

## Time-dependent effects of striatal interleukin-2 on open field behaviour in rats

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### ABSTRACT

There is evidence that immune messengers like cytokines can modulate emotional and motivated behaviours and are involved in psychiatric conditions like anxiety, and depression. Previously, we showed that cytokine gene expression (interleukin (IL)-2 mRNA) in specific brain tissues (striatum, prefrontal cortex) correlated with anxiety-like behaviour (open arm time) in an elevated plus-maze in rats. In a subsequent experiment, a single striatal IL-2 injection showed behavioural trends with the lower dose (1 ng) acting in a behavioural suppressive way, whereas the highest dosage (25 ng) led to activation and anxiolytic-like behaviour. Here, to support and extend our previous findings, we tested Wistar outbred rats after a single unilateral (balanced brain sites) IL-2 injection into the ventral/dorsal striatum followed by an open field test acutely and 24 h later. Analyses for horizontal locomotion showed no differences between groups. However, rats with IL-2-treatment (0.1 ng) showed a dose-dependent avoidance effect (i.e. reduced centre time) compared to the 1 ng group and vehicle controls 24 h later. In addition, suppression of free rearing activity was shown for both IL-2 doses (0.1; 1 ng) compared to saline in the acute test, and partly 24 h later. Thus, in experiment 2, we tested for proactive drug mechanisms to test for delayed effects of IL-2 as observed in experiment 1. In a new sample, rats were returned to their home cages after a striatal IL-2 injection (0.1; 0.01; 0 ng), and tested 24 h and 48 h after the injection in an open field. Neither for the first (24 h) nor for the second exposure (48 h later) did the analyses show any significant behavioural effects. We therefore suggest that emotional-related behaviour can be modulated by striatal IL-2 for at least 24 h. However, such IL-2 effects can only be observed if a mild stressful environmental challenge (i.e., forced open field exposure) is followed immediately after injection. In conclusion, proactive drug effects may be excluded for striatal IL-2 effects on open field behaviour.

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### 1. Introduction

Interleukin-2 (IL-2) is a pleiotropic cytokine that is mainly produced by T-cells and plays a crucial role in initiation and modulation of the cellular immune response in the periphery (Gaffen and Liu, 2004). However, IL-2 is also prominent in the central nervous system where it acts as a neuromodulator in specific brain areas (Hanisch and Quirion, 1996; Nisticò, 1993). Both IL-2-like and IL-2-like receptors are found in the hippocampus, hypothalamus, cerebellum, prefrontal cortex, striatum, septum, and locus coeruleus of humans and other mammals (Hwang et al., 2006; Hanisch and Quirion, 1996; Lapchak et al., 1991); brain regions that are known to play a critical role in psychiatric conditions. In addition, IL-2 influences monoaminergic neurotransmission in the CNS

(Anisman et al., 1996; Lacosta et al., 2000; Petitto et al., 1997; Song et al., 1999; Zalcman et al., 1994), which are often linked to psychological diseases like depression, or anxiety disorders (Booij et al., 2003; Nestler et al., 2002).

Accumulating evidence suggest important relationships between IL-2 and emotional and motivated behaviours (Hanisch, 2001). For example, patients diagnosed with anxiety disorders exhibited lower IL-2 production levels in peripheral blood compared to healthy controls (Koh and Lee, 1998, 2004), whereas patients with panic disorders displayed increased IL-2 serum levels (Rapaport and Stein, 1994). Even more, IL-2 which is known through its application in cancer therapy (Waldmann, 2006), evokes various negative side-effects of emotional and motivational affect during treatment, for example, increasing depressive-like symptoms (Anisman et al., 2005; Capuron et al., 2002).

In animals, systemically administered IL-2 induced depressive-like effects (Anisman et al., 2002), increased locomotor activity (Petitto et al., 1997), and novelty-induced locomotion and exploration behaviour in rodents (Zalcman et al., 1998; Zalcman, 2001). Finally, IL-2 inhibited responding for rewarding lateral hypothalamic stimulation (Anisman et al., 1996, 1998), and demonstrated dose-related effects on

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hedonic processes arising from medial forebrain bundle stimulation (Migueluez et al., 2004).

Only a few animal studies analysing anxiety-related behaviour and IL-2 exist, most with ambiguous results. Neither acute nor repeated IL-2 application led to alteration in elevated plus-maze behaviour in IL-2-treated rats or mice compared to saline (Anisman et al., 2002; Connor et al., 1998; Lacosta et al., 1999; Petitto et al., 1997). On the other hand, it was shown that IL-2/15R $\beta$  knockout mice exhibited a reduced anxiety-like behaviour in the elevated plus-maze when compared to wild type and heterozygous mice (Petitto et al., 2002).

Studies investigating the behavioural effects of centrally administered IL-2 are still scarce. For example, IL-2 microinjected into the medial hypothalamus suppressed defensive rage, while microinjections of IL-2 in the periaqueductal gray (PAG) potentiated defensive rage in cats (Bhatt and Siegel, 2006; Zalcman and Siegel, 2006). Even more, depending on the brain injection area, IL-2 led to soporific effects (Nisticò, 1993), ipsilateral turning and asymmetric body posture (De Sarro et al., 1990), and increased locomotion and exploratory behaviour in rats (Nisticò and De Sarro, 1991).

In our previous studies, we showed that cytokine mRNA expression in specific brain tissues correlated with anxiety-like behaviour in rats gauged by open arm time in the elevated plus-maze. These relationships indicated that cytokines in the brain can be related to avoidance behaviour, and that this relationship is site- (striatum, prefrontal cortex), and cytokine-specific (IL-2; Pawlak et al., 2003, 2005). This hypothesis was functionally tested by microinjections of IL-2 into the striatum, followed by testing on the elevated plus-maze acutely and after a delay of 24 h. Overall, rats showed dose-dependent effects, particularly with a trend for anxiolytic-like effects and more rearings in the higher dose (25 ng), whereas the lowest dose tested (1 ng) led to anxiogenic-like effects and decreased rearings compared to saline controls, although the differences were not significant (Pawlak and Schwarting, 2006). These results suggest that IL-2 may act in a biphasic manner and that IL-2 behavioural effects peak after a yet unspecified delay of time.

To support our previous data, we conducted two further experiments to gain supplementary insights into the actions of central cytokines. In the present study we tested the dose–response curve of microinjected striatal IL-2 and possible delayed drug effects. Therefore, in experiment 1, we investigated the acute and delayed effects of different doses of IL-2 on emotional and motivational behaviour. Based on our previous results (Pawlak and Schwarting, 2006), we hypothesised that a striatal IL-2 microinjection (1 ng; 0.1 ng) has no impact on horizontal locomotion, but dose-dependently increases anxiety-like avoidance behaviour and decreases vertical activity (rearing) compared to vehicle controls. Taking into account that the temporal profile of IL-2 effects is unknown, we tested the rats in an open field, which allows a longer testing time than in the elevated plus-maze. The open field also constitutes an aversive environment in which rodents avoid the open centre, and prefer zones where walls offer protection from potential aerial predators (Prut and Belzung, 2003).

In experiment 2, we tested the hypothesis that the predicted avoidance results of experiment 1 might be explained by a proactive drug effect, that is, independent of immediate exposure to the testing environment. Compared to experiment 1, we tested the lowest and most effective dose of IL-2 again (0.1 ng) and one even lower dose (0.01 ng).

## 2. Experiment 1

### 2.1. Methods

#### 2.1.1. Animals

Forty-eight male outbred adult Wistar Unilever rats (Harlan Winkelmann, Borcheln, Germany) were initially used, weighing between 260 and 298 g (mean: 277.6, standard error:  $\pm$  1.4 g) at the arrival in the lab. They were housed individually in purpose-built acrylic cages

(21 $\times$ 20 $\times$ 38 cm), which were higher than normal single cages to prevent injuries after surgery. The animals were kept under standard laboratory conditions with food and water available *ad libitum* and a 12 h light–dark cycle (lights on: 7:00–19:00 h).

#### 2.1.2. Design

Upon arrival, animals were weighed every day until the end of the experiment. In addition, before surgery all animals were gentled in a self-defined procedure during which each rat was touched and picked up for 5 min (days 3, 5, and 7). Surgery was performed on day 8. After surgery, animals were gentled again for three days (days 12–14), but now with another purpose-defined procedure to prepare them for the microinjection. On day 15 the animals received a single unilateral microinjection. After the injection the animal was returned to its home cage, carried to the dimly lit behavioural testing room and left alone for 5 min. Then, it was tested in an unfamiliar open field (OF 1), and 24 h later the animal was retested without further pharmacological treatment (OF 2). This time, the animal was directly brought to the testing room, where it again was kept alone for 5 min before being tested. The open field test lasted 45 min each. All animal manipulations throughout the study were conducted during the light cycle between 09:00 and 18:00 h. Great caution was exerted to counterbalance the time of the day for the injections of the different doses to avoid possible circadian effects. All experiments were implemented in accordance with the ethical regulations for animal experimentation at the University of Marburg.

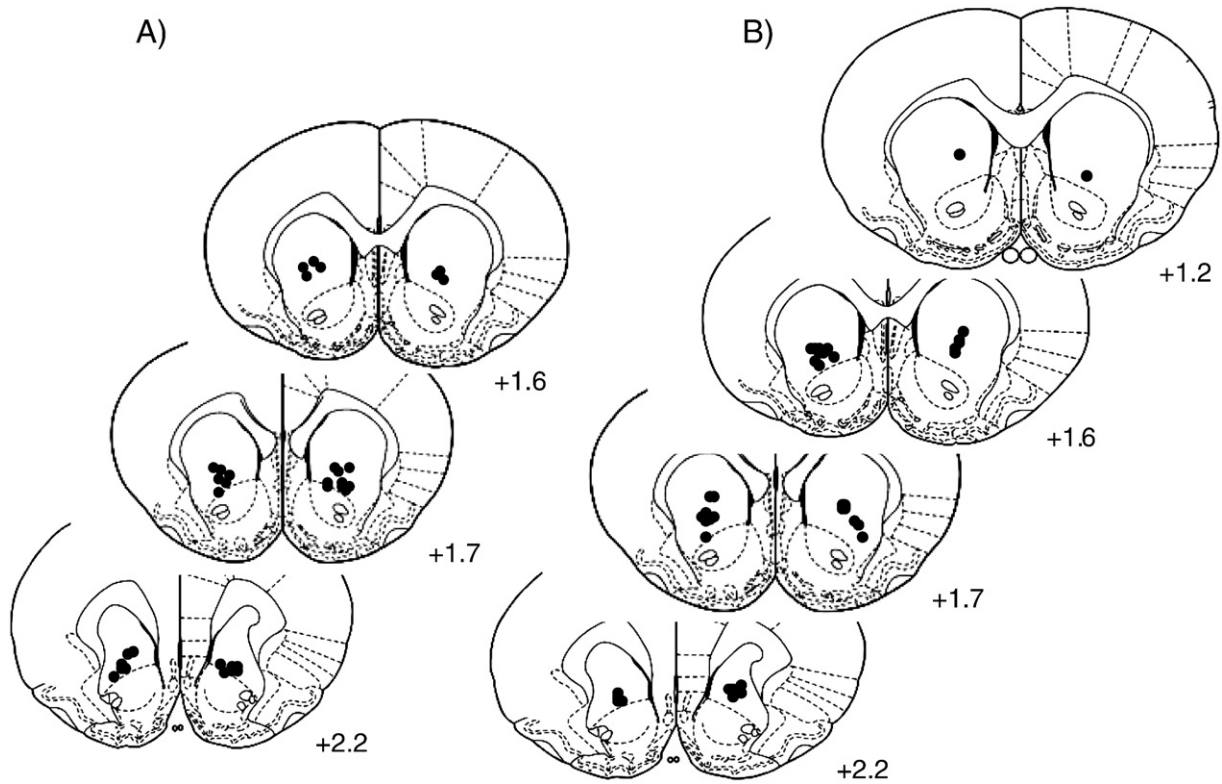
#### 2.1.3. Surgery

On day 8, animals underwent stereotactic surgery to implant one unilateral permanent cannula into the striatum, which was inserted alternately in the right or in the left hemisphere. Rats were anaesthetised with a mixture of 10 mg/kg Xylazine (0.5 ml/kg Rompun<sup>®</sup>, Bayer, Germany) and 100 mg/kg ketaminhydrochlorid (1 ml/kg Ketavet<sup>®</sup>, Upjohn, Germany). The animals were fixed in the stereotactic apparatus (TSE-Systems, Bad Homburg, Germany) and an unilateral guide cannula (C317G, 23 G, inside diameter (ID) 0.32 mm, outside diameter (OD) 0.64 mm, Plastics One, USA) was implanted permanently. The target area was the centre of the striatum as previously used (Pawlak and Schwarting, 2006), with the coordinates anterior = 1.6 mm, lateral =  $\pm$  2.0 mm, ventral = 5.8 mm, relative to bregma (Paxinos and Watson, 1998). The guide cannula was fixed with dental cement (Paladur, Heraeus-Kulzer, Germany), which was kept in place by two stainless skull screws. Removable stainless steel dummy cannulae (C317 DC, OD 0.3 mm, Plastics One, USA) were placed into the guide cannula. Finally, the animal was put into its home cage, brought back into the animal room where it was kept for approximately one h under red light conditions.

#### 2.1.4. IL-2 treatment

One week after the surgery (day 15), the rats were microinjected as follows: The respective animal was brought in a different room other than the testing room using the home cage. Carrier-free recombinant rat IL-2 (R&D Systems, USA) was delivered as a 10  $\mu$ g lyophilised filtered solution in 20 mM of ammonium acetate. Appropriate volumes of sterile phosphate buffered saline (PBS) were added to obtain aliquots containing 1 ng, or 0.1 ng of IL-2. Experimental rats were injected once with one of the two IL-2 doses (1 ng; 0.1 ng), and control animals received injections of PBS vehicle. All procedures were performed with protein low binding pipette tips and tubes, respectively.

All injections were conducted by an injection system consisting of a syringe pump (Syringe Pump 101, World Precision Instruments, Germany), and a syringe (diameter 0.46 mm; 10  $\mu$ l, 1701LT Hamilton, Switzerland), which was attached via flexible polyethylene tubing (ID 0.4 mm, OD 0.8 mm) to the injection cannula (C317I, 30 G, ID 0.15 mm, OD 0.3 mm, Plastics One, USA). The injection cannula was protruding 0.5 mm from the guide cannula. Injections were performed with an injection rate of 0.2  $\mu$ l/min (volume 0.5  $\mu$ l) and were started 30 s after



**Fig. 1.** Coronal sections according to the atlas of Paxinos and Watson (1998) showing the areas of the cannula tip placement in the striatum of A) experiment 1, and B) experiment 2. The figures summarise the correct tip placements from all included animals. The number next to each section refers to its position in the antero-posterior plane relative to bregma (mm). In the individual rat, the cannula was implanted either in the left or right hemisphere.

lowering the injection cannula. After the injection, the injection cannula remained in the guide cannula for further 30 s. During this procedure, the animal was placed on a table where it was hand-held gently.

#### 2.1.5. Behavioural testing: open field (OF)

The OF consisted of a black circular box (inside diameter 79 cm with 50.5 cm high walls), which was monitored by an automated activity monitoring system (EthoVision Pro 3.0, Noldus, Netherlands). All trials were recorded on videotape by a video camera placed ~200 cm above the centre of the apparatus. Behaviour was tested under dimmed white light of 30 lx (centre of the apparatus). The animals were tested 5 min after injection of IL-2 or PBS in OF 1 and re-tested 24 h later, without further drug treatment (OF 2). Each open field test lasted 45 min.

For anxiety-like avoidance behaviour we measured the time spent in the different areas in the OF. We divided the OF into a margin and a centre area. Margin area was considered as a ring near the wall of 9.75 cm width. The remaining middle area of the apparatus was defined as the centre area.

Using EthoVision system, we analysed distance moved, centre time, and entries into the centre. It is commonly accepted, that anxious animals spend more time in the margin area than in the centre (e.g., Clement and Chapouthier, 1998). Therefore time spent in the centre of the open field was taken as an inverse measurement of avoidance behaviour. Distance moved was taken as measure of horizontal locomotor activity to express general activity.

The frequency of rearing behaviour was subdivided into on-wall and off-wall (free) because there is evidence that these two behaviours might be qualitatively different (Lever et al., 2006). One rearing was counted when the rat stood on its hind paws, raised both forepaws off the ground, and stretched its back. The rearing was considered to end when at least one forepaw had reached the floor again. The on-wall

rearing was scored when the animal had at least one forepaw on the wall during rearing time, while off-wall rearing was counted when the animal did not touch the wall with any forepaw at any time during one rearing activity. Rearing behaviour was scored from videotape by observers blind with respect to treatment. We also measured grooming behaviour, which was defined as licking, brushing, or scratching of face and body (duration, frequency). The interrater reliability was analysed with Pearson correlations. For all subjective behavioural analyses correlations between two independent raters ranged between  $r=0.825$  to  $r=0.981$ ,  $P$ -values  $\leq 0.006$ .

#### 2.1.6. Histological evaluation

After OF 2, animals were deeply anaesthetised with i.p. 2.5 ml/kg pentobarbital-sodium (Narcoren®, Merck, Germany) and transcardially perfused with 0.9% saline solution followed by perfusion solution containing 4% formaldehyde. Correct placements of the cannulae were validated by standard histological analysis. Rat brains were removed, postfixed, stored at  $-20$  °C and cut into 30  $\mu$ m thick sections using a cryostat (Leica Microsystems, Germany). Thereafter, we used cresyl violet staining to verify injection sites by two observers blind with regard to treatment, or behaviour.

#### 2.1.7. Data analysis

Behavioural coding was executed by trained observers blind to treatment. Behaviour was analysed in three time blocks of 15 min each. Analyses were performed using ANOVAs with repeated measures for each OF test, with the three time blocks (15, 30, 45 min) as a within-subject factor, and the treatment groups (0 ng; 0.1 ng; 1 ng) as between-subjects factor. All but one three-factor ANOVAs showed significant test day effects which only can be further disentangled by two-factor ANOVAs. However, analyses for each day are essential to the understanding of the underlying mechanisms between acute versus delayed effects. Thus, we report here only two-factor ANOVAs for each

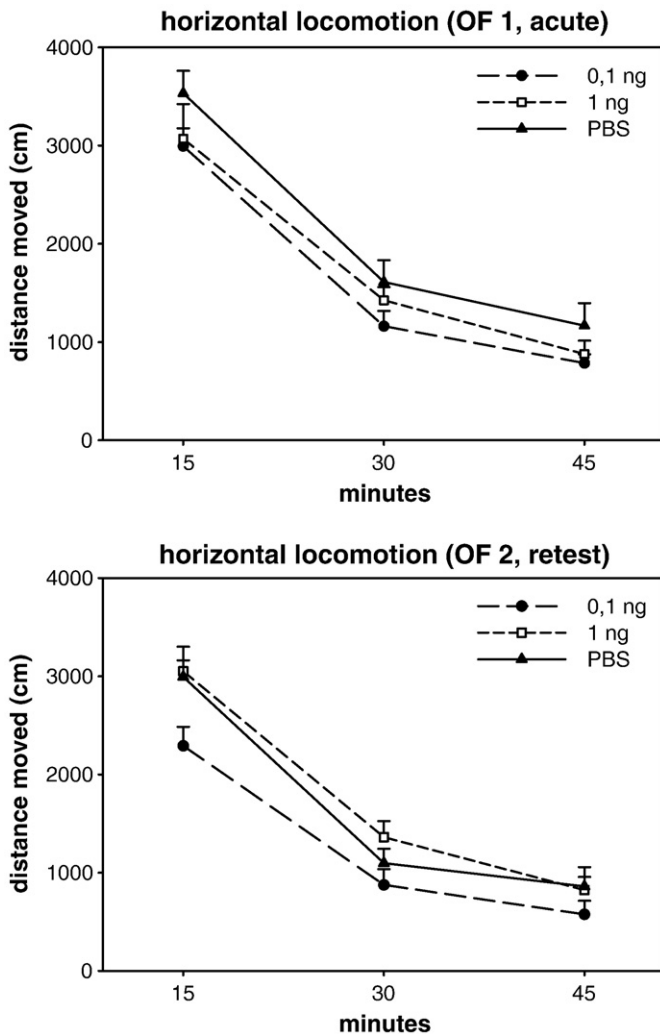
test day separately. Post hoc comparisons were made by least significant difference (LSD) tests.

ANOVA results revealing a statistical trend ( $P < 0.10$ ) were also analysed by post hoc comparisons (Pawlak and Schwarting, 2006). The rationale is that the overall  $F$  is not required in order to conduct multiple comparisons (Howell, 1992; Wilcox, 1987). In some cases we additionally performed univariate ANOVAs to analyse differences between the groups at single time points. Data are expressed as mean  $\pm$  SEM. Post hoc  $p$ -values for locomotion, free rearing, and grooming are two-tailed and taken as statistically significant when  $P \leq 0.05$ , whereas post hoc  $p$ -values for centre time and rearing behaviour are one-tailed according to our predictions based on our previous results (Pawlak and Schwarting, 2006).

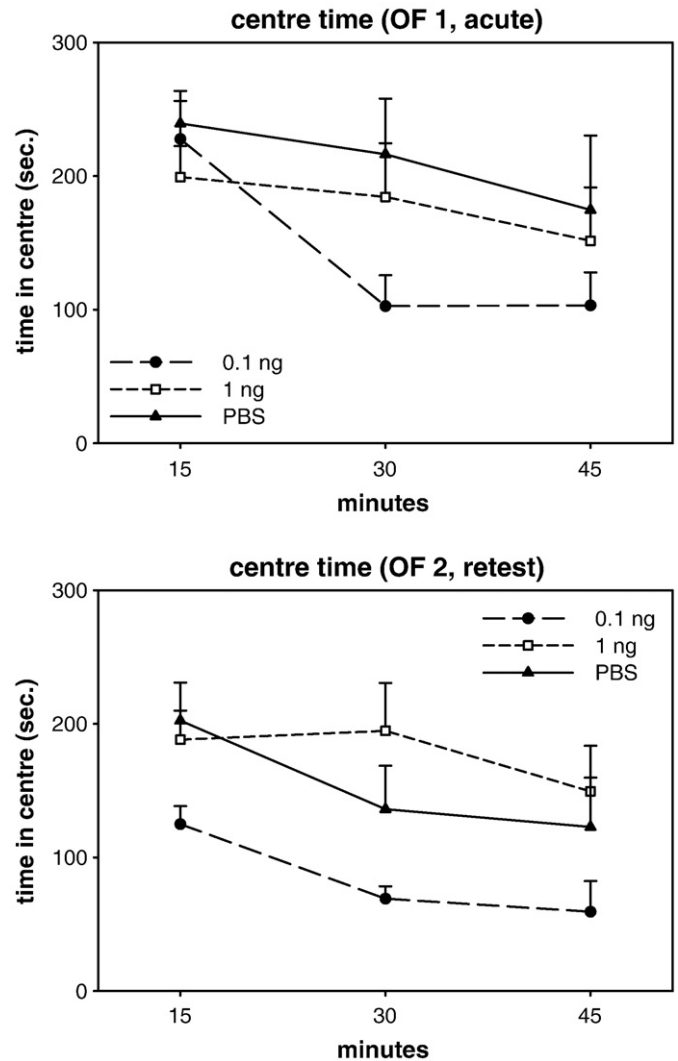
## 2.2. Results

### 2.2.1. Histological evaluation

Only data from rats with correctly placed cannulae were analysed ( $n = 34$ ). For details see Fig. 1A. The final group sizes were: PBS = 10 (left 5 / right 5), 1 ng IL-2 = 13 (7/6), 0.1 ng IL-2 = 11 (5/6) rats.



**Fig. 2.** Experiment 1: Effects of striatal IL-2 on horizontal locomotion (distance moved) in an open field (OF). The OF test (45 min) was performed 5 min (acute) and again 24 h (retest) after a unilateral striatal microinjection of IL-2 (0 ng, 0.1 ng, 1.0 ng). Data are shown in  $3 \times 15$  min blocks and are expressed as mean  $\pm$  SEM. Please refer to the text for the statistical analyses.



**Fig. 3.** Experiment 1: Effects of striatal IL-2 on centre time in an open field (OF). The OF test (45 min) was performed 5 min (acute) and again 24 h (retest) after a unilateral striatal microinjection of IL-2 (0 ng, 0.1 ng, 1.0 ng). Data are shown in  $3 \times 15$  min blocks and are expressed as mean  $\pm$  SEM. Please refer to the text for the statistical analyses.

### 2.2.2. Locomotion (horizontal activity)

The degree of locomotion was measured automatically by the variable distance moved. A clear habituation in locomotion behaviour could be seen in both tests (OF 1, OF 2) with a continuous decrease of distance travelled during the whole test time (OF 1:  $F(2, 31) = 216.8$ ,  $P < 0.001$ ; OF 2:  $F(2, 31) = 268.2$ ,  $P < 0.001$ ; Fig. 2). There was no difference in locomotion behaviour between IL-2 treated and PBS treated rats for OF 1 ( $F(2, 31) = 1.6$ ,  $P = 0.214$ ), but a trend for a dose effect at the undrugged retest 24 h later (OF 2:  $F(2, 31) = 3.0$ ,  $P = 0.063$ ). LSD post hoc analysis for OF 2 revealed significantly more locomotor activity in animals treated with the higher IL-2 dose (1  $\mu\text{g}$ ,  $P = 0.024$ ) compared to the lower dose of 0.1 ng. Further, the 0.1 ng treatment revealed a trend for lower horizontal activity compared to the control group ( $P = 0.082$ ).

### 2.2.3. Anxiety-like avoidance behaviour (centre time)

Both IL-2 treated groups tended to spend less centre time compared to the control group (Fig. 3), although the centre time was not significantly different between IL-2 groups and control group ( $F(2, 31) = 1.1$ ,  $P = 0.358$ ) in OF 1. However there was a trend for an interaction effect between time and group ( $F(4, 62) = 2.3$ ,  $P = 0.067$ ). The OF 2 test was performed on the subsequent day and without any further drug treatment. Analysis

showed a significant dose effect ( $F(2, 31)=4.4, P=0.022$ ). LSD post hoc tests revealed significantly less time spent in the centre for the 0.1 ng IL-2 treated rats compared to the 1 ng IL-2 treated group ( $P=0.004$ ), and a significant reduction compared to the control group ( $P=0.027$ ). Parallel to locomotor activity, on both test days there was a significant time effect (OF 1:  $F(2, 31)=11.4, P<0.001$ ; OF 2:  $F(2, 31)=7.8, P<0.001$ ) for the time spent in the centre, showing that rats spent less time in the centre with progression of time.

#### 2.2.4. Rearing (vertical activity)

In both OFs we observed decreasing general rearing activity during the course of testing (OF 1:  $F(2, 31)=122.7, P<0.001$ ; OF 2:  $F(2, 31)=108.0, P<0.001$ ), Table 1. Neither on OF 1 nor on OF 2 statistical analysis revealed significant differences between the IL-2 groups and the control group (OF 1:  $F(2, 31)=1.1, P=.344$ ; OF 2:  $F(2, 31)=1.5, P=0.231$ ). However, on OF 2 we observed a significant interaction effect for total rearing activity ( $F(4, 62)=3.9, P=0.007$ ). When considering the single test points of the 15 min blocks it shows that there is a significant difference between the groups in the first time block (1–15 min;  $F(2, 31)=3.6, P=0.020$ ; LSD: 0.1 ng < 1 ng IL-2:  $P=0.007$ ; 0.1 ng IL-2 < PBS:  $P=0.038$ ), and during the second time block (16–30 min;  $F(2, 31)=3.4, P=0.024$ ; LSD: 0.1 ng < 1 ng IL-2:  $P=0.018$ ; PBS < 1 ng IL-2:  $P=0.017$ ).

When rearing activity is divided into on-wall and off-wall (free) rearing, both IL-2 treated groups showed less free rearing activity compared to PBS treated animals in OF 1 (Fig. 4), although this analysis did not quite reach statistical significance ( $F(2, 31)=3.1, P=0.061$ ). LSD post hoc tests revealed significantly less free rearing in both IL-2 treated rats compared to PBS (1 ng:  $P=0.043$ ; 0.1 ng:  $P=0.032$ ). Furthermore, analysis on OF 2 revealed a trend for an interaction effect ( $F(4, 62)=2.4, P=0.061$ ) in free rearing activity (Fig. 4). Moreover, post hoc LSD tests showed that free rearing in the 0.1 ng IL-2 group was significantly lower compared to the PBS group in the first time block (1–15 min;  $P=0.050$ ). We found a significant time effect on both test days, showing that free rearing also decreased during the test time (OF 1:  $F(2, 31)=20.8, P<0.001$ ; OF 2:  $F(2, 31)=14.8, P<0.001$ ). Finally, we observed no significant differences between any groups when comparing on-wall rearing (data not shown).

#### 2.2.5. Grooming

Comparing grooming behaviour between groups did not yield any indication for substantial differences neither in OF 1 nor in OF 2 ( $P$ -values  $\geq 0.299$ ; data not shown).

### 3. Summary experiment 1

As predicted, a single unilateral injection of IL-2 into the striatum had no significant impact on locomotor behaviour, which supports our previous results where we also showed no alteration on locomotion behaviour in the elevated plus-maze (Pawlak and Schwarting, 2006). However, the lower dose of IL-2 (0.1 ng) significantly increased

Table 1

	PBS (n=10)		IL-2			
			0.1 ng (n=11)		1 ng (n=13)	
	Acute	24 h	Acute	24 h	Acute	24 h
Rearing total (no.)	80.7±10.9	60.0±8.6	61.9±7.6	48.0±9.0	64.9±9.1	69.8±9.1
Rearing 1–15 min. (no.)	45.6±4.1	40.4±2.6	40.7±4.3	29.6±4.1	39.9±5.7	44.1±4.6
Rearing 16–30 min. (no.)	20.5±4.4	8.8±1.8	13.4±2.8	9.2±2.7	16.9±2.8	18.7±3.9
Rearing 31–45 min. (no.)	14.6±3.7	10.8±5.2	7.8±1.4	9.3±4.4	8.2±2.2	7.0±2.0

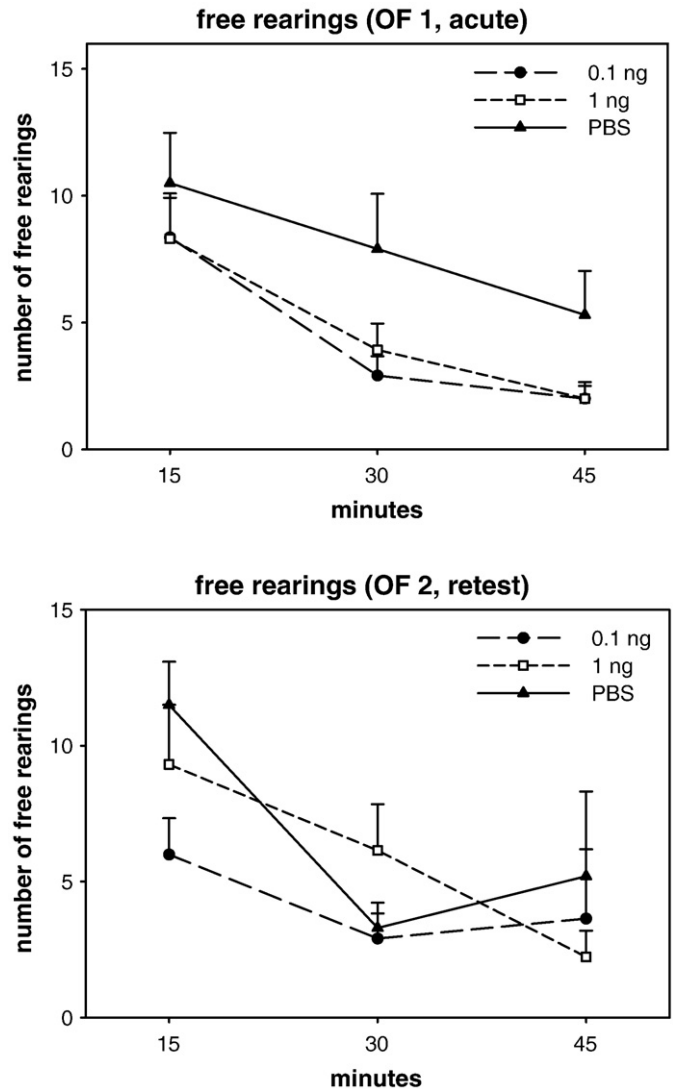


Fig. 4. Experiment 1: Effects of striatal IL-2 on vertical activity (free rearings) in an open field (OF). The OF test (45 min) was performed 5 min (acute) and again 24 h (retest) after a unilateral striatal microinjection of IL-2 (0 ng, 0.1 ng, 1.0 ng). Data are shown in 3×15 min blocks and are expressed as mean±SEM. Please refer to the text for the statistical analyses.

avoidance behaviour since the centre of the open field was continuously avoided particular in the delayed test. Furthermore, a significant interaction was observed in total rearing behaviour in OF 2. Even more, IL-2 treated rats showed blunted free rearing activity in OF 1, and in the beginning of OF 2 for the 0.1 ng IL-2 group, suggesting that IL-2 affects vertical activity, but not horizontal locomotor behaviour.

With regard to aversive stimuli, the group treated with the lowest dose of IL-2 (0.1 ng) showed a trend for an interaction effect in centre time in the acute test, and showed a substantial dose-dependent effect 24 h later, possibly since IL-2 had lasting proactive effects. Proactive drug effects may occur after a delay of time without acute exposure to the testing environment. However, this could not be unambiguously discovered by the present experiment, since we tested the animals 5 min after the injection. Therefore, we conducted a second experiment to test for a possible proactive impact of IL-2. Therefore, rats were tested in an open field 24 h and 48 h after a unilateral striatal IL-2 injection. In experiment 2, we used the most effective IL-2 dose (0.1 ng) of experiment 1, and an even lower dose (0.01 ng) to extend the dose-response effects.

## 4. Experiment 2

Experiment 2 was conducted under very similar conditions to experiment 1. The methodological differences are resumed below. These minimal variations were intended to optimise the procedure.

### 4.1. Methods

#### 4.1.1. Animals

Forty-eight male adult Wistar rats (Harlan Winkelmann, Borchen, Germany) weighing 238–288 g (266.4±1.9 g) at the time of arrival were used. Housing conditions were the same as in experiment 1.

#### 4.1.2. Design

We used a proactive design: On day 15, there was no immediate behavioural testing conducted following the microinjection. Instead rats were returned to their home cages, and tested 24 h (OF 1), and retested 48 h (OF 2) after striatal IL-2 treatment.

#### 4.1.3. Surgery

We implanted smaller cannulae (C315G, 26 G, ID 0.24 mm; OD 0.46 mm, Plastics One, USA) into the striatum, and used appropriate smaller dummy cannulae (C315 DC, OD 0.2 mm, Plastics One, USA) compared to experiment 1. We used a modified injection system with the same syringe pump and Hamilton syringe as in experiment 1, but a smaller flexible polyethylene tube (ID 0.28 mm, OD 0.61 mm, Portex, UK), and a smaller injection cannula (C315I, 33 G, ID 0.1 mm, OD 0.2 mm, Plastics One, USA). With this improved system the volume (0.2 µl) and the injection rate (0.1 µl/min) was reduced to further minimise spreading and to maximise local effects.

#### 4.1.4. IL-2 treatment

The injected doses of IL-2 were partly different compared to the previous experiment. We used one lower (0.01 ng) and one identical IL-2 dose (0.1 ng) as used before in experiment 1, because the latter one yielded the most significant behavioural effects (see experiment 1). In addition, after injection, the injection cannula remained in the guide cannula for 2 min to minimise the ascension of the injected fluid.

#### 4.1.5. Behavioural testing: open field (OF)

We did not assess grooming behaviour this time because of the lack of effects of IL-2 on such behaviour in experiment 1.

## 4.2. Results

### 4.2.1. Histological evaluation

Data of 41 animals with correctly placed cannulae were analysed. For details see Fig. 1B. The group sizes were as follows: PBS=15 (left 7 / right 8), 0.1 ng IL-2=15 (7/8), 0.01 ng IL-2=11 (7/4).

### 4.2.2. Behaviour in the OF

The statistical analyses were identical to experiment 1 and performed with three time blocks of 15 min each as repeated factor. However, due to exclusively non-significant group and interaction effects, we show all behavioural data collapsed for the total time of OF 1 and OF 2, respectively (Table 2).

### 4.2.3. Locomotion (horizontal activity)

Analysis of locomotor behaviour yielded a time effect reaching significance for both OF tests (OF 1:  $F(2, 38)=144.1$ ,  $P<.001$ ; OF 2:  $F(2, 38)=207.2$ ,  $P<.001$ ) showing that distance moved decreased over the time tested. As in experiment 1, group and interaction effects for locomotor behaviour between both IL-2 and control groups were non-significantly different for both test days ( $F$ -values $\leq 0.8$ ,  $P$ -values $\geq 0.556$ ; Table 2).

**Table 2**

Experiment 2: effects of striatal IL-2 on behaviour in an open field (OF)

	PBS (n=15)		IL-2			
	24 h	48 h	0.01 ng (n=11)		0.1 ng (n=15)	
			24 h	48 h	24 h	48 h
Locomotion (cm)	6296.0±395.8	4696.2±348.1	6518.4±481.2	4987.8±475.3	6333.4±338.4	5019.3±373.6
Centre time (s)	594.9±102.2	429.7±125.0	648.8±88.4	456.5±94.4	643.7±47.5	414.5±46.1
Total rearing (no.)	106.0±6.9	81.9±7.1	107.1±5.8	79.7±8.2	116.3±9.1	91.5±8.0
On-wall rearing (no.)	54.6±3.2	41.1±4.3	52.7±6.1	42.4±6.4	49.9±2.8	40.4±3.8
Off-wall rearing (no.)	43.4±5.4	36.0±5.3	45.7±5.1	30.9±4.2	57.9±7.2	43.7±6.6

The OF test (45 min) was performed 24 h and 48 h after a unilateral striatal microinjection of IL-2 (0 ng, 0.01 ng, 0.1 ng). Results are presented as means±SEM.

### 4.2.4. Anxiety-like avoidance behaviour (centre time)

The centre time continuously decreased showing a significant time effect for OF 1 ( $F(2, 38)=8.9$ ,  $P>0.001$ ), and a trend during OF 2 ( $F(2, 38)=3.0$ ,  $P=0.054$ ).

In both tests, we observed no substantial group differences, or interaction effects between both IL-2 and control groups ( $F$ -values $\leq 1.7$   $P$ -values $\geq 0.156$ ). Thus treatment with IL-2 led to no effect on avoidance behaviour in a proactive design (Table 2).

### 4.2.5. Rearing (vertical activity)

Rearing behaviour remained unaffected by proactive treatment of IL-2 for both test days (Table 2). Over the period of test time all rats showed less rearing behaviour (OF 1:  $F(2, 38)=256.4$ ,  $P<0.001$ ; OF 2:  $F(2, 38)=306.0$ ,  $P<0.001$ ). The same pattern emerged when rearing was divided into free (OF 1:  $F(2, 38)=48.8$ ,  $P<0.001$ ; OF 2:  $F(2, 38)=52.3$ ,  $P<0.001$ ), and on-wall rearing (OF 1:  $F(2, 38)=301.7$ ,  $P<0.001$ ; OF 2:  $F(2, 38)=261.0$ ,  $P<0.001$ ). Neither main effects of dose nor interaction effects approached statistical significances ( $F$ -values $\leq 1.69$ ,  $P$ -values $\geq 0.199$ ).

## 5. General discussion

In the present study, and in combination with our previous study (Pawlak and Schwarting, 2006), we have shown that IL-2 had an impact on avoidance behaviour in a hypothesised biphasic manner: In experiment 1 of the present study a low dose of IL-2 (0.1 ng) showed an increase in avoidance behaviour particularly upon undrugged retesting after 24 h, whereas in the foregoing study this cytokine showed a trend towards a decrease in avoidance behaviour in high doses also following a retest 24 h later with 25 ng (Pawlak and Schwarting, 2006).

Further, we observed an interaction between groups in total rearing behaviour in OF 2, but only in free rearing activity did we show a dose-dependent attenuation after acute injection with IL-2 (0.1 ng; 1 ng), and partly after 24 h (0.1 ng) compared to controls. As expected (Pawlak and Schwarting, 2006), IL-2 treatment had no impact on horizontal locomotion as shown before (Petitto et al., 2002), although the striatum is commonly accepted as a critical structure in the regulation of locomotor behaviour (Grillner et al., 2008). In experiment 2, we observed no behavioural effects at all suggesting that such IL-2 effects can only be obtained if a mild stressful environmental challenge (i.e. forced open field exposure) is followed immediately after injection. We therefore suggest that proactive drug effects can possibly be excluded for IL-2 effects on aversive environmental challenges.

Zalcman et al. (1998) showed that particularly IL-2 increased the number of free rearings, but decreased the time spent engaged in ambulatory exploration. At first sight, their results appear to be opposite to our findings, where IL-2 treated animals showed less free rearings compared to the controls. It is known that IL-2 can influence striatal

dopaminergic neurotransmission in vivo and dose-dependently in vitro (Lacosta et al., 2000; Petitto et al., 1997; Song et al., 1999), and there is evidence for a positive relationship between rearing behaviour and dopamine in the ventral striatum (Thiel et al., 1999). Thus, it is possible that systemically administered IL-2 (Zalcman et al., 1998) could have opposite behavioural effects compared to a cranial application, as shown in our study. Other methodological approaches between the studies of Zalcman et al. (1998) and ours should be taken into account, for example, non-species versus species specific IL-2, or the use of mice versus rats. Notwithstanding these different results, it is remarkable that IL-2 has repeatedly shown to alter free rearing behaviour.

Lever et al. (2006) proposed that rearing could be distinguished between unsupported and supported rearing against a surface (e.g., wall). One may speculate that these different types of rearings have also different functional meanings. Along this line, unsupported off-wall (free) rearing may reflect exploratory behaviour coupled with an unknown form of arousal, since it has been suggested that rearing activity may also be determined by emotionality (Gironi Carnevale et al., 1990; Thiel et al., 1998) apart from novel object exploration (Pawlak and Schwarting, 2002). Thus, in the present data our animals treated with striatal IL-2 may have shown an increase of emotionality and decrease of exploration behaviour.

The main results revealed that the 0.1 ng IL-2 group showed a trend for an interaction effect for centre time in the acute test of experiment 1. Even more, 0.1 ng IL-2 dose-dependently reduced centre time 24 h later, which is indicative of avoidance behaviour. On the basis of the pronounced effects 24 h later one could presume that behavioural effects of IL-2 were reached only after a delayed period of time. The present findings are in line with our previous studies, when a higher dose of IL-2 (25 ng) showed a trend for an anxiolytic-like effect 24 h following a striatal microinjection (Pawlak and Schwarting, 2006). This further supports our biphasic hypothesis of IL-2, increasing avoidance behaviour in low doses and decreasing avoidance behaviour in high doses of this cytokine. Moreover, the behavioural modulation had a kinetic component. A trend effect in centre avoidance behaviour was only observed with some delay starting 16–30 min only after 0.1 ng IL-2 injection in OF 1, which continued thereafter. Even more, this behaviour was robust also during the retest 24 h after injection. These avoidance effects on retest were subject to different interpretations.

First, IL-2 appears to start its impact several minutes after injection, but there is only one study which gives evidence about the latency of IL-2 induced behavioural effects (De Sarro et al., 1990). While investigating the effects of IL-2 microinjected into the locus coeruleus, the first behavioural effects appeared within 5–10 min and lasted from 25 to 65 min (De Sarro et al., 1990). However, the authors did not analyse possible delayed effects thereafter, and detailed behavioural data were not provided (De Sarro et al., 1990). Therefore, we conducted the second experiment to test for a possible proactive drug impact by testing the rats 24 h after IL-2 injection in an open field and after 48 h again. The results showed that exploratory and avoidance behaviours were clearly non-significant between IL-2 and PBS-treated groups, suggesting no proactive IL-2 drug effects, at least under our conditions. We suggest that an environmental challenge (e.g., mild stress by forced exposure to an open field as presented here) is necessary to induce delayed emotional and motivational behavioural effects of IL-2. In detail, it is possible that IL-2 induces an aversive state which is associated with the environment (open field) and that this aversive experience come to the fore in the behaviour also during the second exposure to the open field.

Second, IL-2 could have also affected memory of the initial open field experience. When administered repeatedly and systemically, IL-2 led to impaired spatial memory performance in a Morris water maze in mice (Lacosta et al., 1999). However, evidence for impaired memory processes is not supported by our data. Instead, IL-2-treated animals in experiment 1 showed an increase of avoidance behaviour and may therefore indicate an improvement of memory performance. Even

more, IL-2-treated animals may have shown a learned (adaptive) response more quickly as the vehicle treated rats. Notwithstanding our speculations, supportive evidence about the role of the striatum in formation of emotional memory apart from its regulation of motor activity has come from various other studies (e.g., Cahill and McGaugh, 1998; Ferreira et al., 2003; Leppanen, 2006; Morgane et al., 2005).

Apart from memory altering mechanisms, which need to be tested further, IL-2 could have acted in an anxiety-relevant way. IL-2/15R $\beta$  knockout mice exhibited significant reductions in acoustic startle and significant differences in behaviour in the elevated plus-maze, indicating a decreased level of anxiety-like avoidance behaviour (Petitto et al., 2002). Based on our previous work, we hypothesised IL-2 to act in a biphasic manner when striatal microinjections of 25 ng IL-2 led to a trend of increased open arm time as opposed to a 1 ng IL-2-induced nonsignificant decrease of open arm time in an elevated plus-maze (Pawlak and Schwarting, 2006). In line with our previous data, low doses of IL-2 (0.1 ng) were seen to decrease centre time which could be interpreted as anxiety-like avoidance behaviour. Thus, IL-2 appears to affect anxiety-related behaviour in a biphasic manner as hypothesised.

Evidence suggests that IL-2 can initiate a cascade of neurotransmitter activity, for example serotonin (5-HT, 5-hydroxytryptamine), which may modulate anxiety-related behaviour, although the consequences of immune modulators like IL-2 on neurotransmission and their effects on behaviour have only rarely been investigated. Remarkably, an in-vivo reduction of 5-HIAA (5-hydroxyindoleacetic acid) in the nucleus accumbens was demonstrated following a systemic IL-2 injection (Song et al., 1999). Also, Lacosta et al. (2000) found reduced 5-HT levels in the prefrontal cortex after repeated systemic IL-2 injection, while showing the opposite 5-HT effect in the hippocampus post mortem. Accordingly, i.c.v. injected IL-2 led to marked increases of hippocampal 5-HT levels and its metabolite 5-HIAA (Pauli et al., 1998). Finally, we have first indications that systemic IL-2 can affect 5-HT but only moderately dopamine in various brain regions in the rat in-vivo (Karrenbauer et al., 2008). Thus, IL-2 is able to alter neurotransmission in brain regions, which are known to be critical in emotion and motivation.

Strong evidence suggests that cytokines induce behaviour resembling some symptoms of depression (Dantzer et al., 1999), which are thought to be mediated by neurotransmitters, particularly 5-HT. It is well established that 5-HT is crucial for anxiety and related disorders (e.g., depression); patients diagnosed with anxiety or depression disorders are treated successfully with selective serotonin reuptake inhibitors (SSRIs). Moreover, depressive symptoms increased during IL-2 therapy of cancer patients (Capuron et al., 2000; Walker et al., 1997), and these symptoms were accompanied by decreased concentrations of serum tryptophan, which is the main precursor of 5-HT and noradrenalin (Capuron et al., 2002; Wichers and Maes, 2002). Taken together, these findings provide evidence for a critical action of IL-2 in the brain substantially influencing the release of monoaminergic neurotransmitters, which are known to be involved in affective behaviours. Additionally, it is likely that this neurochemical influence has a temporal variable which could explain the delayed, pronounced effects of IL-2 shown here (Karrenbauer et al., 2008).

At first sight, the striatum does not appear to be the typical structure to study aversively motivated behaviour. However, our previous results identified the striatum as a structure in the interrelation of IL-2 and aversively motivated behaviour (Pawlak et al., 2003). In addition, we have shown that rats with high versus low anxiety-like avoidance behaviour differed in their 5-HT post mortem levels in the striatum, but not other brain areas (Schwarting et al., 1998). Furthermore, rats displaying pronounced avoidance behaviour showed a higher *c-fos* mRNA expression in the dorsal striatum compared with rats with low avoidance behaviour (Kabbaj and Akil, 2001). There are now even studies showing local microcircuits in the nucleus accumbens mediating motivated/affective behaviours that are bivalently organised with negative and positive valence along rostrocaudal gradients (Reynolds and Berridge, 2002). Moreover, recent human studies from different groups strongly

suggest that the striatum (ventral and dorsal part) is involved in anxiety and aversive stimulation (e.g., Jensen et al., 2003; Laakso et al., 2003; Lorberbaum et al., 2004; van den Heuvel et al., 2005). Finally, first evidence showed that cytokines increase self-reported fatigue, which was correlated to increased glucose metabolism in the nucleus accumbens and putamen (Capuron et al., 2007). Interestingly, there are some studies that report a motivational impact of IL-2 (Anisman et al., 1996, 1998; Petitto et al., 2002; Zalcman, 2001; Zalcman et al., 1998), but the results reveal controversial relationships between IL-2 and anxiety-related behaviour in animals (Anisman et al., 2002; Petitto et al., 2002). Possible explanations for this lack of effects might be due to methodological factors, for example, the use of human versus rat recombinant IL-2, with the latter used in the present experiment. This detail might be crucial as Naito et al. (1989) showed that rat IL-2 caused an increase in plasma adrenocorticotrophic hormone (ACTH), but human IL-2 had no effect on rats ACTH levels. Another methodological difference between studies is the site of administration, which was primarily systemic (Anisman and Merali, 1999; Lacosta et al., 1999), i.c.v. (Connor et al., 1998), or into the locus coeruleus (De Sarro et al., 1990).

In summary, together with our previous results, we found evidence that a single unilateral IL-2 injection into the striatum biphasically influenced aversively motivated behaviours, enhancing avoidance behaviour in lower (present study) and reducing it in higher doses (Pawlak and Schwarting, 2006). Two behavioural dimensions, time spent in the centre, and free rearings are suggested as indicators for anxiety-like avoidance behaviour and emotionality impact of IL-2. In addition, we did not find evidence for proactive IL-2 drug effects. We therefore suggest that an acute mild stressor (e.g., open field) is necessary to induce emotional and motivational behavioural effects of IL-2, and that this aversive experience comes to the fore in behaviour also during re-exposure to the mild open field stressor. Although our data may contradict previous studies of IL-2 effects on such behaviours, we provide a number of differences to the design of those studies which may account for their previous lack of IL-2 effects specifically on anxiety-related behaviours. Thus, these data provide a basis on which the role of IL-2 in the pathogenesis of some neuropsychiatric diseases could be explored anew.

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