Increased striatal VMAT2 binding in mice after chronic administration of methcathinone and manganese

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1. Introduction

Home-made psychotropic drugs and their self-administration is a major public health concern. In addition to the social issues, these drugs may cause serious neurological conditions leading to severe disabilities. The production and abuse of substances including methcathinone and other synthetic cathinone derivatives are becoming epidemics. Despite legislative restrictions and the control of cathinones, their detection and quantification is limited [1,2].

In several Eastern-European countries, there is increased abuse of home-brewed methcathinone (a.k.a. ephedrone). Chemicals and instructions for making the drug are easily available on the internet. This mixture contains an excessive amount of manganese as a byproduct of the chemical reaction and has been linked as a causative agent with an movement disorder with parkinsonism, early falls, speech disorders, and dystonia [3-5]. The neurological syndrome closely resembles “manganism”, a condition caused by environmental exposure to large concentrations of manganese.

The main reason for the abuse of methcathinone, which is a potent psychostimulant and the beta-keto analog of methamphetamine, is the amphetamine-like stimulation [6]. Methcathinone acts as a preferential catecholamine uptake inhibitor and a dopamine releaser, similar to amphetamine and methamphetamine. It has a clinically similar toxicity profile to amphetamine including euphoria, hallucinations and motor activation [7]. In experiments a divergence between *in vitro* and *in vivo* properties of methcathinone has been noted; the drug is very selective and potent releaser of the catecholamine transporters *in vitro*, but elevates both dopamine and serotonin levels *in vivo* [8].

Vesicular monoamine transporter type 2 (VMAT2) is mostly located in dopaminergic nerve terminals and mediates the response to various dopaminergic toxins including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and methamphetamine [9], [10]. It is vital in maintaining monoamine availability for neurotransmission [11]. Both methcathinone and manganese are capable of interacting with VMAT2, altering its function [12]. In previous imaging studies with [^{11}C]DTBZ in humans, methcathinone and manganese decreased striatal VMAT2 binding in heavy methamphetamine users [13]; in addition increased binding in early methamphetamine abstinence [9] has been reported.

We aimed to reproduce the parkinsonian syndrome caused by the drug mixture in an animal model. We selected a chronic dosing regimen since the onset time of the clinical syndrome is unknown. We measured motor activity to assess the expected movement related symptoms, and [^{11}C]DTBZ was used for positron emission tomography (PET) and autoradiographic evaluation of the dopaminergic system to detect any early alterations at the cellular level. Manganese accumulation in the basal ganglia was evaluated with magnetic resonance imaging (MRI).

2. Results

2.1 Methcathinone and manganese analysis

The average amount of methcathinone in the 100% Test Item solution was 9.87 mg/ml (range 8.82 – 10.54). The average manganese content of the 100% Test Item solution was 0.688 mg/ml.

Manganese concentration in blood measured 24 h after the Test Item injection was 588 µg/l (95% CI 410 - 750). This dropped to 70 µg/l (95% CI -170 – 310, $p<0.001$) 48 h after the injection (Figure 1).

2.2 Motor activity and gait analysis

Results of the motility box testing are summarized in Table 2. Overall, the smaller Test Item dose had a somewhat greater stimulating effect on the animals. Exploratory activity, measured by time spent in the center of the box and on rear feet, was significantly decreased with higher drug doses. The immediate response to the drug remained unaltered during the whole testing period. Within the first 5-15 minutes after the injection of Test Item, the acute response was clearly visible. Animals in all treatment groups demonstrated stereotypical movements in response to the drug. No seizures were observed.

Gait analysis testing did not demonstrate a significant decrease in stride length or any visible disturbance of the stepping pattern during the study period.

2.3 *In vivo* brain uptake of [^{11}C]DTBZ

Representative *in vivo* PET images, time-radioactivity curves for striata and cerebellum, and striatum-to-cerebellum retention ratio curves from the *in vivo* PET imaging were determined (Figure 2). *In vivo* PET scans showed that [^{11}C]DTBZ uptake in the brain peaked during the first minutes after the injection. The striatum-to-cerebellum ratios calculated for the duration of the whole dynamic scan increased in the initial phase, but only minor change was seen from 5 to 20 min. The maximum ratio obtained during this time frame was 2.1±0.3 (n=3) (Figure 2). Based on these findings 10 min after injection was chosen as the time point for *ex vivo* experiments.

2.4 *Ex vivo* [^{11}C]DTBZ tissue counting and digital autoradiography

In the brain, the [^{11}C]DTBZ uptake at 10 min after injection was 3.2 ± 0.2 %ID/g in 100% Test Item injected mice (Group 1), and 3.7 ± 0.2 %ID/g in saline injected mice (Group 4) after 13 weeks of injections, and 3.0 ± 0.3 %ID/g and 3.4 ± 0.1 %ID/g, respectively, after 27 weeks of injections. The striatum-to-cerebellum ratios calculated from the digital autoradiography images after 13 weeks of daily injections were 2.6 ± 0.5 for 100% Test Item injected mice (Group 1, n = 3) and 2.7 ± 0.5 (n = 3) for saline injected mice (Group 4, n = 4). After 27 weeks of injections, the ratio remained similar at 2.8 ± 0.3 (n=4) in Group 4 mice, however, the ratio for Group 1 mice increased to 3.6 ± 0.5 (n=3) and the difference between groups was statistically significant ($p = 0.04$) (Figures 3 and 4).

3. Discussion

The combination of methcathinone and manganese in a homemade drug mixture causes parkinsonian symptoms in human drug abusers after chronic administration. Both substances act as toxins towards the dopaminergic system and there is clear evidence of dopaminergic damage, while at the same time the common anti-parkinsonian treatments fail. We used the drug mixture in a chronic animal experiment over 27 weeks aiming to reproduce a similar movement disorder in animals. We used different dilutions of the Test Item since there is no reported dosage for animal experiments.

The [^{11}C]DTBZ is a well-established marker of VMAT2. We observed an increase of striatal [^{11}C]DTBZ binding after 27 weeks of treatment. This finding is indicative of a lack in striatal vesicular dopamine. A similar increase of DTBZ binding was demonstrated after depleting dopamine with systemic administration of α-methyl-DL-tyrosine methyl ester hydrochloride (AMPT) and administering a single dose of amphetamine [16, 17]. Methcathinone is known to release dopamine from presynaptic vesicular storage and inhibit its subsequent reuptake from the synapse therefore leaving its binding sites available.

The Test Item dosage was based on the results of a pilot experiment (unpublished data). The highest dose (100% Test Item) was chosen so that it would reliably produce an evident increase of locomotor activity. Lower concentrations were also tested to determine how the Test Item behaves over an extended period. Doses that were too high could attenuate motor activity and become lethal as shown with amphetamine [18]. Our chronic treatment regimen failed to reproduce the clinically evident, persistent parkinsonian syndrome with any of the Test Item dilutions. This is possibly due to several reasons. The amount of manganese and methcathinone in the mixture was evidently insufficient. In addition, the dosing regimen consisted only of a single injection each day. However, the acute effects of methcathinone on locomotor activity remained clearly visible throughout the duration of our experiment.

A human PET study by Lee *et al.* in Parkinsonian patients showed that extensive loss of dopamine neurons is functionally compensated for by increased synthesis and release of dopamine from remaining dopamine neurons and by reduced uptake of dopamine into nerve terminals. Thus, in our study the discrepancy between motor symptoms and changes in DTBZ binding may result from changes in other pathways of striatal dopaminergic transmission not revealed by [^{11}C]DTBZ binding [19].

Excess intake of manganese, independent of the route of administration, results in accumulation of the metal in basal ganglia. Studies of active drug abusers using MRI demonstrated increased T1 weighted signal in the globus pallidus and substantia nigra, that is indicative of manganese accumulation. The signal normalizes within a few months after cessation of manganese intake; while clinical symptoms remain unchanged [21, 21]. This feature has also been described in animal experiments with rhesus monkeys [22]. We performed an MRI scan of two Test Item treated animals (data not shown) but did not observe the typical T1 weighted signal increase within 24 h from the last injection despite relatively high doses and high blood concentrations of manganese. A possible explanation is either fast washout of manganese, eliminating the possibility of cellular deposits in the

brain, or the administered amount was insufficient. Blood manganese concentrations measured at 24 h and 48 h after the last injection support the hypothesis of fast washout.

A significant decrease in metabolic activity of the glutamatergic cells throughout the brain and GABA-ergic cells in the basal ganglia has been described in mice after administration of 25 mg/kg of manganese for 21 days [23]. A resulting reduction of paw grip strength was also observed in this study. Another study in mice described manganese accumulation in basal ganglia after administration of 50 or 100 mg/kg of manganese [24]. Manganese doses inducing morphological and clinical changes have been markedly higher than in our study.

The limitations of our study include the lack of direct measurements of striatal dopamine concentration. We used chronic administration of an amphetamine analog to alter striatal dopamine levels, but the dosage was derived from human users and could be too low for rodents. In humans a single small dose of amphetamine is thought to be insufficient to cause a loss of striatal dopamine [25]. We hypothesize that a rodent model of the persistent parkinsonian syndrome is nonetheless feasible by further altering the dosage and administration regimen of the active substances.

The number of various "legal highs" with amphetamine-like psychostimulant properties is constantly increasing. At the same time the toxicity profiles of these substances are not well known and there is a lack of clinical and basic studies to explain the mechanisms of action in the central nervous system. Our findings indicate that chronic treatment with low doses of methcathinone in combination with manganese can induce changes in VMAT2 function.

4. Materials and methods

4.1 Animals and study design

The study was planned as a chronic experiment with a total duration of 27 weeks.
Sixty male C57B6/JRcc Hsd mice (Harlan Laboratories, The Netherlands) aged 5 weeks at the beginning of the study were used. Animals were randomly assigned into one of the four treatment groups (n = 15/group, see Table 1) and housed in smaller groups of seven to eight at standard room conditions with 12h/12h light/dark cycles. They had free access to food and water. All animals were weighed once a week. Weight loss exceeding 20% of body mass was considered the study termination point.
All injections (0.3 ml) were given intraperitoneally once a day for the whole study duration.
For the *in vivo* PET experiments, three male C57Bl/6N mice (aged 10 weeks) from the Central Animal Facility of the University of Turku were used.
Groups were defined by the dose of methcathinone as follows: Group 1 received Test Item containing 100 mg of methcathinone per kilogram of body weight (100% Test Item), Group 2 received (50 mg/kg of methcathinone (50% Test Item), Group 3 received (20 mg/kg of methcathinone (20% Test Item). Group 4 served as the control group and was injected with 0.9% saline (Table 1).

All applicable institutional and national guidelines for the care and use of animals were strictly followed at both participating institutions. The study was approved by the Estonian National Board of Animal Experiments and the Animal Experiment Board of the Province of Southern Finland.

4.2 Substances

Test Item

The Test Item was prepared as previously described [14] to closely resemble the mixture injected by drug abusers. Briefly, 12 pills containing 60 mg of pseudoephedrine hydrochloride (Sudafed, GlaxoSmithKline Pharmaceuticals, Poland) were stripped of the coating layer by thorough washing. The pills were crushed and dissolved in 60 ml of boiling tap water. The oxidizing agent was1 g of potassium permanganate ($KMnO_4$, Grindex Ltd, Latvia) and 0.45 ml of vinegar was added to complete the reaction. The solution was rapidly cooled to room temperature, filtered through regular filter paper to remove visible debris and stored at +4˚C. Dilutions were made using 0.9% saline.

Methcathinone content analysis

The Direct Analysis in Real Time (DART) method was used to measure the methcathinone content in the 100% Test Item. Three separately prepared samples of the mixture were analyzed. The system consisted of a DART ion source (Control software version 1.55, IonSense Inc, Saugus, MA, USA) and the Accurate Time of Fight (AccuTOF) mass spectrometer (JEOL JMS-T100LC, Tokyo, Japan) operating in positive-ion mode and controlled by “Mass Center main” software (Version 1.3.7, JEOL Ltd., Akishima, Japan). Helium (4.0L/min) was used as the ionization gas of the DART ion source. All measurements were performed with the orifice 1 voltage 15V, gas heater temperature of 400˚C, discharge electrode needle at 3000V, electrode voltage at 177V, and grid electrode voltage at 384V. Internal mass calibration was achieved using a dilute solution of polyethylene glycol (PEG) 600 in methanol. Mass to charge ratio (m/z) range of 100-1000 was used in recording mass

spectra. The recorded mass spectral data were processed with the Mass Center software (JEOL, Tokyo, Japan); background subtraction and intensity centralizing were carried out in peak detection.

Manganese content analysis
Manganese concentration in the 100% Test Item was analyzed using a SpectroCirosCCD Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) (Spectro Analytical Instruments GmbH&Co, Kleve, Germany) as described in detail earlier [14]. Manganese levels in the blood samples were determined by atomic absorption spectrometry. Blood for testing was drawn from the left ventricle of the heart immediately after the animal was sacrificed. Animals from Group 1 (n=3) and Group 4 (n=4) were used. Samples were analyzed by electrothermal atomic absorption method (ETAAS) SpectraAA 220Z (Varian, Mulgrave, Australia) with Zeeman-effect background correction. Certified reference materials (Seronorm Trace elements Whole Blood L1 LOT MR4026 and L2 LOT 0503109) were used. In cases of high manganese content, 0.2 ml of blood was diluted to 1.0 ml with milliQ water, while, for normal levels, 0.1 ml of blood was diluted to 1.0 ml with milliQ water.

4.3 Imaging studies
Radiosynthesis
The [^{11}C]DTBZ was synthesized at the Radiopharmaceutical Chemistry Laboratory of the Turku PET Centre and the final product was formulated in 0.9% NaCl / 0.1 M phosphate buffer (3/2, v/v). The specific radioactivity of [^{11}C]DTBZ was 307.9 $\pm$ 228.8 MBq/nmol (n = 6, range from 36.7 to 623.7 MBq/nmol) at the end of synthesis. The radiochemical purity of the product exceeded 95% in all syntheses.

In vivo PET imaging
Dynamic *in vivo* PET imaging (13 frames: 5 x 60, 1 x 300, 5 x 600 s) of healthy, untreated C57Bl/6N mice (n=3) was performed to determine the optimal time point for subsequent *ex vivo* brain autoradiography experiments. Imaging was performed using the Inveon multimodality PET/CT scanner (Siemens Medical Solutions, Knoxville, TN, USA) as described earlier [15]. The scan started immediately after intravenous injection of [^{11}C]DTBZ (injected dose 9.6 ± 4.5 MBq, injected mass 16.1 ± 15.0 ng). Striata and cerebellum were selected as regions of interest (ROIs) using Inveon Research Workplace Image Analysis software (Siemens Medical Solutions, Knoxville, TN, USA). A CT template from the same mouse and a co-registered general mouse brain MRI template were used as anatomical references. The uptake of ^{11}C-radioactivity in the brain was evaluated as striatum-to-cerebellum ratios from the time-activity curves.

Ex vivo [^{11}C]DTBZ *tissue counting, digital autoradiography and image analysis*
Animals from Group 1 (100% Test Item) and Group 4 (control) were used for *ex vivo* [^{11}C]DTBZ experiments after 13 weeks (Group 1 n = 4; Group 4 n = 4) and 27 weeks of treatment (Group 1 n = 3; Group 4 n = 4).
The animals were killed by CO_2 inhalation 10 min after the injection of [^{11}C]DTBZ. The intravenously injected radioactivity per animal was 25.4 ± 4.7 MBq (injected mass range 33 to 580 ng). The brains were dissected, measured for radioactivity, frozen and sectioned for digital autoradiography as described earlier [15].

The obtained autoradiography images were analyzed for count density (photostimulated luminescence per unit area, PSL/mm^2) ratios between defined ROIs for striata and cerebellum with a computerized image analysis program (Aida 4.22, Raytest Isotopenmessgeräte, GmbH, Straubenhardt, Germany). The ROIs were drawn on at least three different sections from every animal. Count densities for background areas were subtracted from each ROI value, the mean values were calculated and the striata-to-cerebellum ratios were obtained.

4.4 Locomotor activity testing

Motility box

Locomotor activity (evaluated as the time in locomotion and travelled distance) and exploratory behavior (evaluated as the time spent on rear feet and in the center of the box) were tested at baseline, and at 13 and 27 weeks. Six animals from each group were tested. Animals were placed alone into a photoelectric motility box (448 mm x 448 mm x 450 mm) connected to a computer (ActiMot/MoTil, TSE Technical & Scientific Equipment GmbH, Germany) immediately after receiving the injection of Test Item or saline. The illumination level of the transparent test boxes was approximately 400 lux. Data were captured for the first 60 min. The box center was defined as 60% of the centermost area of the floor. Group means were calculated for further statistical analysis.

Ink test

Ink testing was carried out every four weeks seven times during the experiment. Nine animals from each treatment group were included. Testing was carried out before giving the injections of Test Item or saline. After coating the animals' feet with non-toxic colored ink (red ink for front feet and blue ink for rear feet) the mice ran along a 50 cm tunnel that was lined with white paper. Resulting footprints were used to measure the stride length between front and rear feet on both sides. Five steps were measured in each case and the average step length was calculated for each animal. Group means were used for statistical analysis.

4.5 Data analysis

Results are expressed as means and standard deviations with 95% confidence intervals. Statistical analysis was performed with GraphPad Prism, version 5.01 (GraphPad Software, San Diego, CA, USA). Manganese concentrations were analyzed by Kruskall-Wallis test and motility box data were analyzed by using two-way ANOVA. Autoradiography results from the mice treated with Test Item (Group 1) or saline (Group 4) were compared between groups using unpaired two-tailed Student's t-test. The limit for statistical significance was set at $p<0.05$.

5. References

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Figure Legends

Figure 1. Blood manganese concentrations. Results for mice treated with the 100% Test Item (Group 1, n = 3) 24 and 48 h after the last injection at 27 weeks and Control (Group 4, n = 4).

Figure 2. Representative *in vivo* PET images of a non-treated C57Bl/6N mouse (time frames 10-20 min after [^{11}C]DTBZ injection) from the levels of striata and cerebellum (A), and time-radioactivity curves as percentage of injected dose/g tissue (%ID/g) obtained from VOIs drawn to the striata and cerebellum (B). Striata-to-cerebellum ratio curve was determined for the duration of the 60 min scan and it stayed constant from 5 min onwards. The 10 min time-point was chosen for the *ex vivo* brain autoradiography studies (C).

Figure 3. *Ex vivo* autoradiography images of brain slices showing ^{11}C-radioactivity uptake 10 min after [^{11}C]DTBZ injection. Left slices are from the cerebrum at the level of striatum (S) and cortex (CTX), and lower slices are from the cerebellum (CB). A mouse treated with 100% Test Item (27 wk, upper panel) and a saline treated mouse (27 wk, lower panel), Red/yellow color represents high uptake and blue represents low uptake of radioactivity. Color scales of the images are normalized to the injected dose (ID) per animal.

Figure 4. *Ex vivo* brain autoradiography results after 13 weeks (Group 1 [treated], n = 3; Group 4 [control], n = 3) and 27 weeks (Group 1, n = 3; Group 4, n = 4) of treatment. Accumulation of ^{11}C-radioactivity in striata (Str) compared with cerebellum (CB) 10 min after the [^{11}C]DTBZ injection.

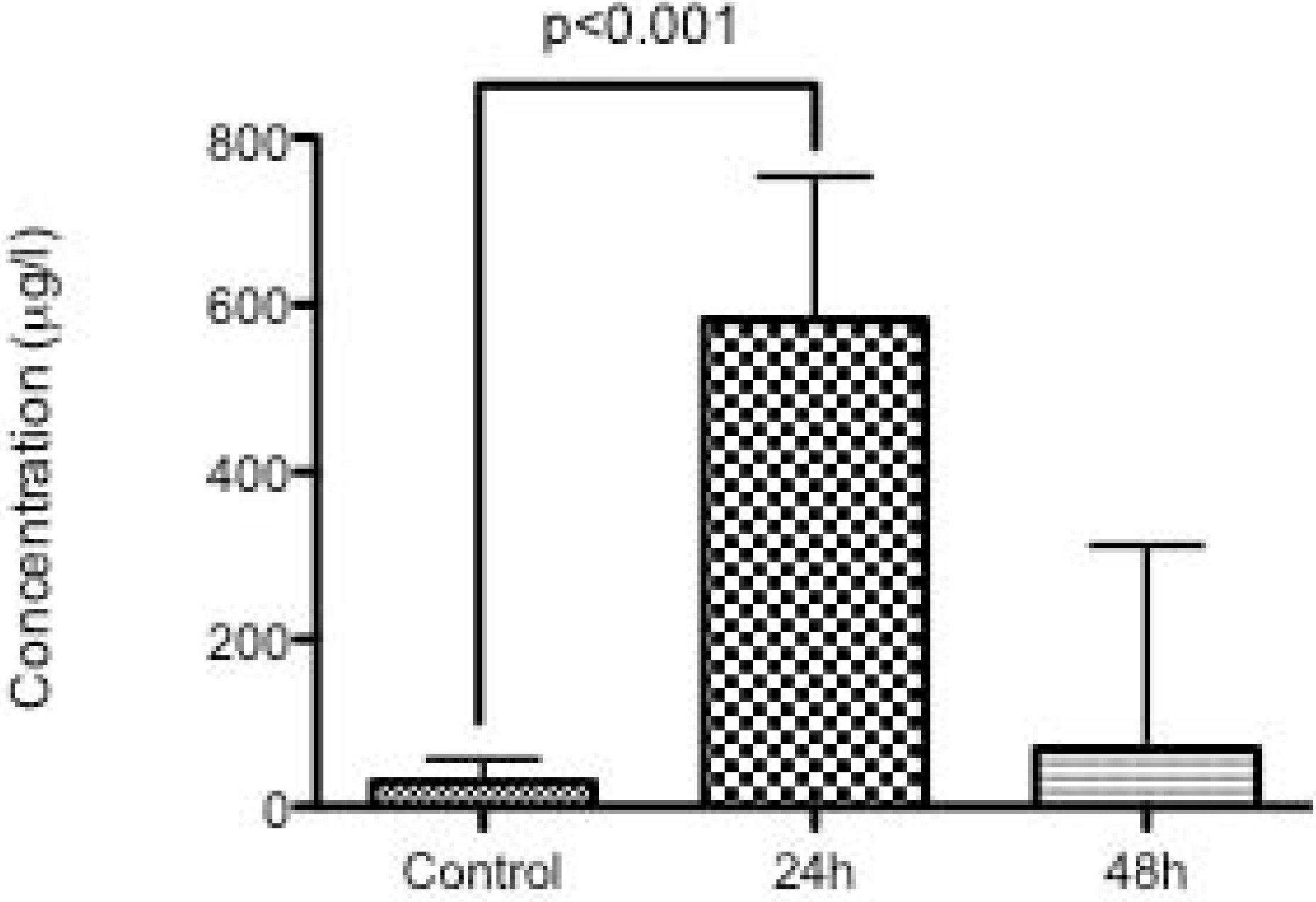
Table 1. Description of the treatment groups and numbers of animals used for testing.

	Group 1	**Group 2**	**Group 3**	**Group 4**
	100% Test Item	50% Test Item	20% Test Item	Saline
Treatment, average substance dose				
Methcathinone (mg/kg)	98.7	49.3	19.7	-
Manganese (mg/kg)	6.88	3.44	1.38	-
Mean weight (g)				
Week 1	29	31	31	33
Week 27	28	28	30	28
Number of animals used for testing				
Group total	15	15	15	15
Motility Box	6	6	6	6
Gait analysis	9	9	9	9
Ex vivo autoradiography	6	-	-	7
Brain tissue counting	7	-	-	8

Table 2. Motility box test results. Six animals from each group were tested 3 and 6 months after the treatment began. Results were compared with the control group (Group 4) at the same time point.

	Distance (m)		Time in center (s)		Time on rear feet (s)	
	3 months	6 months	3 months	6 months	3 months	6 months
Group 1	203.8	343.0*	3.2***	23.5***	142.8	58.7***
Group 2	241.0	395.3***	3.0***	49.8***	190.5	136.2**
Group 3	425.7***	362.7**	23.7*	61.0*	198.0	337.5
Group 4	209.2	214.8	77.2	138.2	318.2	384.3

*=p<0.05; **=p<0.01; ***=p<0.001.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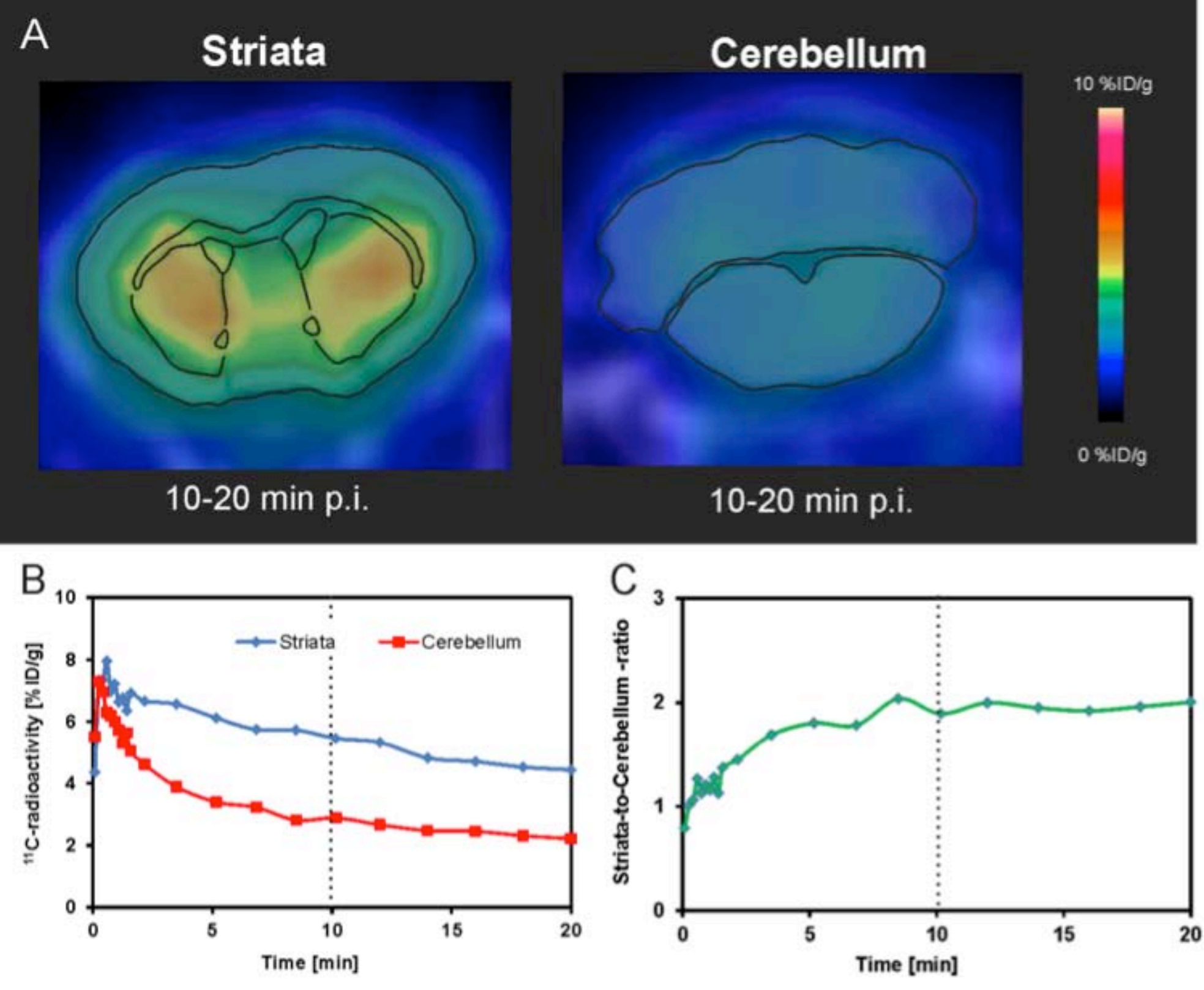
A
Striata
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10-20 min p.i.
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Striata
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Time [min]
C
Striata-to-Cerebellum -ratio
Time [min]

Test item
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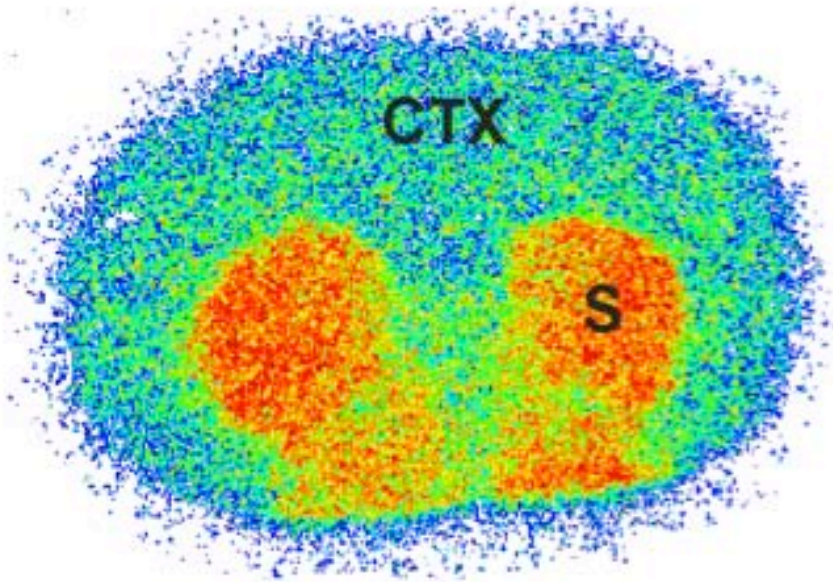
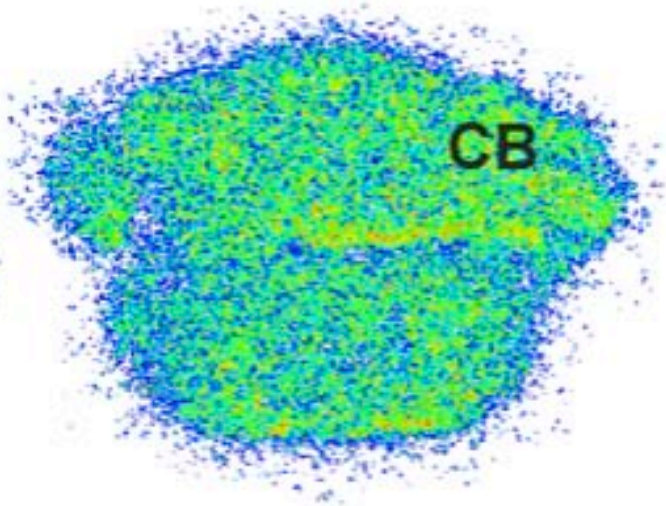
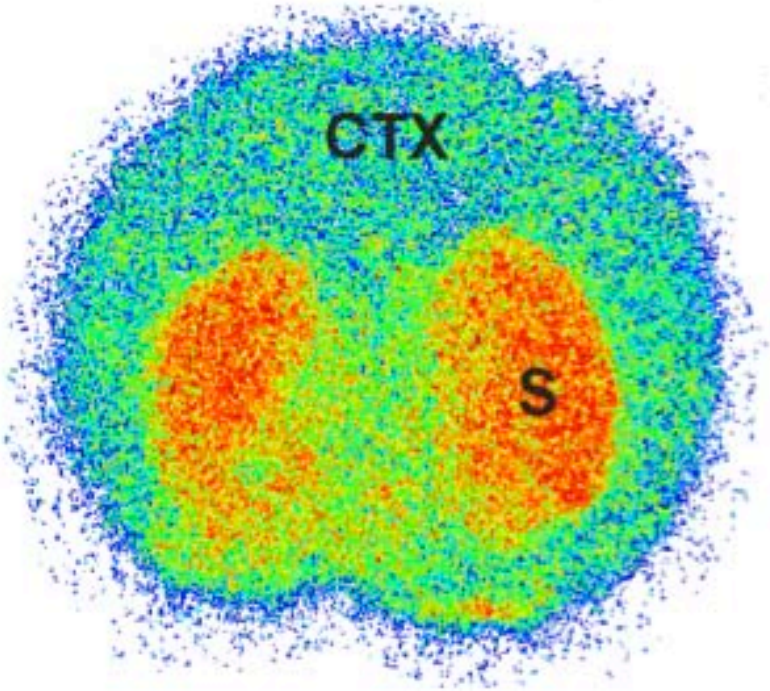
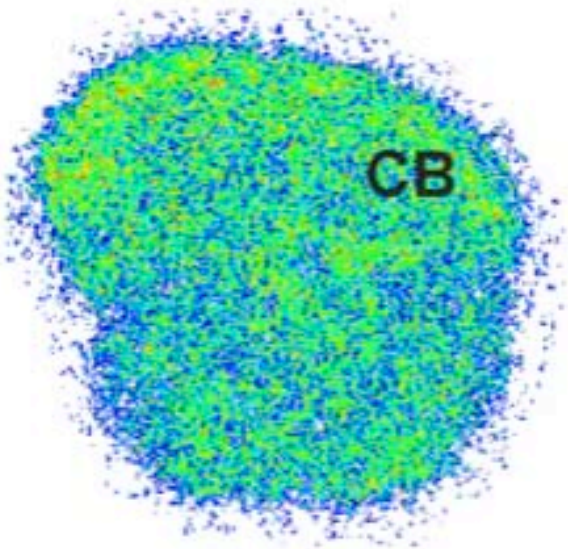
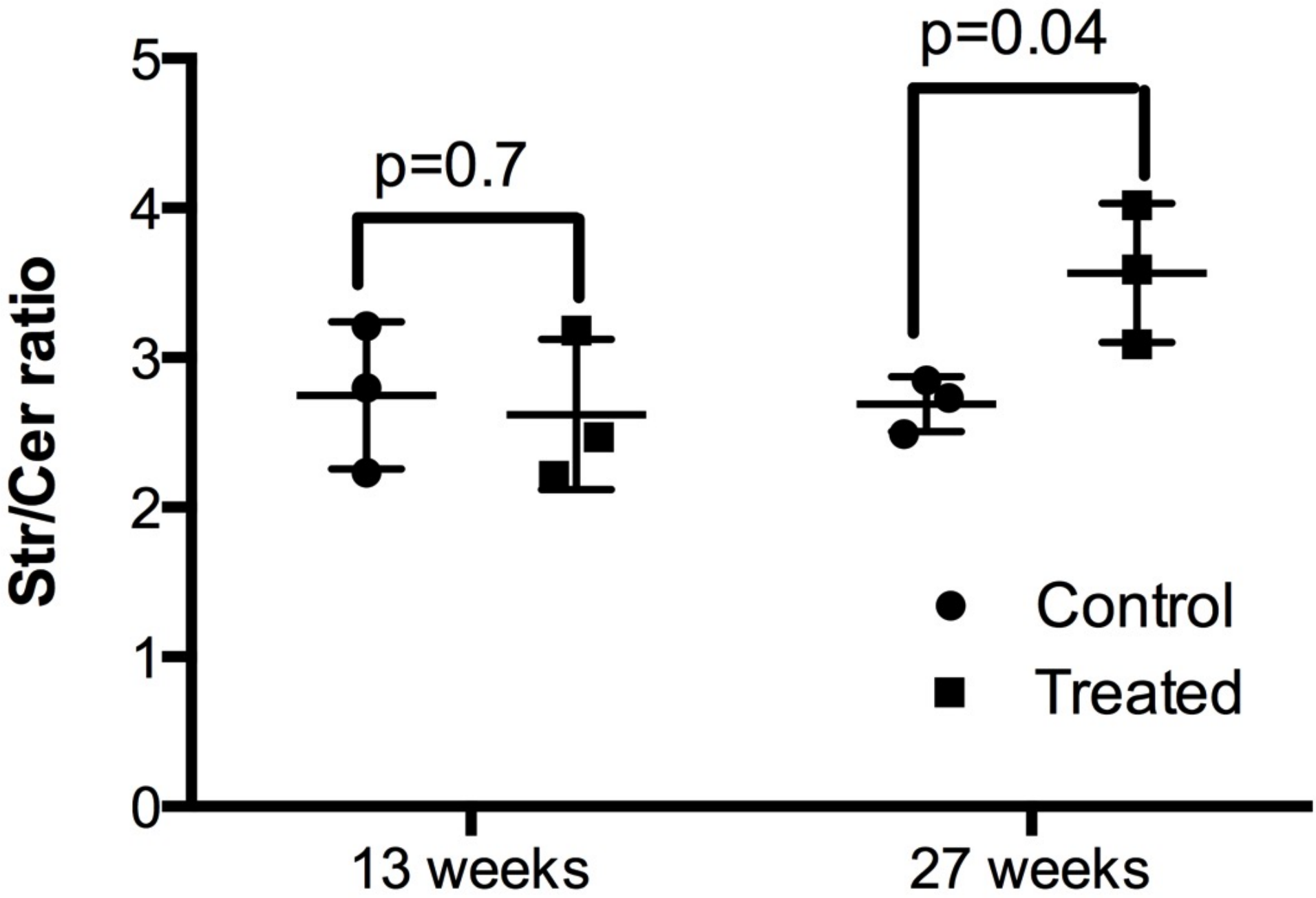




Saline
ID = 31.6 MBq






p=0.04
p=0.7
5
4
3
2
1
0
Str/Cer ratio
Control
Treated
13 weeks
27 weeks