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Long-term methadone treatment impairs novelty preference in rats both when present and absent in brain tissue

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ABSTRACT

Behavioral consequences of long-term methadone treatment have received little attention either in humans or experimental animals. In this work, we show that methadone (2.5–10 mg/kg) administered (sc) once daily for three weeks with repeated withdrawal on Saturday and Sunday impairs the novelty preference in rats. One hour after the last injection, when methadone was still present in brain tissue, the rats were too affected by the sedative effects of the drug to perform the test. This was confirmed by an almost total lack of locomotor activity or exploratory behavior. One day after the last injection, the methadone treated rats showed a 70% reduction ($p<0.05$) in novelty preference compared to rats administered saline. No methadone was detected in the brain tissue at this time. Moreover, there were no differences in locomotor activity or total exploratory behavior between the groups, indicating a specific impairment of cognitive functioning. In brain tissue, the methadone concentration versus time profile was shifted to the left after long-term treatment, indicating a change in uptake and distribution of the drug. The area under the two concentration versus time curves was, however, similar. Methadone disappeared completely from the brain within one day. Together, these results suggest that long-term methadone treatment may have a negative impact on cognitive functioning in rats, regardless of whether methadone is present in brain tissue.

1. Introduction

Methadone, a synthetic opioid which acts on opiate receptors, is used in maintenance therapy of heroin dependence as it is effective in retaining patients in treatment and preventing relapse (Gerstein, 1992). However, chronic use of opioids, whether prescribed or used illicitly, has been associated with alterations in various cognitive functions (e.g. Ersche and Sahakian, 2007; Gruber et al., 2007). Over the last decade, it has been shown that methadone patients perform significantly worse on attention, information processing, short-term memory, long-term memory, verbal intelligence, visual perception, visual memory, and problem solving compared to both abstinent previous heroin abusers and healthy controls (Darke et al., 2000; Mintzer et al., 2005; Prosser et al., 2009; Specka et al., 2000; Verdejo et al., 2005). As a consequence, it has been claimed that methadone maintenance therapy might lead to additional impairment above that resulting from earlier heroin abuse. However, it is difficult to distinguish the effects of methadone itself from other factors associated with the life-style of the patients (e.g. nutrition, health care, additional drug abuse).

Since interpretation of epidemiological studies is challenging and ethical considerations impede long-term controlled randomized methadone studies in a population of healthy non-drug abusing humans, preclinical animal experiments might give important information. Surprisingly, neither behavioral nor toxicological studies in animals seem to have been given much priority. As far as we know, there are only three studies about the cognitive effects of methadone exposure (Hepner et al., 2002; Tramullas et al., 2007, 2008). These studies show that, in mice and rats, both acute and chronic administration of methadone affect performances which are dependent on the hippocampus formation, a brain structure important for functions like attention, spatial orientation and consolidation of memory (Broersen, 2000).

The results, both in humans and animals, may be influenced by the presence or absence of methadone in brain tissue. In methadone maintenance patients, it is reasonable to assume that cognitive testing is always performed under the influence of methadone, since the half-life of methadone in humans is long ($T_{1/2}=15$–55 h; see Wolff et al., 1997) and the patients receive daily doses. In mice and rats, the metabolism rate of methadone is probably much faster, but the pharmacokinetic profile of methadone in the brain tissue of rodents is poorly described. However, it is important to know whether a possible impairment of cognition is dependent on the presence of methadone in the brain, as such dependence would indicate a reversible drug effect rather than more permanent changes caused by long-term treatment.

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In this study, we used a novelty-test to explore the effect of long-term methadone treatment on cognitive functioning in rats. We also examined the pharmacokinetic profile of methadone in blood and brain tissue after acute and long-term administration, and we measured methadone concentrations in brain immediately after termination of the novelty-test to confirm presence or absence of methadone at the time of the behavioral testing.

2. Materials and methods

2.1. Subjects

The subjects were 94 male Wistar rats (Møllegaard Breeding Laboratories, Denmark) weighing 282 ± 2 g when the experiments started. The rats were housed individually with free access to commercial rat pellets and water. The climatized vivarium (21 °C) was illuminated from 8 am to 8 pm. The animals included in the behavioral study (n = 35) were handled individually (3 min/day) for 3 days before treatment started and every day during the treatment period. The experiments were approved by the Norwegian National Animal Research Authority.

2.2. Drugs

S,R-Methadone-HCl (MW 345.91; Sigma, Norway) was dissolved in 0.9% saline and stored at room temperature. The rats were injected once daily Monday to Friday for three weeks with increasing doses. In a previous study, chronic administration of methadone with repeated withdrawal showed greater deleterious effects in the Morris water maze compared to a continuous chronic treatment regime (Tramullas et al., 2007). Concern due to acute lethality and/or accumulation of the drug made us chose an escalating treatment regime intended to induce a gradual tolerance for the drug, like what is usually done in maintenance patients starting methadone treatment. The first week the dose was 2.5 mg/kg the two first days and 5 mg/kg the next three days. The last two weeks the dose was 10 mg/kg. The control group was injected with 0.9% saline. All injections were subcutaneous (sc) in a volume of 1 ml/kg. The injections were given in the morning (8–10 am) and administered biweekly alternating at the right and left side of the lower back. The maximum dose, 10 mg/kg, was chosen based on LD50 for methadone in rats which is 30 mg/kg and 9 mg/kg for oral and iv administration, respectively (www.bionichepharmausa.com).

2.3. Novelty-test

The behavioral testing was carried out using a modified version of the novelty-test described by Myhrer (1989). In brief, the floor of a plexiglass cage (56 × 34 × 20 cm) was divided into 18 equal squares (9 × 11 cm). Three identical aluminum cubes (5 × 5 × 5 cm) were evenly distributed in the cage in fixed positions (neutral objects). The novel object was a smaller cube (4.5 × 4.5 × 4.5 cm) with two uneven sides. All objects were painted light gray. The testing room was provided with a fan producing white noise, and the only light was a 15 W bulb 60 cm above the apparatus. The day before testing, the rats were allowed individually to explore the empty apparatus for 15 min before injection of methadone or saline.

Separate groups of rats were tested 1 h or 1 day after the last injection and each rat was only tested once. The test was divided into two phases. In phase 1, the rats were presented with the three neutral objects. In phase 2, the neutral object in the middle position was replaced with the novel object. The two phases lasted for 5 min interrupted by a 10 min break in the home cage. The following behaviors were recorded by observation: number of seconds in contact with each object (exploratory behavior), number of squares traversed (locomotor activity), and number of rearings. Novelty preference was scored as the difference between exploration of the novel versus the mean of the two neutral objects in phase 2. Exploration of an object was defined as directing the snout toward the object at a distance of 1.5 cm or less. Bodily touch other than the snout was not considered to be exploratory behavior. Prior to testing each rat, the apparatus and objects were carefully washed to eliminate olfactory cues.

2.4. Pharmacokinetic studies and determination of methadone in brain tissue

Rats (n = 59) were administered methadone or saline for three weeks as described above. These animals were only used for pharmacokinetic studies. On the last day all rats were given methadone (10 mg/kg). At specified times after injection (5–480 min), the rats were decapitated and blood was collected from the neck. Blood samples were immediately transferred to microcentrifuge tubes containing sodium fluoride (final conc. 4 mg/ml) dissolved in heparin (100 IU/ml), and frozen in liquid N_2. After blood sampling, the brain (except cerebellum) was quickly removed, washed in cold 0.9% saline, blotted on a filter paper and frozen in liquid N_2.

To determine brain methadone concentration during testing, brains were also removed from animals participating in the behavioral study immediately after termination of the novelty-test.

2.5. LC–MS/MS analysis

The presence of methadone in blood and brain tissue was measured using a modified version of the LC–MS/MS analysis described previously (Karinen et al., 2009). The brains were weighed and homogenized in cold dH_2O (1:2). Brain homogenate and blood was diluted with dH_2O (1:1), added internal standard (5 μM methadone d_9/morphine d_9) and 85% acetonitrile/15% methanol (1:5), mixed well and placed in the freezer for 10 min. The samples were centrifuged (4500 rpm, 10 min) before the organic phase was transferred to 5 ml glass tubes, evaporated to dryness under N_2 at 50 °C, reconstituted with 100 μl 35% acetonitrile/65% 5 mM ammonium format buffer (pH 3.1) and transferred to 0.3 ml plastic autosampler tubes. Methadone was separated at 50 °C on a XTerra C18 column (Waters, Milford, MA) using gradient elution with mobile phase consisting of methanol and 5 mM ammonium format buffer (pH 3.1). The gradient started with 10% methanol which was increased to 90% over the next 8 min. Flow rate was 0.2 ml/min. Cut-off for both blood and brain tissue was 0.01 μM. The masses monitored were m/z 310.05 > 265.10 for methadone and m/z 319.05 > 268.10 for methadone d_9. The between-day variations were less than 9%.

2.6. Statistical analysis

Data are presented as mean ± SEM. Statistical analysis of the behavioral data was carried out using an unpaired Student's t-test, or one-way analysis of variance (ANOVA) and group comparisons with Newman–Keuls post-hoc test. The pharmacokinetic data were analyzed with a general linear model with treatment as fixed factor and time as random factor. Analysis of the weight data was performed using a general linear model with treatment as fixed factor, weight at test day as dependent variable and weight at day 1 as covariate. p-values less than 0.05 were stated as statistically significant.

3. Results

The daily injections of methadone caused a short-lived (15–30 min) state of hyperactivity followed by sedation lasting for 3–5 h, leaving the rats immobile. This characteristic behavior as a consequence of the injections did not change during the treatment period, indicating no obvious development of tolerance. No traditional withdrawal signs (e.g. nausea/vomiting, diarrhea, wet-shakes, shivering, lacrimation) were observed, either Monday to Friday or in the drug-free weekends. The methadone rats did not put on as much weight as the saline rats during

the treatment period, so the saline rats had a 13% higher weight compared to the methadone rats on the testing day ($F(1,57) = 63.849$; $p \leq 0.05$; Fig. 1). Eight rats died during the treatment period.

3.1. Novelty-test

The results presented in Table 1, upper part, show that 1 h after the last methadone injection, the rats were too affected by the acute sedative effects of the drug to perform the novelty-test. The methadone rats ($n = 8$) had almost no locomotor or exploratory activity ($p \leq 0.05$) compared to the saline injected rats ($n = 7$). Therefore, no comparison of preference for the novel object could be made. LC–MC/MS analysis revealed presence of methadone in the brain at this time (4.01 ± 1.07 nmol/g).

One day after the last injection, the rats treated with methadone ($n = 8$) explored the novel object significantly less ($F(1,15) = 8.048$; $p \leq 0.05$; Table 2) than the saline rats ($n = 9$), while there was no difference in the exploration of the neutral objects, leading to a significant difference of 70% in the preference for novelty ($F(1,15) = 6.379$; $p \leq 0.05$; Table 2). There were no differences in locomotor activity or in the number of rearings between the groups in each phase. The difference ($F(1,15) = 7.878$; $p \leq 0.05$) in total exploration in phase 2 (Table 1, lower part) reflects the difference in exploration of the novel object. No methadone was detected in the brain tissue at this time.

3.2. Pharmacokinetic

The drug-concentration profiles of methadone in blood up to 480 min after administration revealed no differences between previously drug naïve rats and those exposed to long-term administration (Fig. 2A). In brain tissue, there was no overall difference between previously drug naïve rats and those exposed to long-term treatment, while a difference was seen in the interaction between drug-concentration and time ($F(6,39) = 3.351$; $p < 0.01$; Fig. 2B).

4. Discussion

Using a novelty-test, we showed that long-term treatment (3 weeks) with racemic methadone impairs novelty-seeking in rats both 1 h and 1 day after the last injection, although in different ways. At 1 h, when methadone was still present in the brain, the rats were too affected by the sedative effects of the drug to perform the test. They were immobile, did not pay attention to their surroundings and showed almost no exploratory behavior. Because of this condition, it is difficult to ascribe the finding to a real impairment of cognitive functioning. One day after the last injection, the methadone treated rats showed a 70% lower novelty preference compared to rats administered saline. No methadone was detected in the brain tissue at this time. There were no differences in locomotor activity or total exploratory behavior between the two groups, which argue in favor of a specific impairment of cognitive functioning.

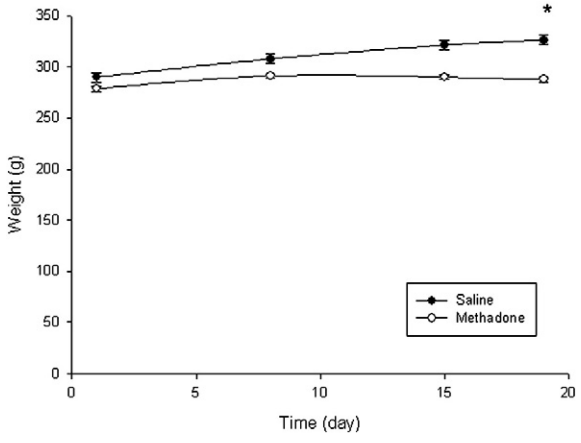

Fig. 1. Weight curves for methadone-and saline-treated rats during the treatment period.

Because rats naturally have a strong preference for examining changes in the environment, alteration in their exploration time of a novel object is considered a sensitive method for measuring cognitive functions. Performance of the novelty-test has been shown to be dependent on hippocampus and associated areas (Myhrer 1988, 1989). The reduced novelty preference seen 1 day after the last injection may be a result of interference with the working memory, which may lead to reduced ability to remember which object has recently been examined and therefore random exploration of the objects. There is a possibility that the difference may be ascribed to the different amounts of attention paid to the novel object, but recognition memory is also linked to hippocampus (Broadbent et al., 2004).

In methadone maintenance treatment, an average human methadone dose is 80–120 mg/day (Joseph et al., 2000). The rats in our study were administered 2.5–10 mg/kg/day. Given that the half-life of methadone is 16–48 h in humans (Wolff et al., 1997) and 1.5–4 h in rats (Misra et al., 1973; Fig. 2), the 24-h doses are comparable for the two species. The difference is that rats will have a high methadone concentration for a relatively brief time-period followed by a drug free period before the next injection, while the concentration in humans would be more stable. Our rats were also given two drug free days every Saturday and Sunday, reducing the total drug load compared to humans.

The 5 and 10 mg/kg doses had a pronounced effect on the rats during the first few hours after injection. This was reflected in low locomotor and exploratory activity during the test at 1 h, indicating no obvious tolerance development during the treatment period which may be related to the high dose administered. It is possible that tolerance to the behavioral effect would have been observed if a lower dose had been administered, or if the observation of the animals had taken place later in relation to the last methadone injection. For comparison, tolerance to different sensorimotoric performances was shown in mice when tested 2 h after the last methadone injection (10 mg/kg/day) on day 37 (Tramullas et al., 2007).

The absence of abstinence symptoms one day after the last injection is in accordance with previous findings. Tramullas et al. (2008) have reported that 6 weeks of treatment with methadone (chronically or repeated withdrawal) did not affect locomotor activity in mice when measured in the passive avoidance test 10–12 h after the last injection. Moreover, in the Morris water maze, methadone rats showed impaired cognition at the same testing time but there was no difference in the motor performance between the groups (Tramullas et al., 2007). This may, at least in part, be because of slow elimination of methadone from blood and brain (see Fig. 2), thereby relative mild abstinence symptoms compared to e.g. heroin. However, we cannot exclude the possibility that our rats showed abstinence symptoms during the night (when they were not observed) and that we tested them after the abstinence phase.

There appeared to be no development of metabolic tolerance, as no clear evidence was found for an increase in drug metabolism. There were no overall differences in the presence of methadone after single and long-term administration, either in blood or brain. However, the drug-concentration profile over time was shifted to the left in brain tissue after long-term administration compared to single injections, indicating a change in the uptake and distribution of the drug. The same tendency was seen in blood although not significant. The higher maximum concentration in the long-term treated rats cannot be explained by accumulation of drug since no methadone was detected in the brain tissue of rats novelty-tested 1 day after the last injection, corresponding to $t = 0$ in Fig. 2. Because the curves for the long-term treated rats did not follow first order kinetic, it is not possible to

Table 1
Mean measures of activity and total exploratory behavior in rats tested 1 h and 1 day after the last injection of a three weeks injection regime (see text for details).

Group	Testing time	N	Total exploration (s)		Total locomotion (number of crossings)		Total rearing (s)	
			Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2
Saline	1 h after last injection	7	38.9 ± 4.12	30.4 ± 4.54	69.7 ± 4.13	63.7 ± 8.87	21.3 ± 1.42	21 ± 2.45
Methadone		8	1 ± 0.60*	0.9 ± 0.74*	15 ± 5.28*	14.4 ± 3.94*	1 ± 0.53*	3.8 ± 2.51*
Saline	1 day after last injection	9	44.2 ± 2.83	43 ± 4.57	65.3 ± 4.94	64 ± 5.78	17.9 ± 0.81	17.6 ± 1.83
Methadone		8	40.3 ± 3.29	26.9 ± 3.25*	62.6 ± 6.44	48.4 ± 6.78	16.3 ± 1.16	14 ± 2.09

* Significant difference compared to its respective saline groups, $p \leq 0.05$.

calculate $T_{1/2}$ for methadone in our study. It looks, however, as if the half-life may be shorter in long-term treated rats compared to drug naïve. Early publications reported that chronic methadone treatment may have no effect (Alvares and Kappas, 1972; Peters, 1973; Raitano and McMillan, 1983) or may induce its own metabolism (Misra et al., 1973; Spaulding et al., 1974). These discrepancies may be due to differences in the exposure regimes.

The methadone rats did not gain weight during the second and third weeks of the treatment period, resulting in lower weight on the testing days compared to saline treated rats. This is in accordance with earlier findings which have shown that chronic methadone treatment causes a reduction of body weight in adult rats (Vajda et al., 1975). Since no differences were seen in the number of rearings, locomotor activity or total exploratory activity (phase 1) between the groups tested 1 day after the last injection, it seems unlikely that the general condition of the rats affected the impaired novelty preference seen at this time.

As in humans, the rats showed individual variations in how well they tolerated the administered methadone. During the injection period 8 animals died, probably due to respiratory failure (Corkery et al., 2004). Six of the deaths occurred after the first injection in the second or third week when the rats had been off methadone for two days (Saturday and Sunday).

The effect of long-term methadone consumption on cognitive functions has received limited attention compared to other opioids and drugs of abuse. Our findings support and complement the studies of both Hepner et al. (2002) and Tramullas et al. (2007) who showed that methadone disrupts working memory performance in the Morris water maze, a task which is dependent on hippocampus. Hepner tested rats 15 min after a single injection of methadone, suggesting that the drug affects attention processes when present in brain tissue. Tramullas and co-workers placed chronically exposed rats and rats treated with repeated withdrawals in the maze 12 h after the injection on days 21 to 32 of the treatment period, and found cognitive impairments in both groups. Methadone was detected in blood at the time of their testing, implying presence also in brain tissue. Our results are therefore the first to demonstrate an effect of long-term methadone treatment after complete clearance of the drug from the brain. The impaired novelty-seeking we observe 1 day after the last injection cannot be due to ongoing receptor activation affecting short-term intracellular signaling pathways, but is probably a result of sustained neuronal modifications. It should be noted that the effect was unlikely related to methadone withdrawal or tolerance which did not appear to develop in our animals by use of the present drug administration protocol.

In studies with methadone maintenance patients the results are ambiguous. Improvements (Gruber et al., 2006), impairments (Verdejo et al., 2005; Mintzer et al., 2005) and no changes (Prosser et al., 2006) in cognitive functions have been reported. However, because of small sample sizes, inappropriate control groups, the presence of various uncontrolled life-style factors and lack of objective abstinence symptom evaluations, it is difficult to interpret the actual importance of methadone itself. The improvement seen in some of the human studies may partly be a result of associated reductions in intake of alcohol and other drugs, an improved less harmful life style, and stabilization of substitution medication. In animal experiments we can isolate the effect of methadone by excluding life-style factors and simultaneous use of other drugs. However, future animal studies should also include methadone treatment of previously heroin exposed animals.

Table 2
Mean measures of exploratory behavior in phase 2 measured using a novelty-test 1 day after the last injection of a three week injection regime (see text for details).

Group	N	Phase 2		
		Novel (s)	Neutral/2 (s)	Novelty preference (s)
Saline	9	25.3 ± 3.27[a]	8.8 ± 1.12	16.5 ± 3.06
Methadone	8	12.5 ± 2.87[b]	7.1 ± 0.65	5 ± 3[b]

[a] Significant difference compared to exploration of the neutral objects (Neutral/2), $p \leq 0.001$.
[b] Significant difference compared to the saline group, $p \leq 0.05$.

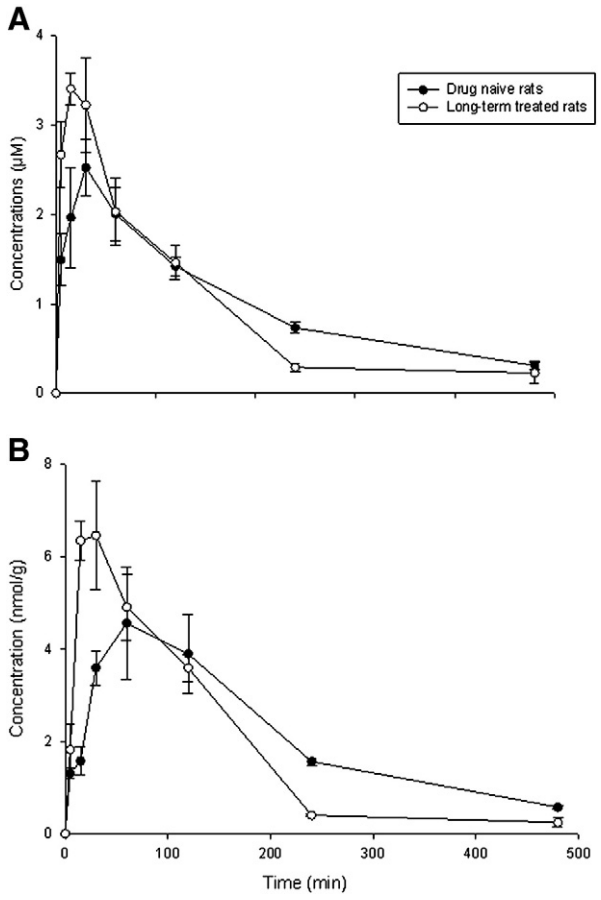

Fig. 2. Concentrations of methadone in blood (µM; A) and brain tissue (nmol/g; B) as function of time (min) after drug administration to previously methadone naïve and long-term methadone treated rats, respectively (see text for details).Values are mean ± SEM, n = 3–7.

Chronic morphine treatment affects learning and memory in rodents (Sala et al., 1994; Spain and Newsom, 1991). These impairments have been connected to changes in neuronal mechanisms such as inhibition of cholinergic activity (Li et al., 2001), apoptosis (Emeterio et al., 2006), reduced synaptic plasticity (Pu et al., 2002), reduced neurogenesis (Eisch et al., 2000), and changes in central signaling proteins (Lou et al., 1999). In humans, cognitive testing has indicated that long-term (ab) use of heroin and morphine may be associated with long-lasting impairments in different brain functions (e.g. Ersche and Sahakian, 2007; Gruber et al., 2007). Also a variety of neuropathological changes have been found (Buttner et al., 2000). It seems reasonable that long-term methadone exposure will affect similar mechanisms as morphine and heroin, but this remains to be examined.

In conclusion, cognitive deficits caused by long-term methadone treatment may influence the daily life of patients and the outcome of rehabilitation programs. Since studies in methadone maintenance patients are difficult to control and randomized studies in healthy non-drug-abusing human populations are unethical, experimental animal studies may serve as a valuable background for additional pre-clinical and future clinical studies in a field with important knowledge gaps.

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