, and an enhanced one 49  
improves learning and memory (for review see Gold 2003). 50  
Moreover, a selective loss of cholinergic neurons in the 51  
brain of Alzheimer's patients was already well documented 52  
decades ago (Davies and Maloney 1976). In line with that, 53  
cholinomimetics (cholinesterase inhibitors) are an accepted 54  
therapy in Alzheimer's disease (Birks 2006). Accordingly, 55  
if the outcome of a centrally administered BTX is similar 56  
to the effects on the peripheral cholinergic nerves, cogni- 57  
tive impairment could be expected. Contrary to such 58

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59 expectations, some authors recently suggested that a central  
60 administration of BTX A could be useful in the therapy of  
61 seizures and several other CNS disorders (Bozzi et al.  
62 2006; Verderio et al. 2007). Therefore, further investiga-  
63 tion of the centrally applied BTX-A seems increasingly  
64 important.

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

## 70 Methods

### 71 Animals

72 A total of 32 male Wistar rats (Zagreb University School of  
73 Medicine, Zagreb, Croatia), 3-month-old and weighing  
74 250–300 g at the beginning of treatment, were used in two  
75 experiments. Additional 23 rats were used for preliminary  
76 experiments in which dose dependency of BTX-A effects  
77 was examined.

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

### 89 Drugs

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

### 99 Drug administration

100 Rats were randomly divided into groups (5–6 per group in  
101 Experiment 1, 10–11 per group in Experiment 2) and given  
102 general anaesthesia (chloral hydrate 300 mg/kg, i.p), fol-  
103 lowed by an icv injection of either saline as vehicle or

**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
<i>N</i>	8	8	8
BTX-A 0.5U			
Mean	70,50	80,25	54,00
SE	4,787	6,909	9,958
<i>N</i>	4	4	4
BTX-A 2U			
Mean	56,00	76,75	48,5
SE	7,012	11,736	7,577
<i>N</i>	4	4	4
BTX-A 4U			
Mean	65,14	62,714	43,86
SE	7,731	8,909	5,152
<i>N</i>	7	7	7
Kruskall-Wallis test			
Chi-square	3,154	4,653	<b>7,507*</b>
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 (\* $p < 0.05$ )

104 selected BTX–saline solutions. An icv-injection of botu-  
105 linum toxin type A was applied bilaterally into the left and  
106 the right lateral ventricle, according to the procedure  
107 described by Noble et al. (1967). Drug concentration and  
108 solution volume were adjusted according to the animal  
109 body weight, and a volume of 4  $\mu$ L per 300 g body weight  
110 was administered (2  $\mu$ L/ventricle). The same procedure  
111 was applied for dose-dependent pre-screening.

### 112 Acquisition of motor skills

113 Motor learning was assessed using the Rotarod test. The  
114 apparatus consisted of a horizontal rod (7 cm in diameter,  
115 10 cm long), situated 30 cm above the landing platform.  
116 The animals were placed on the rod with their head  
117 directed against the direction of the rotation so they had to  
118 progress forward to maintain equilibrium. Duration of their  
119 holding without falling down was measured. The test was  
120 run up to 180 s.

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

124	sessions started on the day following the last acclimation	administration was conducted. Time period between the	171
125	session. Each (daily) training session consisted of four	end of the training phase and the surgical procedure was	172
126	trials with an inter-trial interval of 10 min. The training	2 days. The MWM performance was evaluated 3, 10, 20,	173
127	sessions were performed over four consecutive days. This	30, 40, 60, 120, and 150 days after the BTX-A application.	174
128	assessment was part of Experiment 1 and was applied	During a probe trail (cut-off time 60 s), the total time each	175
129	6 months after the drug administration.	rat swam in the former platform quadrant was recorded.	176
130	Spatial learning and memory tests	A larger pool was used than in Experiment 1 (1.75 vs.	177
131	Spatial memory was evaluated in the hidden platform	1.2 m), so the absolute magnitudes of time spent in the goal	178
132	version of the MWM task. Rats were required to learn	quadrant were not comparable between the experiments.	179
133	spatial location of a hidden platform in a square pool filled	Statistics	180
134	with clear water (25°C). A circular transparent platform	All values are expressed as the mean $\pm$ SE. In all three	181
135	(10 cm in diameter) was placed 1.5 cm below the water	data sets (Rotarod test; MWM 1 and MWM 2), the	182
136	surface in the middle of the northeast quadrant. There were	Friedman test was used to analyse data in each group	183
137	large high-contrast visual cues throughout the room and on	within the time course. Depending on data set character-	184
138	the pool walls.	istics, either the Kruskal–Wallis or the Mann–Whitney <i>U</i>	185
139	The following MWM procedure was the same for all	test was used for comparison of the data among groups at	186
140	experiments:	the same time point. A <i>p</i> value less than 0.05 was con-	187
141	A rat was placed in the pool with its head facing the pool	sidered statistically significant.	188
142	wall. A different starting point was used for each trail in	<b>Results</b>	189
143	pseudo-random order. If the rat did not find the platform	Dose-dependent pre-screening experiment	190
144	within 120 s, it was gently guided by hand to the platform.	To investigate whether BTX-A affects memory and pos-	191
145	When finding the platform, rats were allowed to remain	sible dose-dependency of such effects; a MWM task was	192
146	there for 30 s. For each point of testing, rats were trained in	assessed in four groups: (1) saline-treated; (2) BTX-A	193
147	this task for four consecutive days, performing block of	0.5 U/kg; (3) BTX-A 2 U/kg and (4) BTX-A 4 U/kg.	194
148	three trials per day. An inter-trial interval of 5–10 min was	During the probe test, the time rats spent swimming in the	195
149	given. On the fifth day, rats were given a retention probe	goal quadrant was recorded at multiple points: 0.5, 1 and	196
150	test in which the platform was removed from the quadrant.	3 months after the treatment. The Kruskal–Wallis test	197
151	For dose-dependent pre-screening, following procedure	showed marginally significant difference in group perfor-	198
152	matched one described for Experiment 1, except that test-	mance for probe tests conducted 3 months post-treatment	199
153	ing was done within 3 months and the cut-off time in probe	( <i>p</i> = 0.51), while differences on the probe tests conducted	200
154	test was set at 120 s.	prior to that point were not significant (Table 1).	201
155	<i>Experiment 1</i>	Experiment 1	202
156	No training in the task prior to drug administration was	<i>Motor learning</i>	203
157	conducted. The MWM performance was evaluated at day	To examine whether the central administration of the BTX-A	204
158	15 after the surgery, 1 month after the surgery and from	impairs acquisition of skilled behaviour, performance on the	205
159	that point once per month for 12 months in total. During a	Rotarod test of the BTX-A- and the saline-treated rats was	206
160	probe trail (cut-off time 90 s), the total time each rat swam	assessed. Results of four trials per training session were	207
161	in the former platform quadrant was recorded.	averaged and treated as one trial. The changes in performance	208
162	<i>Experiment 2</i>	over four training sessions, analysed with the Friedman test,	209
163	Prior to drug administration all animals were trained in the	were statistically significant both in the saline-injected group	210
164	MWM task for 10 sessions (10 consecutive days), each	( $\chi^2 = 8.846$ , <i>df</i> = 3, <i>p</i> < 0.05) and the BTX-A injected	211
165	session consisting of three trials, following the general	group ( $\chi^2 = 14.455$ , <i>df</i> = 3, <i>p</i> < 0.01). Differences in the	212
166	procedure described above. At the end of the training	groups' performance were not statistically significant for any	213
167	phase, all animals were able to find the platform within	of the training sessions, as confirmed with the Kruskal–Wallis	214
168	10 s. After the training phase, animals were randomly		
169	assigned to the control (saline-infusion) group ( <i>N</i> = 11) or		
170	the BTX-A-infusion group ( <i>N</i> = 10), and an icv drug		

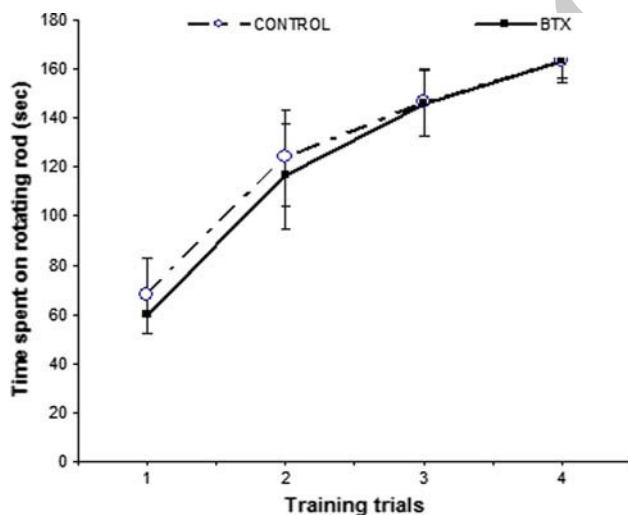
215 test ( $\chi^2_{\text{day}1} = 0.409$ ,  $df = 1$ ,  $p > 0.05$ ;  $\chi^2_{\text{day}2} = 0.186$ ,  $df = 1$ ,  
 216  $p > 0.05$ ;  $\chi^2_{\text{day}3} = 0.001$ ,  $df = 1$ ,  $p > 0.05$ ;  $\chi^2_{\text{day}4} = 0.012$ ,  
 217  $df = 1$ ,  $p > 0.05$ ).

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

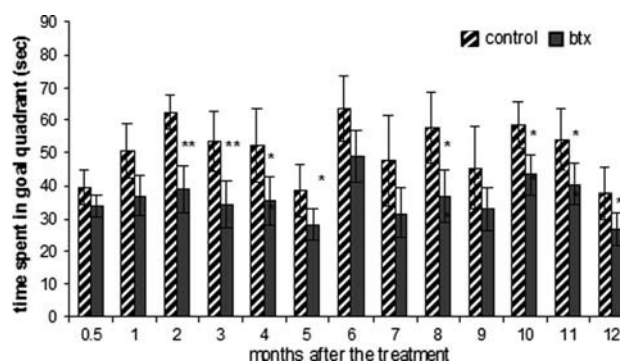
### 223 MWM—no previous training

224 To investigate onset and duration of the BTX-A-induced  
 225 memory impairment, a MWM task was assessed and during  
 226 a probe test, the time rats spent swimming in the goal  
 227 quadrant was recorded at multiple points: day 15 after the  
 228 treatment and from that point once per month for  
 229 12 months in total. A clear trend of impaired performance  
 230 in the BTX-A group is notable in all probe trials (Fig. 2).  
 231 The Friedman test showed that changes in performance  
 232 within each group over the time course were not statisti-  
 233 cally significant, neither for the BTX-A ( $\chi^2 = 13.555$ ,  
 234  $df = 12$ ,  $p > 0.05$ ) nor the saline-injected group  
 235 ( $\chi^2 = 13.872$ ,  $df = 12$ ,  $p > 0.05$ ), i.e. results for each  
 236 group were stable over time.

237 The groups' performance on each of the multiple probe  
 238 trials was compared using the non-parametrical Mann-  
 239 Whitney  $U$  test. The between-group difference was con-  
 240 firmed statistically significant on the 2nd to 5th month of  
 241 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  $\pm$  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  $\pm$  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 ( $p = 0.052$ )  
 242 was found on the 1st month of testing, and performance at  
 243 other time points (15th day, 6th, 7th, 9th month) was non-  
 244 significant ( $p > 0.05$ ). Non-significant difference in some  
 245 of the points of testing could be a result of random varia-  
 246 tions, due to small number of animals per group and rela-  
 247 tively high variability of tested behaviour. As random  
 248 variations are inconsistently related to the true results of  
 249 measurement, they should cancel themselves when results  
 250 are aggregated over multiple data-points, resulting with  
 251 significant difference in all aggregated time points.

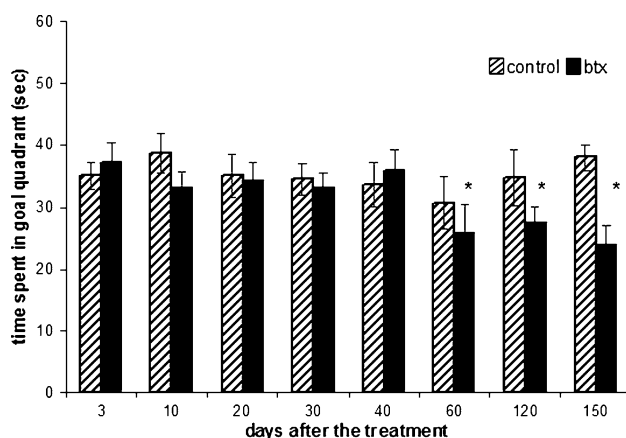
252 To test this hypothesis, four quartiles were calculated  
 253 from the original data set, i.e. individual results for each  
 254 3 months were averaged and treated as one. No changes in  
 255 the amount of time that rats spend in the goal quadrant  
 256 were found in any of the groups (Friedman test;  
 257  $\chi^2_{\text{saline}} = 2.28$ ,  $df = 3$ ,  $p > 0.05$ ;  $\chi^2_{\text{btx-A}} = 2.80$ ,  $df = 3$ ,  
 258  $p > 0.05$ ) and group differences at all four quartile points  
 259 were found significantly different (Kruskal-Wallis test;  
 260  $\chi^2_{\text{quartile}1} = 7.50$ ,  $df = 1$ ,  $p < 0.01$ ;  $\chi^2_{\text{quartile}2} = 4.03$ ,  $df =$   
 261  $1$ ,  $p < 0.05$ ;  $\chi^2_{\text{quartile}3} = 7.50$ ,  $df = 1$ ,  $p < 0.01$ ;  $\chi_{\text{quar-}$   
 262  $\text{tile}4}^2 = 5.66$ ,  $df = 1$ ,  $p < 0.05$ ), showing impaired perfor-  
 263 mance in the BTX-treated group.

### 265 Experiment 2

#### 266 MWM—training in task prior to the treatment

267 To examine whether an icv injection of BTX-A could  
 268 affect retrieval of the previously learned spatial informa-  
 269 tion, rats were trained in the MWM task prior to treatment.  
 270 The MWM test was performed after the drug administra-  
 271 tion. During a probe test, the time that rats spent swimming  
 272 in the goal quadrant was recorded 3, 10, 20, 30, 60, 120 and  
 273 150 days after the treatment (Fig 3). For the saline group,





**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  $\pm$  SEM,  $n = 10$ –11. The mean difference was tested using the Kruskal–Wallis test (\*\* $p < 0.01$ ; \* $p < 0.05$ )

274 the Friedman test showed no significant changes in performance over time ( $\chi^2 = 11.634$ ,  $df = 1$ ,  $p > 0.05$ ); but  
 275 for the BTX-A-injected group a decrease in performance  
 276 after 2nd month of testing was found ( $\chi^2 = 15.165$ ,  $df = 7$ ,  
 277  $p < 0.05$ ). The Kruskal–Wallis test showed significant  
 278 difference in group performance for probe tests conducted  
 279 on 2nd ( $\chi^2 = 4.492$ ,  $df = 1$ ,  $p < 0.05$ ), 3rd ( $\chi^2 = 4.576$ ,  
 280  $df = 1$ ,  $p < 0.05$ ) and 5th ( $\chi^2 = 11.148$ ,  $df = 1$ ,  $p < 0.01$ )  
 281 month after the treatment, while group performance on the  
 282 probe test conducted prior to 2nd month after the treatment  
 283 were not significant ( $\chi^2_{\text{day}3} = 0.549$ ,  $df = 1$ ,  $p > 0.05$ ;  
 284  $\chi^2_{\text{day}10} = 1.541$ ,  $df = 1$ ,  $p > 0.05$ ;  $\chi^2_{\text{day}20} = 0.013$ ,  $df = 1$ ,  
 285  $p > 0.05$ ;  $\chi^2_{\text{day}30} = 0.150$ ,  $df = 1$ ,  $p > 0.05$ ;  
 286  $\chi^2_{\text{day}40} = 0.245$ ,  $df = 1$ ,  $p > 0.05$ ).

## 288 Discussion

289 It is generally accepted that modulation of the central  
 290 cholinergic system influences cognitive function, primarily  
 291 attention processes and capacities (Everitt and Robbins  
 292 1997; Sarter and Bruno 1997), and consequently learning  
 293 and memory (Torres et al. 1994; Sarter et al. 2003).

294 One of the most frequently used laboratory tools for  
 295 investigating spatial memory in rats is the MWM task (for  
 296 review, see D'Hooge and De Deyn 2001). The standard  
 297 version of the MWM task requires an animal to learn to  
 298 escape from water onto a hidden platform, using distal  
 299 extra-maze cues to map the platform location. This spatial  
 300 version of the task is considered to be largely dependent on  
 301 the neuronal integrity of the hippocampus (Morris et al.  
 302 1986).

303 Effects of central cholinergic system manipulations are  
 304 extensively investigated using the MWM (for review, see  
 305 Myhrer 2003). In general, impaired performance in the  
 306 MWM task was related to a systemic administration of  
 307 cholinergic antagonists (Miyamoto et al. 1989; Cozzolino  
 308 et al. 1994; Fishkin et al. 1993; Puumala et al. 1996;  
 309 Jackson and Soliman 1996; von Linstow Roloff et al.  
 310 2007; Herrera-Morales et al. 2007), or icv infusions of  
 311 toxic agents, such as cholinergic immunotoxin 192IgG-  
 312 saporin (Nilsson et al. 1992; Lanza et al. 1995; Garcia-  
 313 Alloza et al. 2006) and cholinergic neurotoxin AF64A  
 314 (Opello et al. 1993). Surprisingly, however, botulinum  
 315 toxin, usually assumed the most potent cholinergic neu-  
 316 rotoxin, was investigated only sporadically. So far, only  
 317 two studies on experimental animals addressed the effect  
 318 of BTX on cognitive performance. The only experimental  
 319 finding that indicates a long-term cognitive impairment  
 320 after a central application of BTX was done with clinically  
 321 less important BTX-B. Ando et al. (2002) found that  
 322 entorhinal injections of BTX-B lead to an impaired per-  
 323 formance on several memory tasks and a long-lasting  
 324 reduction of LTP formation in aged rats. In a short-lasting  
 325 experiment (9 days), Luvisetto et al. (2004) found that an  
 326 intracerebroventricular (icv) injection of BTX-B and  
 327 BTX-A (7.5 pg/animal) in mice is associated with  
 328 impaired performance on the novel recognition test, but  
 329 had no effect on the avoidance acquisition. Both studies  
 330 indicate that the BTX-related cognitive impairment  
 331 develops relatively shortly after the drug administration  
 332 (1–2 weeks). Although different species, doses, types of  
 333 BTX, mode of application as well as nature of cognitive  
 334 test used do not allow direct comparison between those  
 335 two studies, it is evident that the central administration of  
 336 botulinum toxin in animals contributes to some sort of  
 337 cognitive decline.

338 In spite of these preclinical observations of potentially  
 339 deleterious effects of the central administration of BTX,  
 340 some authors recently suggested that centrally applied  
 341 BTX A could be useful in therapy of seizures and some  
 342 other CNS disorders (Bozzi et al. 2006; Verderio et al.  
 343 2007).

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

356 the effect and its potentially long duration appeared more  
357 intriguing.

358 In the follow-up experiment (Experiment 1), rats' per-  
359 formance on the MWM test was monitored for 12 months  
360 after the drug administration. Results showed that a single  
361 icv injection of 2 U/kg of BTX-A leads to impaired per-  
362 formance. The effect lasted up to 12 months, with no sign  
363 of recovery; and became significant between the 1st and  
364 2nd month after the toxin administration. However, due to  
365 the high intra-group variability and the fact that the  
366 Friedman test did not show significant changes in perfor-  
367 mance over time for neither group, we cannot exclude the  
368 possibility that significant differences in performance  
369 between the BTX-A- and the saline-injected rats could  
370 exist prior to the observed ones (Fig. 2). Onset of memory  
371 impairment between 1st and 2nd month seems especially  
372 important because other BTX\_A effects like muscle  
373 weakness or antinociceptive effect become evident within  
374 few days and after 2 months they are not visible any more.  
375 (Aoki 2002; Bach-Rojecky et al. 2005).

376 Additionally, using the Rotarod test, we investigated the  
377 effect of BTX-A on motor skill acquisition. Motor learning  
378 is a model of procedural learning, which is known to lar-  
379 gely depend on the basal ganglia (Salmon and Butters  
380 1995). In spite of the important role of cholinergic nerves  
381 in the central motor control performance, the Rotarod  
382 performance was not affected by the employed dose of  
383 BTX A (Fig. 1). Some research suggests that selective  
384 ablation of cholinergic neurons in the striatum impairs  
385 procedural learning only in reward-related tasks, but not in  
386 simple motor tasks (Kitabatake et al. 2003) which could  
387 account for our findings.

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

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

405 This is the first report of long-term memory deficits  
406 induced by the central administration of BTX-A. In the  
407 neuromuscular junctions, BTX damages the function of  
408 cholinergic nerve endings by cleavage of SNAP-25, which

409 prevents release of acetylcholine (Jankovic 2004). For the  
410 reasons which are not completely understood, near non-  
411 functioning neuromuscular junctions, the sprouting of  
412 cholinergic nerve endings takes place (Meunier et al.  
413 2002). Accordingly, in interpreting our results, the first  
414 assumption could be that in the CNS, like at neuromuscular  
415 junctions, the function of cholinergic nerves is prevented,  
416 but if this were the case, we should expect cognitive deficit  
417 to be detectable much earlier. On the other hand, the lack  
418 of expected effects on motor performance cannot be  
419 explained by the difference in the distance of the basal  
420 ganglia and hippocampus from the cerebral ventricles into  
421 which the toxin was injected, but it could be a consequence  
422 of different vulnerability of cholinergic neurons depending  
423 on their length and myelination (Braak et al. 2006).  
424 However, slow onset of cognitive decline could hardly be  
425 explained with direct and acute loss of cholinergic func-  
426 tion. Highly speculative possibilities may be related to a  
427 misguided slow sprouting after damage of the axonal  
428 function. However, there is evidence that BTX does not  
429 only affect the release of acetylcholine but, at least in vitro,  
430 also some other neurotransmitters like glutamate, GABA,  
431 dopamine, serotonin, etc. (Ashton and Dolly 1988; Berg-  
432 quist et al. 2002; Verderio et al. 2007; Najib et al. 1999).

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

## 448 Conclusion

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

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

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