Towards a mouse model of depression : a psychoneuroendocrine approach

Dalm, S.

Chapter 3

Non-invasive stress-free application of glucocorticoid ligands in mice

Sergiu Dalm
Vera Brinks
Maaike van der Mark
E. Ron de Kloet
Melly S. Oitzl

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Abstract

Most drug delivery procedures induce stress which might interfere with the pharmacological action of the drug and behavior. Stress is deduced from high and long-lasting elevations of the hormone corticosterone. We set out to develop a non-invasive, stress-free method of drug delivery in mice. Validation consisted of delivery of glucocorticoid ligands via oats to male C57BL/6J mice.

Oat consumption induced a small increase in corticosterone concentrations after 15 min (< 50ng/ml) that returned to low resting levels at t=30 (< 10ng/ml). Gavage and intraperitoneal vehicle injections resulted in long-lasting corticosterone elevations (> 100ng/ml at t=30 and ~ 50ng/ml at t=60 min after delivery). Adding corticosterone to oats resulted in 3-fold higher plasma corticosterone in the 15.0mg/kg-group (± 250ng/ml) compared to the 4.5mg/kg-group at t=30 and t=90. Application of the glucocorticoid receptor antagonist RU38486 (200mg/kg) elevated the plasma corticosterone levels for at least eight hours. Additional swimming increased corticosterone even further. Presumably, already the small oat-consumption-induced increase of corticosterone requires negative feedback via glucocorticoid receptors.

In conclusion, the context-dependent and dose-controlled application of drugs via oats avoids confounding strong stress system activation and makes it suitable for studies on learning and memory processes.

Introduction

Assessment of pharmacological drug profiles, but also studies on mechanisms underlying cognition and behavior, require the controlled application of drugs. Most procedures related to administration of drugs to small laboratory animals like mice, require invasive methods. Already hand-restraint will lead to a concomitant, non-controlled and unwanted activation of the stress system (Balcombe et al. 2004). By definition, any kind of stress, even a mild stressor, is a potential confounding factor of drug effects. Specifically, in relation to the well known effects of stress on cognitive processes (Lupien and McEwen 1997; Joels et al. 2006) a non-invasive, stress-free and dose- and time-controllable drug-delivery is of crucial importance, but often disregarded or discarded as neglible.

The stress response in humans and rodents is controlled by the Hypothalamic-Pituitary-Adrenal (HPA) axis. In mice and rats, corticosterone is the glucocorticoid hormone secreted from the adrenals in response to stress, i.e., any event that disturbs the psychological and physiological homeostasis of the organism. The effects of corticosterone are mediated in the brain by two nuclear receptors: the high affinity mineralocorticoid receptor (MR) and the low affinity glucocorticoid receptor (GR). Neuroendocrine regulation via MR controls basal HPA axis activity and sensitivity to a stressor. GR is activated after high circulating corticosterone levels, exerting negative feedback and facilitation of the essential recovery from the stress response (De Kloet et al. 1998). Measurement of circulating plasma corticosterone concentrations is an accepted tool to assess stress-induced activation of the HPA axis.

Drug-delivery via food or drinking water is an easy to perform, non-invasive procedure for mice, however lacking dose- and time-controlled deliveries (Ruzek et al. 1999). Although the route of administration (*per os*) fits a stress-free application form, dose- and time-control has to be accomplished differently. Therefore, we address the potential of using a treat or bait to deliver drugs. This has previously been shown effective in other species like birds, where mealworms injected with corticosterone were supplied in close context with the requested behavioral response (Breuner et al. 1998). As a treat, we decided to use oats as mice like to eat them and the structure of oats facilitates the soak up of solutions. To validate the method, we administered glucocorticosteroids ligands to manipulate HPA axis activity and subsequently measured blood plasma corticosterone concentrations.

The aim of the experiments was to devise a non-invasive stress-free, dose- and time-controlled procedure for effective delivery of glucocorticoid agonists and -antagonists to mice. In context with the procedures of drug administration, corticosterone

concentrations were measured in blood plasma at various time points. First, we tested the hypothesis that mice would readily consume the oats, without concomitant increase in endogenous corticosterone levels. Vehicle applied via a gavage or intraperitoneal injection was expected to result in higher corticosterone concentrations. Second, we investigated the dose- and time-dependency of corticosterone treatment in oats. Finally, the effect of the GR antagonist RU38486 was determined.

Materials and Methods

Animals and housing

Ten weeks old male C57BL/6J mice were purchased from Janvier Bioservices (Netherlands). Upon arrival, the mice were single housed with food and water *ad libitum* and allowed to acclimatize for two weeks to the testing room. The room was temperature (19 – 21 °C) and humidity (50 - 60%) controlled; lights on from 0700 to 1900h (12-12h light-dark cycle). Animals were repeatedly handled, weighed and tested between 0900 and 1400h. The experiments were approved by the Local Committee for Animal Health, Ethics and Research of the University of Leiden. Animal care was conducted in accordance with the EC Council Directive of 24 November 1986 (86/609/EEC).

Familiarization to oat administration

One week prior to the start of the experiments, a feeding-cup (2.3cm diameter x 2.5cm high; Figure 1A) was glued to the floor in a corner of the home cage, opposite the nest location. For familiarization, three flakes of oats (Speltvlokken, Biologische teelt, Graanpletterij de Halm, Netherlands; ± 140mg) were placed in the cup on three consecutive days, every other day at 0900h. The grid of the cage was lifted and the sawdust was removed from the cup using an air puff generated with a pipette. Next, the oats were placed into the cup using forceps to minimize human odor transfer. Thereafter, the cage was closed and the mouse was allowed to eat the oats undisturbed. All the oats were consumed within 10 min (Figure 1B/C).

Drugs

Oat delivery: One day prior to the experiment, three flakes of oats were placed in a glass vial and the solutions containing corticosterone, GR antagonist or dissolvent were applied. The glass vials containing the oats were kept at room temperature over

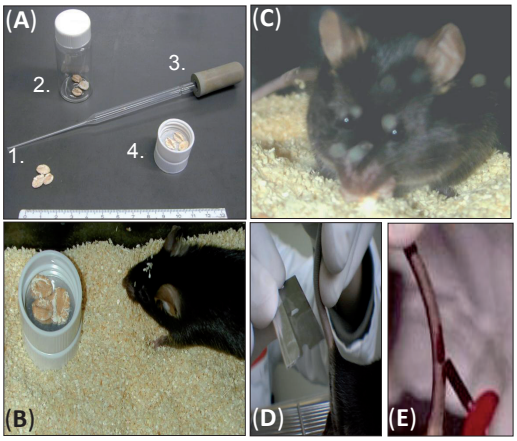

Figure 1
Tools for drug administration via oats and subsequent blood sampling via tail incision. **(A)** prerequisites used during preparation: 1) oats, 2) glass vial, 3) pipette, 4) feeding cup; and ruler giving the size of the objects in cm; **(B)** three flakes of oats are placed with forceps in a feeding cup in the home cage of the mouse; **(C)** mouse eats oats; **(D)** incision of the tail with a razor blade to allow **(E)** blood sampling.

night. Within 16 hours, the solution was absorbed by the oats and they were dry when presented to the mice.

Corticosterone was dissolved in CORT-HBC complex (Sigma-Aldrich Cole; Germany) in 5.6 and 18.7ng/ml and 0.9% NaCl-HBC as vehicle (VEH). From these solutions 20µl was applied to three oats resulting in a corticosterone dose of 4.5 and 15.0mg/kg for the treatment groups (Ruzek et al. 1999).

The GR antagonist RU38486 (100mg/ml; kindly provided by Corcept Pharmaceuticals, CA, U.S.A.) was dissolved in 1ml 0.9% NaCl containing 0.25% carboxymethylcellulose and 0.2% Tween20 (VEH). From this solution 50µl was applied to the oats (mice received 200mg/kg RU38486).

Gavage injection: The mouse was hand restraint and a gastric feeding needle (length = 3cm; 19 gauge = 1mm diameter; BioService, Belgium) was used to apply a volume of 200µl/25g bodyweight *per os.*

Intraperitoneal (i.p.) injection: The mouse was hand restraint and kept on its back within the palm of the hand. The injection (needle: 25 gauge = 0.5mm) was given in the lateral aspect of the lower left quadrant of the belly.

Adrenalectomy

To start surgery with low stress system activity, mice were transported to the operation room two hours earlier. Between 0900 to 1400h adrenalectomy (ADX) was performed using the dorsal approach under isoflurane anesthesia. The percentage of isoflurane used to induce anesthesia was 4%, and was decreased to 2% at the moment of surgery. The adrenals were removed and surgery was completed within 5 min. Mice received

two bottles, one containing 0.9% saline, and the other containing water, upon return to their home cage.

Blood sampling and corticosterone assay

Corticosterone concentrations were measured in blood samples obtained via tail incision (Figure 1D/E). Briefly, a small incision with a razor blade at the base of the tail allowed collection of < 50µl blood within 60s after opening of the animal's cage (Durschlag et al. 1996; Dalm et al. 2005). We refined the method to minimize possible confounding factors of the incision procedures and concomitant blood volume reduction (Grassler et al. 1990). The mouse is only touched at the tail, not fixated in the hand (Figure 1D/E). Only a minute volume of blood was collected, i.e., < 50µl, with at least 60 min between two blood samples. To stop bleeding, the tail was gently pressed into the sawdust of the cage. Separate groups of mice are used for the time-course measurements. The entire procedure lasted less than 60s. Data on Oats+VEH treated mice in Figure 2 support the efficacy of our blood sampling method. We conclude that the blood sampling procedure did not significantly contribute to the observed pharmacological effects of our experimental design.

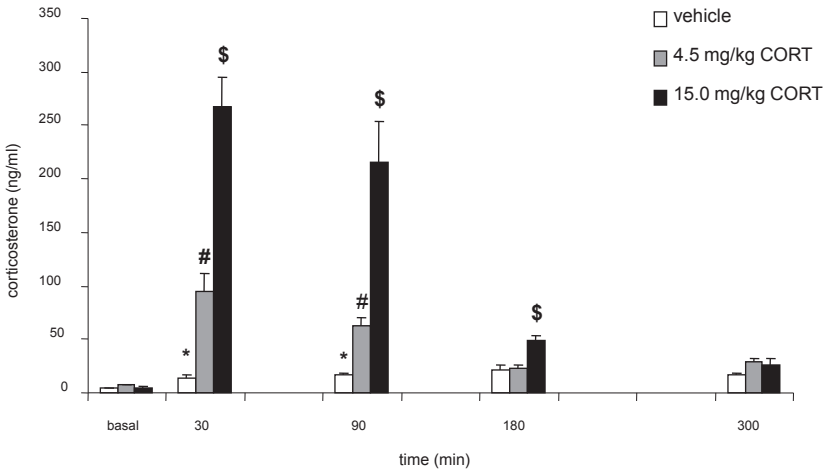

Figure 2

Dose-controlled delivery of corticosterone (CORT: 0, 4.5 and 15.0mg/kg bodyweight) via oats (indicated by arrow at 0900h) to adrenalectomized mice. Plasma corticosterone concentrations in ng/ml on the day before (basal), and t=30, 90, 180 and 300 min after oat consumption. Note that all corticosterone values of ADX mice are in the range of basal corticosterone secretion. Data are presented as mean ± S.E.M.; $p < 0.05$: * vs. other groups.

At the end of the experiments, blood samples were taken from trunk blood. Blood was collected individually in capillaries, coated with potassium-EDTA (Sarstedt, Germany), stored on ice and centrifuged with 13000 rpm at 4°C for 10 min. Plasma was stored at –20°C. Corticosterone was analyzed using a commercially available radio immunoassay kit ^{125}I-corticosterone (MP Biomedicals, Inc., NY, USA; sensitivity 3ng/ml).

Blood glucose levels in response to oat consumption

In response to food consumption the blood glucose levels rise (Gagliardino et al. 1984). Basal glucose levels were determined one day before three flakes of oats (± 140mg) were presented in the feeding cup of the mice (two groups of mice (n = 8/group). Blood samples were taken after 5, 15 and 60 min after oat presentation (group 1) and after 10 and 20 min (group 2). A droplet of blood was applied to a test strip and within seconds the glucose content of the blood was displayed in mmol/l (Accu-Chek Compact, Roche Diagnostics, GmbH, Mannheim, Germany). Then, the mouse was returned to its home cage (total duration < 15s).

Experiment 1: Methods of drug-delivery and HPA axis activity

To determine whether and to what extent the methods of drug-delivery (including moving the cage, eating oats, the vehicle, gavage and intraperitoneal injections) affect HPA axis activity, we estimated corticosterone concentrations at multiple time points.

Oats procedure: One day before the experiment started, blood samples were taken to determine basal corticosterone concentrations. Mice were distributed randomly to three groups (n = 10 – 11/group): (1) Delivery control procedures: get the cage from the shelf, lift grid, touch feeding cup with forceps, close cage and return it to the shelf; (2) Consumption of pure oats; (3) Response to vehicle: consumption of oats with absorbed dissolvent used for the GR antagonist RU38486. Blood was collected from the same mice either at 30 or 60 min after the oat procedures on two consecutive days.

Gavage injection*:* Two groups of mice (n = 8/group) were injected *per os* with 200µl VEH (see above).

Intraperitoneal injection: Two groups of mice (n = 8/group) were injected intraperitoneal with 200µl VEH.

Handling procedures related to gavage and intraperitoneal injections (mice, n = 8): get the cage from the shelf, lift grid, pick up the mouse from the cage at the base of the tail, place mouse on grid, restrain the mouse as preparation for either gavage and intraperitoneal injection for 5s, return mouse to cage.

Experiment 2: Dose-controlled corticosterone administration via oats

To differentiate the amount of exogenously administered corticosterone in the blood from the endogenously secreted hormone, mice were adrenalectomised (ADX). In contrast to rats, ADX-mice remain to secrete basal low concentrations of corticosterone from scattered cell groups in the vicinity of the adrenals (Hummel 1958). ADX mice lack the stress- or circadian-induced increase in corticosterone secretion, keeping a basal secretion of corticosterone between 5 and 25ng/ml. To verify the quality of ADX, basal blood samples were taken in the evening five days after surgery. Two days later at 0900h, mice (n = 8/group) received three flakes of oats containing 4.5 or 15.0mg/kg corticosterone or vehicle. Blood samples were taken at t=30, 90, 180 and 300 min following oat consumption.

Experiment 3: GR antagonist RU38486 delivery via oats

Activation of GR regulates the negative feedback on corticosterone secretion during the circadian peak and in response to stress (De Kloet et al. 1998). Blockade of the GR inhibits negative feedback. Consequently, corticosterone concentrations increase or remain elevated. Mice ate oats with the GR antagonist RU38486 or vehicle (factor: treatment). One hour later, half of the mice had to swim in a bucket filled with warm water (30cm diameter x 40cm high; 26 ± 1°C) for 1 minute, to activate the HPA axis (factor: condition). The mouse was removed from the water using a grid and returned to its home cage which was placed underneath a heating lamp (250Watt) for 3 min. Control mice remained in their home cage. We hypothesized that blockade of GR by RU38486 would result in high concentrations of corticosterone in mice exposed to swim stress.

According to treatment and condition, four groups were formed (n = 8/group): (1) Oats + RU38486 + swim; (2) Oats + VEH + swim; (3) Oats + RU38486 + no-swim; (4) Oats + VEH + no-swim. Basal corticosterone concentration was determined between 0900 to 1000h, one day before the start of the experiment. The next day, mice received three flakes of oats containing 200mg/kg RU38486 or vehicle at 0900h. One hour after consumption at t=60 min, a blood sample was taken. Then, mice returned to their home cage or swam for 1 minute. Subsequent blood samples were taken at t=*90*, 120, *180* and 240 min after swimming. *Italic time points* indicate separate groups of mice. Between blood sampling, mice remained in their home cage.

Separate groups of mice were fed with Oats + RU38486 or Oats + VEH (n = 6/treatment) and blood samples were collected: (1) 15 min after oat delivery to estimate a possible short-lasting rise in corticosterone secretion due to oat consumption; and (2) to

further assess the duration of GR antagonism: one day before (control) and 8 hours after oat delivery during the circadian evening surge (at 1700h, i.e., two hours before lights off, (Dalm et al. 2005), and 24 hours after oat delivery.

Statistical analysis

Data was analyzed by one- or two-way analysis of variance (ANOVA; factors: treatment and/or condition), when appropriate with repeated measurements followed by Tukey *post-hoc* test. Total corticosterone values (AUC: area under the curve) were compared by t-test. Data are presented as mean ± S.E.M. Significance was accepted at $p < 0.05$.

Results

Experiment 1: Methods of drug-delivery and HPA axis activity

At baseline, corticosterone concentrations of all groups were in the range of low basal levels (Table 1: $F_{(5,55)}$=0.649, p = 0.663). Depending on the applied procedure, corticosterone concentrations increased after 30 and 60 min (time*group: $F_{(10,100)}$=11.406, p = 0.001). However, none of the procedures related to oat administration, nor eating of pure and vehicle-treated oats and procedures related to gavage and intraperitoneal delivery altered the plasma corticosterone concentration.

Corticosterone secretion in response to the vehicle delivered via oats, gavage and intraperitoneal injection increased over time and depended on the method of delivery (time*group: $F_{(4,18)}$=9.731, p = 0.001). While vehicle delivery via oats had

Table 1:

Basal morning resting and procedure-induced corticosterone concentrations (ng/ml) in blood plasma at 30 and 60 min after delivery. Data are presented as mean ± S.E.M.; $p < 0.05$: * **bold** vs. all other procedures and time points; *italic* vs. basal.

Delivery procedure	basal	30 min	60 min
pure oats	7.0 ± 0.8	8.8 ± 1.1	7.3 ± 1.1
oats + vehicle	8.3 ± 1.2	9.3 ± 1.2	7.5 ± 2.3
procedures – oats	6.3 ± 0.4	6.9 ± 0.6	5.4 ± 0.6
i.p. injection (vehicle)	7.9 ± 0.9	**115.8 ± 16.8***	**90.1 ± 16.3**
gavage injection (vehicle)	7.8 ± 1.2	**133.9 ± 36.8***	**43.3 ± 9.8**
procedures gavage and i.p.	8.1 ± 0.9	*25.0 ± 1.9*	*31.3 ± 14.2*

no effect, gavage and intraperitoneal injections resulted in significant elevations of corticosterone at 30 and 60 min (p = 0.001 and p = 0.005, respectively).

Blood glucose levels in response to oat consumption

Basal levels of blood glucose (8.53 ± 0.38mmol/l) were in the expected range for C57BL/6 mice at 10 weeks of age (Saravia et al. 2002). We observed that mice consumed the oats within 10 min after presentation. Blood glucose increased in response to oat consumption (t=5 and 10 min: 9.70 ± 0.30 and 11.46 ± 0.21mmol/l) and remained at the same level from 10 to 60 min after oat presentation (time: $F_{(3,35)}$=20.700, p = 0.001).

Experiment 2: Dose-controlled corticosterone administration via oats

Exogenous corticosterone delivered via oats dose-dependently increased plasma corticosterone concentrations (Figure 2: treatment ($F_{(1,21)}$=97.941, p = 0.001). The significant time*treatment interaction effect ($F_{(4,84)}$=20.865, p = 0.001) is due to the ADX group: ADX mice that received Oats + VEH had the expected low basal corticosterone concentrations of 5.90 $\pm$ 1.22ng/ml over all time points (p > 0.05). Consumption of oats containing corticosterone resulted in elevated concentrations at 30 and 90 min (p < 0.05): the dose of 15mg/kg corticosterone resulted in a 3-fold higher plasma concentrations than 4.5mg/kg (p < 0.05). When compared to 4.5mg/kg corticosterone and vehicle, corticosterone levels after 15mg/kg corticosterone were still significantly elevated 3 hours after consumption (p = 0.001).

Experiment 3: GR antagonist RU38486 delivery via oats

Mice consumed oats containing RU38486 or vehicle (treatment), one hour before swimming or not swimming (condition). Figure 3 depicts that corticosterone concentrations were significantly affected by treatment ($F_{(1,28)}$=701.424, p = 0.001) and condition ($F_{(1,28)}$=10.463, p = 0.001). In all but the VEH no-swim group, plasma corticosterone concentrations increased over time (time effect: $F_{(5,140)}$=65.825, p = 0.001). Corticosterone was significantly higher in the RU38486 than in the VEH mice (treatment*time $F_{(5,140)}$=57.058, p = 0.001) and significantly elevated by swimming (condition*time $F_{(5,140)}$=2.453, p = 0.036).

Swimming further potentiated the corticosterone concentrations (condition: $F_{(1,14)}$=4.667, p = 0.049), also expressed by significantly higher AUC values (mean ± SEM mg/ml; swim: 69.18 ± 3.29 vs. no-swim: 58.16 ± 2.94; p = 0.026). Corticosterone

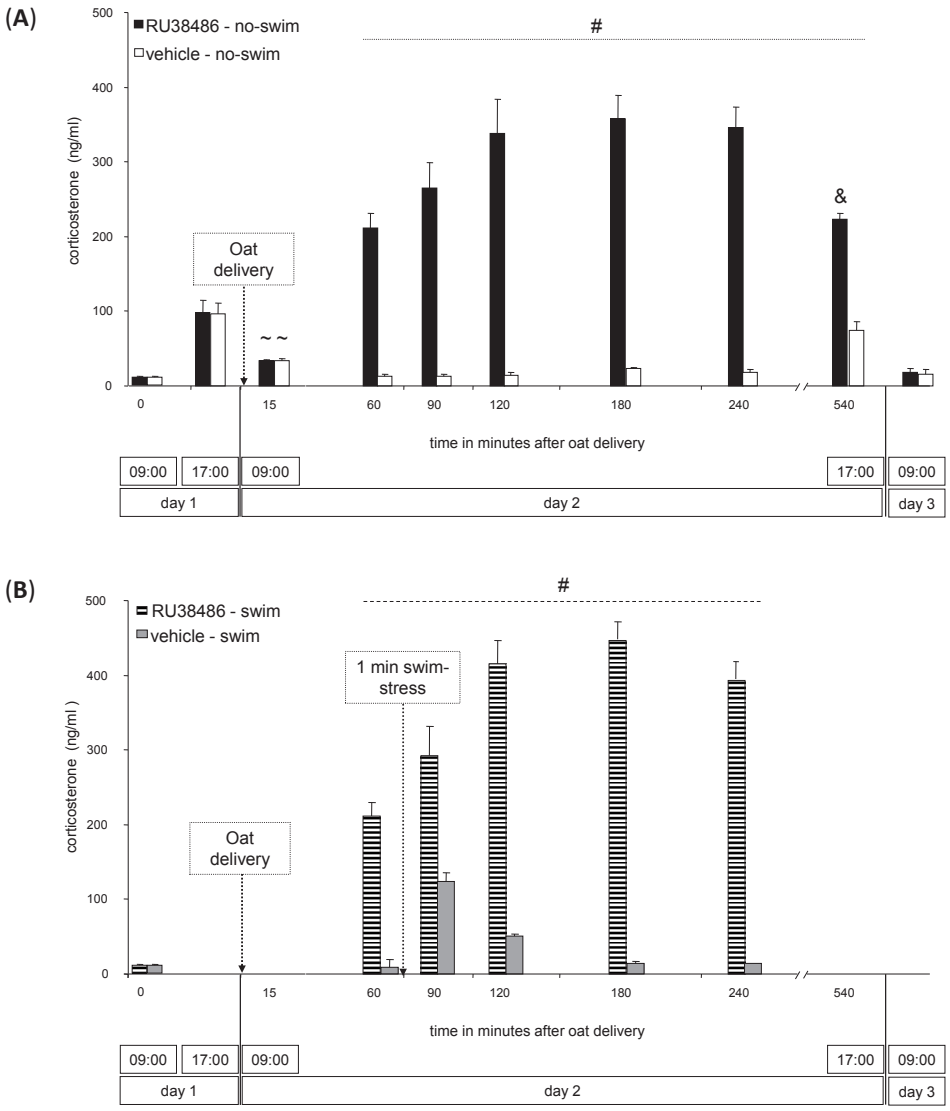

Figure 3

Plasma corticosterone in ng/ml before (day 1 at 0900 and 1700h) and after consumption of oat with the GR antagonist RU38486 or vehicle (indicated by arrow at 0900h) on days 2 and 3. Mice of both groups were **(A)** not exposed to a 1-min swim or **(B)** exposed to a 1-min swim stress (indicated by the arrow at 1000h), 60 min after consumption of the oats. Blood samples were taken from different groups of mice t=15, 60, 90, 120, 180, 240 and 540 minutes (1700h) after oat-consumption on day 2. On day 3, another sample was taken at 0900h. Data are presented as mean ± S.E.M.; $p < 0.05$: ˜ t=15 vs. basal: t=0 day 1 at 0900h; # RU38486 vs. VEH; & day 2 vs. day 1: evening corticosterone at 1700h.

concentrations before testing were low and comparable between the groups (data not shown; $F_{(3,31)}$=0.560, p = 0.646).

Already the consumption of RU38486 containing oats increased plasma corticosterone (Figure 3A; t=60 min after oat delivery; $p < 0.05$). The additional 15 min time point after oat consumption revealed a slight increase in corticosterone secretion in both RU38486 and VEH groups (vs. baseline: p = 0.001). Apparently preventing GR action at this time underlies the massive subsequent increase in plasma corticosterone in the RU38486 mice, while corticosterone returns to baseline values in the VEH group with intact GR function.

Swimming alone increased the total corticosterone values calculated as AUC (Figures 3A/B; vehicle groups: swim 7.41 ± 0.70 vs. no-swim 3.10 ± 0.27mg/ml; p = 0.001). Interestingly, 180 min after swimming, corticosterone levels were even lower than in no-swim controls (p = 0.013), and comparable to controls at 240 min.

RU38486 delivery via oats kept the levels of plasma corticosterone elevated for at least 8 hours as the evening concentrations (1700h) were still higher compared to VEH (p = 0.001) and the evening value one day before the oat delivery (p = 0.001). Comparably low resting corticosterone concentrations were found 24 hours after delivery of oats ($p > 0.05$).

Discussion

We have devised a non-invasive stress-free method of drug delivery in mice by validation on the glucocorticoid stress-system activity. Here we demonstrate that (1) ligands of the glucocorticoid system can be delivered via oats, (2) the effects are not confounded by long-lasting stress system activation induced by the method of administration and thus allow (3) drug delivery in close context with a test situation, e.g., a behavioral task. Furthermore, the procedure is easy to perform which minimizes variability of drug effects induced by the researcher and drug-application technique.

Any disturbance in the homeostasis of the organism induces HPA axis activity, which is expressed as an increase in circulating concentrations of corticosterone in mice and rats (De Kloet et al. 1998). The magnitude and duration of HPA axis activation is an accepted indicator for the degree of stress applied. In the present study, procedures related to drug delivery via oats did result in a minute increase in corticosterone. In contrast, procedures related to both, gavage and intraperitoneal injections resulted in substantial and long-lasting increased corticosterone concentrations. Whereas high-long

lasting increases of corticosterone are generally considered as “stressful”, we defined the minute increase in corticosterone as “stress free”. The devised method of drug delivery via oats reduced the magnitude and duration of stress system activation, could and should be used for other compounds as well.

Method of drug delivery

Non-invasive drug delivery in rodents can be realized via food or water. Mice are nocturnal animals, which mainly eat and drink during their behaviorally active (dark) period. Next to the fact that the dose of the drug delivered via free access to consumables cannot be controlled, most laboratories perform experiments during the inactive (light) period of the mice. To force dose- and time-controlled consumption, depriving mice of food and water is in itself a stressor (metabolic stress) with wide-spread consequences, also on HPA axis activity and disturbance of circadian activity patterns (Sommerville et al. 1988; Duclos et al. 2005). To avoid food deprivation-induced stress and to motivate eating during the light period, we selected oats as a treat. Mice like eating oats and readily overcome neophobia. Providing the oats at a fixed location in the home cage excluded stress induced otherwise by a novel environment or touch by a human experimenter. In the present study, all mice eat the oats containing the different glucocorticoid ligands within 10 min. The period of corticosterone and GR antagonist delivery via oats can be extended for at least one week (V. Brinks, S. Dalm, *unpublished*). However, drugs might have a bad taste or smell, such that mice might not eat the drug-containing oats (or only once). Hence, we recommend testing a possible neophobia or taste-aversion response to the oats + drug, like we have done, before the start of the experiment.

Choosing the appropriate vehicle reduces masking of wanted effects of administered drugs of interest. The selection of the most appropriate vehicle is based on the properties of the substance under investigation. Brown and colleagues (Brown et al. 2000) tested several vehicles, including water, corn oil and 1% methylcellulose/0.2% Tween80. They demonstrated that gavage administration of corn oil at 20ml/kg induced a stress response in a volume dependent fashion, whereas water and 1% methylcellulose/0.2% Tween80 did not. We also showed that the dissolvent of the glucocorticoid antagonist RU38486 in oats did not influence corticosterone concentrations.

To assure dose-controlled delivery via oats, mice are housed solitary. Housing conditions can significantly influence the behavior of mice, and this relates to enriched vs. poor environment, single vs. group housing, gender and strain effects (Ouagazzal et al. 2003; Chourbaji et al. 2005). Male mice, due to their territorial aggression should be

preferentially housed solitary with some environmental enrichment like paper towels (Van Loo et al. 2004). Social housing is the optimal way of housing female laboratory mice (Van Loo et al. 2007). To allow dose-controlled consumption of oats, introducing separations into the home cage of group-housed mice might be an option, but could induce stress due to disturbance of the home-environment as well. How feasible such a procedure is, has to be tested.

Effects of glucocorticoid ligands

Corticosterone. Administration of different doses of corticosterone to adrenalectomised mice allowed us to mimic the natural surge of corticosterone that has been described in response to a novel cage (Grootendorst et al. 2001b). The higher dose of 15mg/kg induced an approximate 3- fold increase in blood corticosterone concentrations when compared to the lower dose of 4.5mg/kg. Interestingly, 5 hours after administration of both doses, plasma corticosterone concentrations were similar. These findings will allow to choose appropriate doses of corticosterone in future studies. Based on the action mechanism of corticosterone (De Kloet et al. 1998) we may assume that in adrenally intact mice the two doses of corticosterone will have activated GR to a different degree, leading to enhanced negative feedback. Here, in ADX mice we may conclude that corticosterone also initiated GR actions. The decrease in plasma concentrations, however, is due to clearance from the organism, for both doses of corticosterone within 3 to 5 hours.

Glucocorticoid antagonist. In the current study RU38486 was administrated systemically one hour before a 1 minute swim trial. Given the corticosterone response, swimming strongly activates the HPA axis and can be considered as a stressor (Figure 3B). Mice that received RU38486 and swam for 1 minute, indeed showed the expected increase in corticosterone concentrations, lasting at least 8 hours, but less than 24 hours. Surprisingly, mice that consumed the RU38486-treated oats, but did not swim, also showed strongly increased corticosterone concentrations. This is in contrast to previous studies using intracerebral injections in rats showing that the effects of GR antagonism on corticosterone regulation occur in response to stress and at the peak of circadian corticosterone secretion, but not during the period of low basal corticosterone secretion (Ratka et al. 1989; van Haarst et al. 1996). Also systemic injections of GR antagonists in rats did not change basal corticosterone secretion (Spencer et al. 1998; Spiga et al. 2007). There are known species-dependent stress responses, and mice and rats habituate differently to laboratory handling and injections procedures (Balcombe et al. 2004). We assumed that oat-consumption itself might have activated the HPA axis within

this 30 min time frame and verified this in a follow-up experiment. Indeed, 15 min after oat consumption corticosterone concentrations were slightly increased. The slightly elevated blood glucose within 10 min is in accordance with the post-prandial increase in plasma glucose seen after consumption of carbohydrate-rich food. Glucocorticoids and glucose strongly interact (Gagliardino et al. 1984; Peters et al. 2004). We suggest that already the rather slightly elevated corticosterone will activate some GR to regulate negative feedback and thereby controlling the oat-consumption-induced secretion of corticosterone. The systemic application of the GR antagonist RU38486 involves an effective GR blockade throughout the entire organism and corticosterone levels kept rising. We conclude that the loss of GR activation potentiated corticosterone secretion due to the combined actions of glucose and corticosterone. This and the question of a genomic or non-genomic (via nuclear or membrane receptors) action of the GR antagonist cannot be resolved at present and should be addressed in further studies. The fast time course (within 1h) might point to a non-genomic action, however Morsink et al. (Morsink et al. 2007) reported in hippocampal tissue of rats genomic actions of corticosteroids via GR. Relevant is the fact that the initially unexpected increase of corticosterone due to oat consumption now additionally proves the efficacy of RU38486 as GR antagonist.

The dose of 200mg/kg RU38486 used was 16-fold higher compared to the study of Ratka (Ratka et al. 1989) and might be considered as extremely high. However, high doses of RU38486 prove very successful in patients suffering from neuropsychiatric disorders (up to 2000mg/BW (DeBattista and Belanoff 2006). Furthermore, mice lack the α_1-acid glycoprotein, which in humans binds about 95% of circulating RU38486 (Heikinheimo et al. 1987; Heikinheimo et al. 1989). Thus in mice, low concentrations of RU38486 are rapidly cleared from the body. In order to understand the mechanism underlying the beneficial effects of long term GR antagonism on endocrine and behavioral regulation, high dose of RU38486 will increase the likelihood of GR antagonism and as a consequence disturb negative feedback. This is clearly demonstrated by the long-lasting elevation of endogenous corticosterone. While exogenous corticosterone is cleared from the system after 3 to 5 hours, corticosterone levels remain extremely high during this time domain. This is indicative for continuous secretion of corticosterone due to the blockade of negative feedback actions via GR (Dalm 2006).

With respect to the use of oats as a reward or carrier for drugs in studies on learning and memory, we have to keep in mind that glucose is also known to modulate cognitive functions (Messier 2004). However, to be effective, glucose has to be administered in much higher concentrations than induced by oat consumption.

Moreover, the number of oats could be reduced to minimize effects on corticosterone and glucose secretion.

Drug delivery via oats has several advantages over other methods of application. It is (i) non-invasive; the short-lasting slight increase in plasma corticosterone after oat consumption, is not comparable to injection-induced effects, neither in quantity nor quality; (ii) easy to use; (iii) allows administration in close-context and can be used repeatedly; (iv) no food or water deprivation is needed (Mitev et al. 1993); (v) time of administration is not confined to the dark, behaviorally active period; (vi) the vehicle is inherent to the drug effects; (vii) it resembles more the conditions in human drug delivery. Of course, there are also arguments against this kind of peripheral drug delivery. First, the drug effect is not selective as it reaches the entire body and may not pass the blood-brain-barrier. This holds true for other systemic methods of drug delivery and is also related to pharmacodynamic and –kinetic characteristics of the drug. Second, timing of drug-delivery will depend on the number of oats presented, but can be achieved within 10 min or less. It might not be suitable to study fast drug effects, while it does allow to study drug effects in close-context to, for instance, behavioral performance. Third, administration via oats requires that mice are single housed, at least during the time of drug delivery. Separating mice with partitions in their home cage might be a possibility. However it is likely, that interference with the home cage environment will introduce an extra stress factor. It might be possible to adapt mice to such handling by a series of habituation trials. Fourth, taste and smell of the drug could influence its consumption, but these are aspects that have to be tested before the experiment. When to use this method of drug delivery via oats? One has to balance the pros and cons, but it is the scientific question that is central to the design of the experiment.

Conclusions

We consider drug delivery via oats as method of choice as it allows to dissociate the effect of the administration procedure from the properties of a drug. Since we have demonstrated that intraperitoneal and gavage injections lead to long-lasting corticosterone exposure that most likely will affect memory processes (Sandi et al. 1995; Sandi et al. 1997), we specifically propose oats for context-dependent stress-free drug delivery.

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